FLOWER HEAD DEVELOPMENT IN THE ASTERACEAE FAMILY

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ABSTRACT OF THESIS:

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The flower head of the Asteraceae family is its iconic trait, yet little is known about its development. Understanding the fundamentals of the flower head development will help construe the evolution of one of the most successful plant families. In this thesis, we carried out an investigation into the role of auxin in flower head development and patterning in *Matricaria inodora* and *Senecio vulgaris*. Auxin is one of the most crucial plant hormones and has been implicated in almost all stages of growth and development. In *Matricaria inodora* and *Senecio vulgaris* auxin was found to be involved in flower head development and pattern formation. Manipulation of the endogenous auxin *in planta* showed homeotic conversions of disc florets to phyllaries or ray florets. Analysis of lateral organ identity genes revealed a concentration dependant response of the identity genes to auxin. The homeotic change of lateral organs in a concentration dependant manner is one of the key traits of a morphogen that had never been documented *in planta* before. We suggest that auxin acts as a morphogen in the developing flower head to control development and pattern formation. Visualisation of auxin distribution using a Beta-glucuronidase marker gene further confirmed the presence of an auxin gradient in the developing flower head. Auxin appears to have a secondary role in the petal outgrowth and shape in ray florets. In summary, auxin appears to be controlling the development and pattern formation in the flower head through the concentration dependant recruitment of lateral organ identity genes.

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Research Contributions

Chapter 3: A novel morphogen-like role of auxin in determining capitulum pattern formation in Asteraceae

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Chapter 4: A morphological study of flower head development in the Asteraceae

Nicholas Zoulias, Minsung Kim*

Chapter 5: Virus induced gene silencing as a genetic tool in the Asteraceae family

Nicholas Zoulias, Helena Garcês, Minsung Kim*

List of Abbreviations

ARF	Auxin Response Factor
cDNA	Complimentary DNA
CYC	*CYCLOIDEA*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	double-stranded RNA
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
GUS	Beta-glucuronidase
IAA	Indole-3-acetic acid
LFY	LEAFY
MADS-box	MCM1, AGAMOUS, DEFICIENS, SRF -box
mRNA	messenger RNA
MYB	Myeloblastosis
NPA	1-Naphthylpthalamic Acid
PCR	Polymerase Chain Reaction
PDS	Phytoene desaturase
PIN1	Pin-Formed1
qPCR	quantitative PCR
RA	Retinoic Acid
RAREs	Retinoic Acid Response Elements
RISC	RNA-induced silencing complex

RNA	Ribonucleic acid
RXR	Retinoid X receptors
SEM	Scanning Electron Microscopy
siRNA	small interfering RNA
TCP	TEOSINTE BRANCHED1, CYCLOIDEA, and PCF transcription factor
VIGS	Virus Induced Gene Silencing

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Chapter 1

Introduction

Two of the most fundamental questions in developmental biology are: organogenesis and the pattern formation of organs. In plants, flower development is an excellent system to study both organogenesis and pattern formation because four distinct organs develop from a group of homogenous floral meristem. Studies on flower development are also critical to further understand plant reproduction, such as fruit and seed development, which is important for agricultural applications. Understanding how these developmental processes evolved allows us to understand the coeval complex forms, which were formed by successful adaptations to certain niches in the environment. The pseudanthium (false flower) is a highly-modified inflorscence with complex pattern formation and organogenesis. A pseudanthium is a collection of flowers (known as florets) and bracts (modified leaves) that are clustered together generally mimicking a single flower (Hutchinson, 1964). Some pseudanthia can consist of as few as two florets, and some more than a thousand. Pseudanthia are very successful inflorescence form, having appeared across several distinct orders throughout angiosperm evolution. Figure 1 shows a phylogenetic tree of angiosperm orders, with the orders having pseudanthium in them highlighted in yellow (Group, 1998). The tree show that pseudanthia appear in both monocot and eudicot evolution several times as well as appearing in both basal and more recently evolved orders. The most iconic and well known order containing pseudanthia is the Asterales, and within the Asterales the family recognised exclusively by its pseudanthium is the Asteraceae.

1.1 The Asteraceae family

The Asteraceae family is one of the largest families of flowering plants with close to 23,000 species over 1,500 genera (Bremer, 1994). As such a large and diverse family, the Asteraceae includes several important crop species (such as sunflower, lettuce and artichoke), and many other species that are either culturally or horticulturally important (Bremer, 1994). The Asteraceae family is hypothesised to have originated in South America (Barreda et al., 2010). This is supported by both fossil evidence and by the fact that that the most basal tribe, Barnadesieae,

of the Asteraceae is endemic to South America. A recent discovery of a rare flower head fossil in Patagonia, South America gave an insight into both early floret forms and evolutionary separation from other angiosperms. The new fossil evidence suggests that the divergence of the Asteraceae family occurred around 47.5×10^6 years ago , compared with molecular analysis which put the divergence around 38-42 $\times 10^6$ years ago (Kim et al., 2005, Barreda et al., 2010). Whilst the family is very diverse there is a single unifying feature. All the members of the Asteraceae produce a compact pseudanthium called capitulum or flower head. Flower head of the Asteraceae family provides a unique research opportunity for plant biologists, developmental biologists and evolutionary biologists. It allows for the comparison of single flowers against flower heads (analogous structures performing the same function), as well as providing information on lateral organ development and evolution.

1.1.1 Structure of the flower head

The flower head of an Asteraceae is usually composed of three different lateral organs: Phyllaries, ray florets and disc florets. These lateral organs are almost always formed in the order of phyllaries, ray floret and disc florets. The phyllaries (bracts), are modified leaves and mimic the sepals of a solitary flower. Ray florets are bilaterally symmetric, while disc florets are radially symmetrical (Koch, 1930). Whilst the order of lateral organs in the flower head rarely changes, the number of layers of each lateral organ varies from species to species. Figure 2A shows a typical Asteraceae flower head (radiate), it consists of a single layer of phyllaries followed by a layer of ray florets surrounding the central disc florets. In contrast, the flower head shown in Figure 2D has a single layer of phyllaries followed by multiple layers of ray florets and no disc florets in the centre of the flower head. Figure 2C shows a discoid flower head that consists of only phyllaries and disc florets, which is in direct contrast to the radiate flower head form shown in Figure 2B. Other unusual flower head forms include the ‘compound’ flower head such as that of *Leontopodium alpinum* (Edelweiss) which is made up of a small collection of discoid flower heads

that mimic a single flower. The ‘compound’ flower head has an extra level of complexity in *Oedera capensis*, in that there are positional cues to ensure that only the flower heads on the outside produce ray florets on their margins. Conversely, there is the very minimal flower head of *Calycadenia hooveri* that contains just a single ray and disc floret.

As well as having different lateral organ patterning and flower head forms, there is a variety in the floret types. Both ray and disc florets are composed of 5 petals and are distinguished on the arrangement of the petals. Ray florets are always highly zygomorphic (bilaterally symmetric) and are usually found in a 3+2 petal arrangement where three ventral petals are elongated and fused to form the ligule and the two distal petals are reduced. Dandelion (*Taraxacum officinale*) flower heads are formed from ray florets that have a 5+0 petal scheme, where all 5 petals are fused to form the ligule (Fig.2 D), which is typical of the Cichorieae tribe. Disc florets are normally actinomorphic (radially symmetric) and have the 5 petals arranged in a star shape. The petals of a disc floret can vary in length from very short to long and lobbed. Flower heads of *Gerbera hybrid* contain a series of chimeric florets which are intermediates between ray and disc florets.

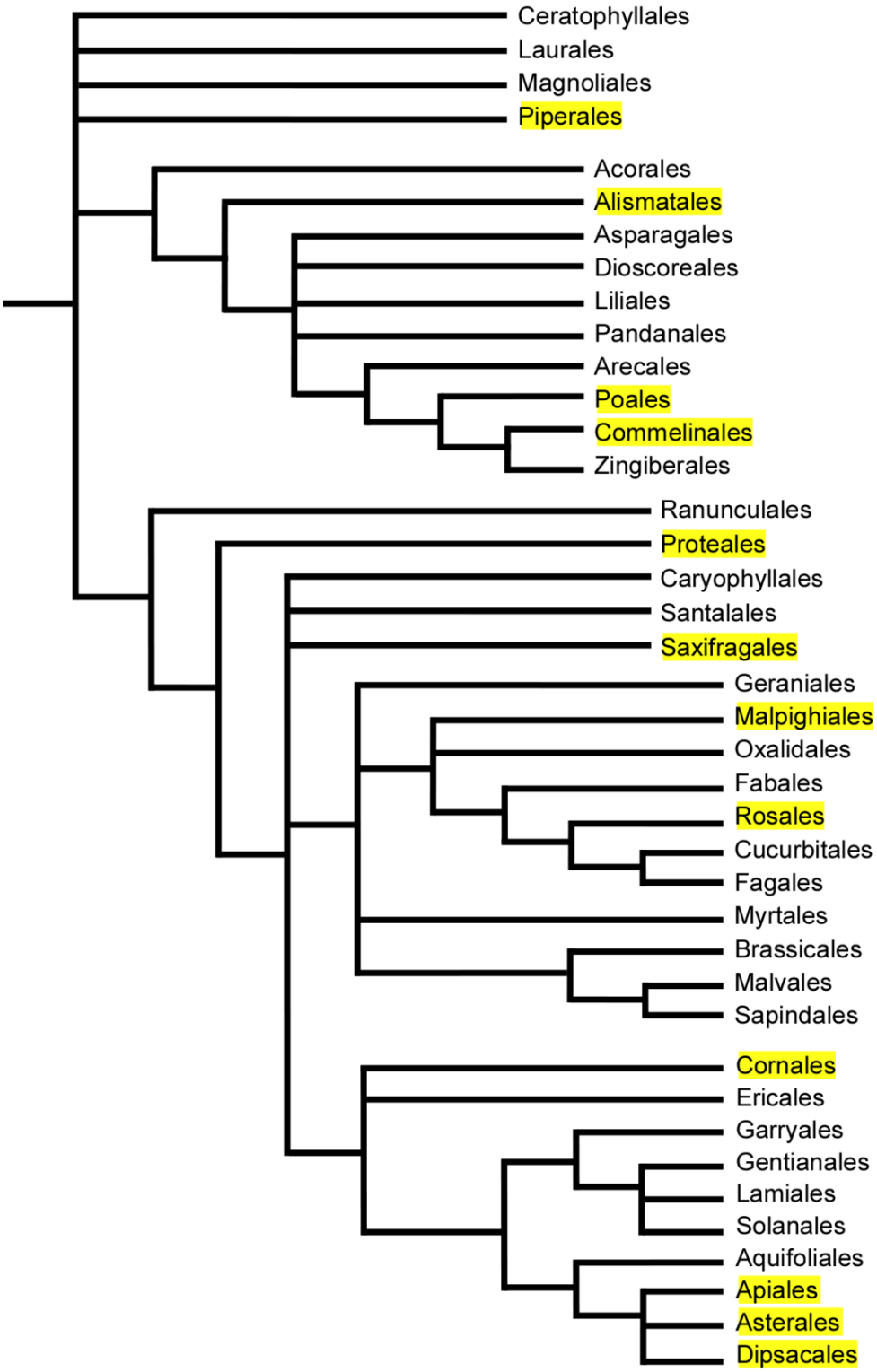


Figure 1: Phylogenetic tree of ordinal angiosperm classification adapted from The Angiosperm Phylogeny Group, 1998. The tree has been adapted to show orders which contain pseudanthia, highlighted in yellow.

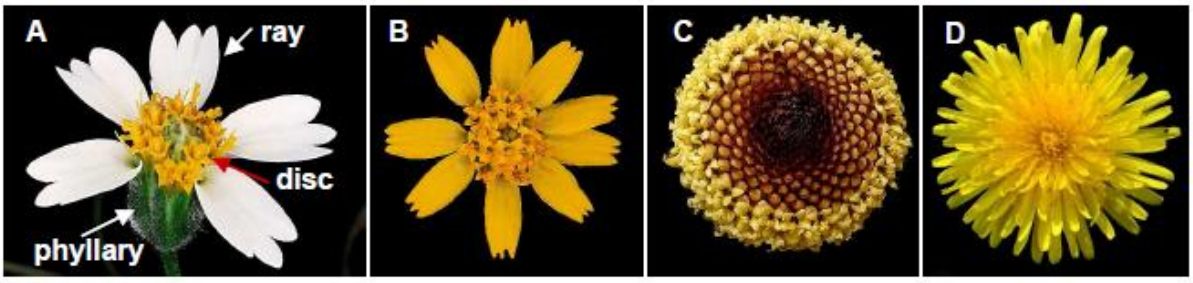


Figure 2: Different Asteraceae flower head forms. (A) Typical flower head with phyllaries, ray and disc florets. (B) Radiate, (C) Discoid and (D) Ligulate flower head.

1.2 Organ patterning

In animals, it is well understood how a positional cue can be translated into organogenesis, whereas in plants this process remains very poorly understood. One of the central dogmas in animal development is the role of morphogens as positional cues for patterning. A morphogen is a signalling molecule that governs the pattern of organ and/or tissue development, as well as governing the differentiation of specialist cells within the tissue. Morphogens diffuse from the point of production to form a concentration gradient across a developing tissue, allowing for concentration dependant responses (Driever and Nüsslein-Volhard, 1988b, Driever and Nüsslein-Volhard, 1988a). Although the definition of a morphogen is constantly evolving, in order for a signalling molecule to be considered a morphogen there are two criteria that must be met. Firstly, the morphogen must function in a concentration dependent manner, that when modified, alters the developmental fate of its target cells. Secondly, a morphogen must act directly on its target cells rather than through a series signalling intermediates. Morphogens are usually thought to be spatially distributed, a theory which is summerised by the French flag model. In the French Flag model, cells differentiate in response to their perceived position in the morphogens gradient (Wolpert, 1969).

In *Drosophila*, *Bicoid* mRNA and protein acts as a morphogen to control anterior-posterior polarity in developing embryos (Driever and Nüsslein-Volhard, 1988b, Driever and Nüsslein-Volhard, 1988a). When *bicoid* mutants undergo embryogenesis they are no longer able to define their anterior (head) and instead form two posteriors (tails), which is lethal to the organism (Driever and Nüsslein-Volhard, 1988b, Driever and Nüsslein-Volhard, 1988a). *Bicoid* is a classic example of spatial morphogens of which concentration is higher in the anterior region but decreases to near zero towards the posterior. However, more recent evidence has suggested that some morphogens work in a more spatial-temporal manner (Jaeger et al., 2008). That is to say, as well as the concentration gradient, that the time the developing cell receives the signal is also able to impact the developmental outcome. *Bicoid* is not alone in acting as a morphogen in flies, other molecules such as retinoic acid (RA) can also act as morphogens (Aulehla and Pourquie, 2010, White et al., 2007). RA's morphogen mechanism interactions are more complicated than *Bicoid's*; RA is one of three morphogen gradients all interacting on the same developing tissue, rather than the single gradient mechanism seen in *Bicoid*. There have been studies that show that the development of the somite-forming unit and differentiation of the paraxial mesoderm is mediated by RA (Aulehla and Pourquie, 2010). In a *Drosophila* embryo, a RA gradient is established, which switches on downstream genes in a dosage dependant manner (Aulehla and Pourquie, 2010). The switched on downstream genes in turn determine cell fates and organogenesis in the developing tissue.

Plants are large multicellular organisms with complex patterns and tissue formation but as of yet no morphogens have been identified. RA signalling and the way in which it is mediated in *Drosophila* has many similarities to the plant hormone, auxin. Auxin, which was first investigated by Charles Darwin, has been implicated in almost all areas of plant growth and development (Darwin, 1882, Okada et al., 1991, Reinhardt et al., 2000, Friml et al., 2002, Tanaka et al., 2006, Cheng and Zhao, 2007, Koenig et al., 2009). Auxin is involved in trophic responses to light and gravity (by cell expansion), leaf initiation and margin control (acting as a signalling molecule)

(Arteca, 1996). In the developing *Drosophila* embryos the RA gradient is detected in cells by retinoic acid receptors (RARs) for which RA acts as the ligand (Aulehla and Pourquie, 2010). The RARs form heterodimers with retinoid X receptors (RXRs), which in turn bind to DNA regions called retinoic acid response elements (RAREs) (Yu et al., 1991, Chambon, 1996). The binding of RA activated RAR-RXR heterodimers to RAREs causes changes in transcriptional regulation in the genes containing the response element, *e.g.,* the well-known Hox genes (Aulehla and Pourquie, 2010). Similar mechanisms analogous to this RA pathway can be seen in the auxin signalling pathways (details discussed in the next section). Similarities seen between the different animal morphogens and auxin suggest that auxin may act as a morphogen *in planta*.

1.3 Auxin

Auxin is one of the most extensively investigated plant hormones, and was the first major plant hormone (phytohormone) that was discovered (Arteca, 1996). Plant hormones act as signals to mediate responses to change in the plant's environment. Plant hormones also help in mediating developmental changes, such as the initiation of lateral roots and flowering (Okada et al., 1991, Reinhardt et al., 2000, Ottenschläger et al., 2003). The first experiments to be recorded on an unknown chemical signal (hormone) were performed by Charles Darwin in 1882. He observed the way that a coleoptile of canary grass (*Phalaris canariensis*) could respond to light. Through a series of light-dark experiments on different sections of the coleoptiles he concluded that only the very tip of the coleoptiles could respond to light and thus that they must control the rest of the plant (Darwin, 1882). The tip of the coleoptiles' are now known as the shoot apical meristem (SAM) and are known to mediate auxin production in response to unidirectional light. Auxin accumulates on the 'dark side' of the coleoptile where it acidifies the cell wall, loosening it and allowing for cell expansion so that it can bend towards the light (Ding et al., 2011). A few years after Darwin published these results, the major form of auxin [indole-3-acetic acid (IAA)] was discovered in

fermentation media, but it would be half a century before IAA was found in plant tissue (Went, 1935, Arteca, 1996). In the years of research to follow there were several different 'auxins' isolated from plants. In 1954, it was agreed to call any compound synthesised by plants and that was molecularly similar to IAA, an auxin (Arteca, 1996). In the past 60 years there has been a plethora of research into the role of auxin in plants, focusing on its effects upon biosynthesis and metabolism, transport, growth and development and stress responses (Koenig et al., 2009, Swarup et al., 2005, Tanaka et al., 2006, Vanneste and Friml, 2009, Cheng et al., 2006).

1.4 Polarised auxin transport

1.4.1 PIN-FORMED auxin efflux transporters

As auxin plays a major role in many stages of plant growth and development, the ability of plants to control the movement and accumulation of auxin is crucial. However, the importance of auxin polar transport in plant development and growth has been debatable because of the physiological properties of auxin. In the apoplast of plants about 15% of IAA is in the undissociated form (IAAH) that can freely diffuse across plasma membranes. Thus it was suggested that carrier mediated transport of auxin may be of minor importance, instead the biosynthesis and the diffusion across amenable concentration gradients were more important (Rubery and Sheldrake, 1973, Raven, 1975). This theory was eventually quashed by experimental evidence of IAA diffusion. The first experiments on auxin diffusion looked at the uptake in suspensions of cell cultures and found that auxin uptake was non-linear and pH dependant (Rubery and Sheldrake, 1974).

Together with the physiological property that IAA dissociates into to its anionic form in the cytoplasm of cells and is therefore trapped and not free to diffuse, it became clear that auxin transport throughout plants was reliant on carrier mediated transport (Rubery and Sheldrake, 1973, Rubery and Sheldrake, 1974). The first information into polarised auxin transport came from floral mutants (Okada et al., 1991). Auxin has been shown to be involved with many aspects

of development, yet there is conflicting information on auxin's ability to initiate flowering. It has been reported that auxin is able to inhibit the induction of flowering in Bromiliads (Kęsy et al., 2008). In contrast to the inhibition of flowering in Bromiliads, studies in model plants have shown that auxin is necessary for the transition from vegetative growth to reproductive growth and flower formation (Okada et al., 1991, Thingnaes et al., 2003). It has been reported that there is a link between the accumulation of endogenous auxin and flowering time (Thingnaes et al., 2003). But the most striking evidence that shows auxin's importance in flower formation has come in the form of mutagenesis studies. The mutation of auxin transporters, synthesis genes, and auxin response factors in Arabidopsis all lead to the formation of pin-shaped inflorescence (Fig 3B-D) (Bennett et al., 1995, Cheng and Zhao, 2007, Okada et al., 1991, Przemeck et al., 1996). These pin-shaped inflorescences lack the ability to form floral buds from the inflorescence meristems. The pin-shaped inflorescence phenotype gave its name to the first gene mutated to form the phenotype as *PIN-FORMED1* (*PIN1*). The PIN family is a family of directional auxin efflux transporters critical to normal plant growth and development (Grunewald and Friml, 2010).

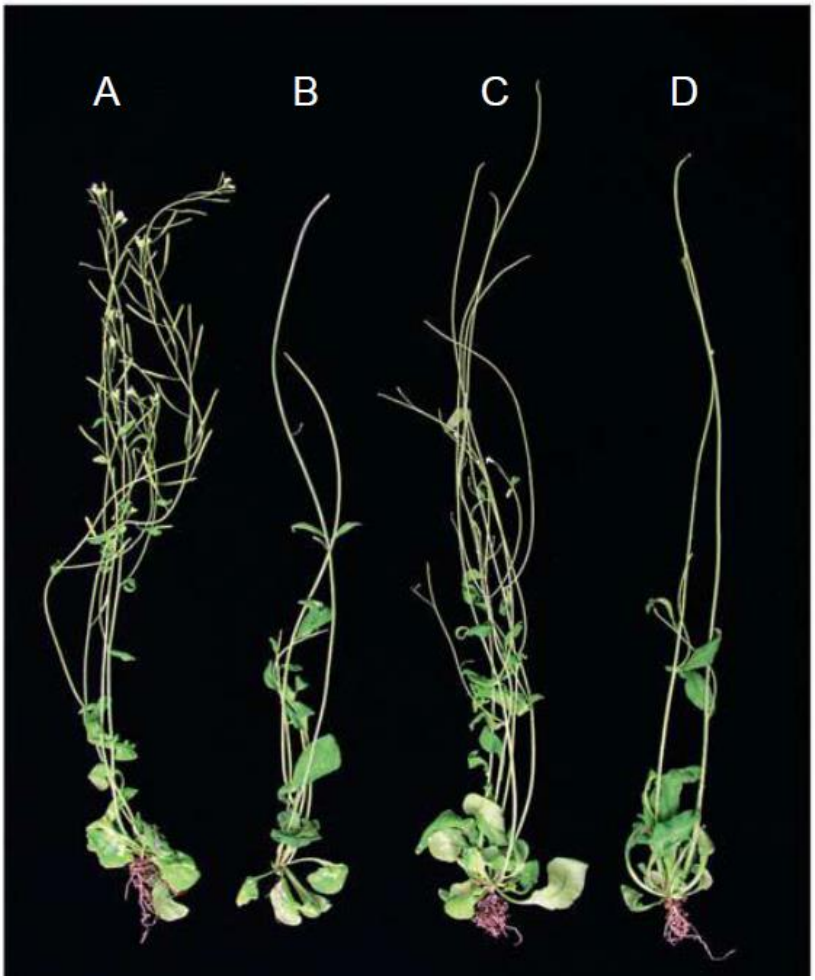


Figure 3: Pin-shaped inflorescence mutants all lacking the ability to transport or sense auxin. From left to right; wild type (A), *pin-formed1* (B), *pinoid* (C), and *monopteros* (D). Taken from Cheng and Zhao (2007).

Since the isolation of *PIN1* from floral mutants (Okada et al., 1989, Okada et al., 1991), eight *PIN* genes have been identified in the *Arabidopsis* genome, and these have been the subject of a number of studies. The PIN proteins can be categorised into two groups: long and short PIN proteins (Fig. 4A). The long PIN proteins are involved in intercellular auxin transportation and are localised to the plasma membrane (PIN1-PIN4 and PIN7) (Křeček et al., 2009). Short PIN proteins are involved with cellular auxin homeostasis and are usually localised to the endoplasmic reticulum (ER) (PIN 5, 6 and 8) (Mravec et al., 2009, Ding et al., 2012, Cazzonelli et al., 2013). PIN6 was originally classed as a long PIN based on its predicted structure even though it has a shortened hydrophilic loop when compared to the hydrophilic loop of other long PINs. After a

recent expression study in tobacco, PIN6 was found to be localised to the ER, indicating that it behaves like a short PIN protein (Friml and Jones, 2010, Mravec et al., 2009).

Recent phylogenetic studies across streptophyte algae, non-seed plants and angiosperms, have suggested that PIN proteins were originally expressed in the ER of the streptophyte algae, and later localisation to the plasma membrane occurred during land plant evolution (Feraru et al., 2012, Viaene et al., 2013). The main feature of the long PIN proteins is that they all have a central hydrophilic loop (Fig. 4B), which separates two hydrophobic regions (that are approximately 5 transmembrane helices in length each) (Křeček et al., 2009). PIN protein sequences do not contain an ATP-binding domains and their energy source for operation is still unknown (Gälweiler, 1998). The transcription of all long PIN proteins is up-regulated by auxin, whereas PIN5 (a short PIN protein) is down-regulated by auxin (Křeček et al., 2009, Mravec et al., 2009). All PIN proteins have a highly conserved sequence in the hydrophobic helices, these conserved regions are not tolerant of any insertions or deletions and are thought to be critically important for function (Křeček et al., 2009). In contrast to the highly conserved hydrophobic helices, the hydrophilic domains only show similarity within group members (long or short), due to the absent hydrophilic loop in short PINs (Fig. 4B) (Křeček et al., 2009).

1.4.2 Long PIN-FORMED transporters

The long PINs are thought to be the major efflux transporter of auxin in plants (Fig. 5), with PIN1 being the most extensively investigated. As mentioned previously, PIN1 was first discovered due to the pin-formed inflorescence in *Arabidopsis* and it has since been the focus of numerous studies (Okada et al., 1991, Gälweiler, 1998, Geldner and Palme, 2001, Friml et al., 2003, Ganguly et al., 2010, Bilsborough et al., 2011). Besides being necessary for the formation of floral buds, PIN1 is involved in embryogenesis, leaf initiation (phyllotaxis), leaf margin shaping, branching, vascular development and leaf margin patterning (Gälweiler, 1998, Ganguly et al., 2010, Koenig et al., 2009, Bilsborough et al., 2011, Kierzkowski et al., 2013). Phenotypes seen in the *pin1* mutant

and to some extent the other long *pin* mutants can be phenocopied by the application of transport inhibitors. One of the most well-known transport inhibitors, naphthylpthalamic acid (NPA), can phenocopy the *pin1* mutant faithfully when plants are grown on media containing NPA (Geldner and Palme, 2001). Both NPA grown and *pin1* inflorescences can be rescued by the localised application of auxin onto the pin-formed inflorescences (Reinhardt et al., 2000).

In *Solanum lycopersicum* (tomato) plants, the SAM grown on NPA becomes pin-formed shape but new leaf primordia can be initiated by the local application of auxin (Koenig et al., 2009). But in many processes PIN1 works in coordination with other PINs. For example, during embryogenesis the coordination of PIN1, PIN3, PIN4 and PIN7 is necessary to create the correct distribution of auxin, which is essential to the embryo development (Friml et al., 2002, Blilou et al., 2005, Ganguly et al., 2010, Sabatini et al., 1999). In the root, the maintenance of the root apical meristem (RAM) is dependent on the coordination of PIN proteins. PIN1 localises to the basal side of the root cortex cell, transporting auxin towards the RAM, whilst PIN2 transports auxin in both apical and basal directions ensuring the homeostasis of auxin in the region. PIN3, PIN4, and PIN7 act to keep to the concentration of auxin very high in the quiescent centre (QC) by laterally transporting auxin to the QC cells (Friml et al., 2002, Blilou et al., 2005, Křeček et al., 2009, Grunewald and Friml, 2010). The auxin that is transported away by PIN2 is recycled into the basal PIN1 stream of auxin via further lateral transport by PIN3 and PIN7. Thus, the loss of one of the PIN transports has strong phenotypic effects on the growth and development of the RAM and root.

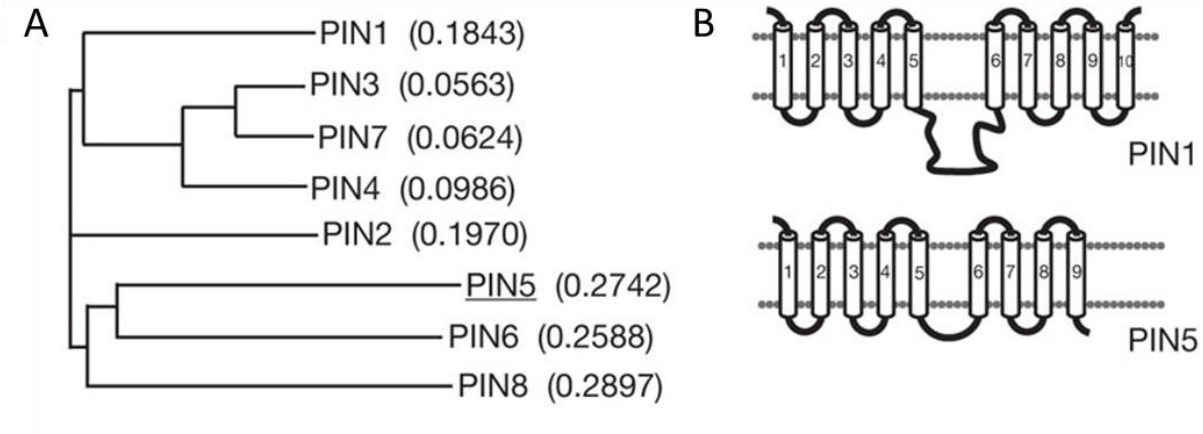


Figure 4: Phylogenetic analysis of *Arabidopsis* PIN family (A) reveals two distinct clades of long PIN proteins (PIN1-4, 7) and short PIN proteins (PIN5, 6, and 8). The typical long hydrophilic loop seen in PIN1, a long PIN (B), separating the two transmembrane domains. In contrast to PIN1, short PIN proteins have short hydrophilic loops as seen in the diagrammatic scheme of PIN5. Figure adapted from Mravec et al. (2009).

1.4.3 PIN-FORMED cycling

Original models of auxin efflux have PIN proteins as static but PIN proteins all appear to have the ability to alter their cellular position depending on tissue type, developmental and tropic signals. For the long PIN proteins there is now a fairly well understood mechanism behind apical/basal transcytosis (Fig. 5). It involves several different pathways but two of the most well understood are the PID and GNOM-Dependent pathways (Friml, 2010). PINOID (PID) is a serine/threonine protein kinase that is capable of phosphorylating PIN proteins. The *pid* mutant phenocopies (Fig. 3C) the pin inflorescence seen in *pin1* mutants (Fig. 3B), which suggested that it was involved the regulation of PIN proteins (Bennett et al., 1995, Okada et al., 1991). Recent work by Kleine-Vehn et al. (2009) revealed that PID phosphorylation allows for basal to apical transcytosis of PIN proteins (Bennett et al., 1995). Transcytosis occurs because phosphorylated PIN proteins do not get sorted to the GNOM-Dependent pathway, when internalised into vesicles, but instead get sorted to the trans-golgi network for apical targeting (Kleine-Vehn et al., 2009). Protein

phosphatase 2A (PP2A) is able to antagonise the phosphorylation action of PID, allowing for PIN proteins to be sorted back to the basal membrane via the GNOM-Dependent pathway. The GNOM-Dependent pathway is also responsible for the sorting of PIN proteins to the lytic vacuole for destruction. These two pathways are integral in the plants ability to produce an apical or basal differential distribution of auxin (Kleine-Vehn et al., 2009, Grunewald and Friml, 2010).

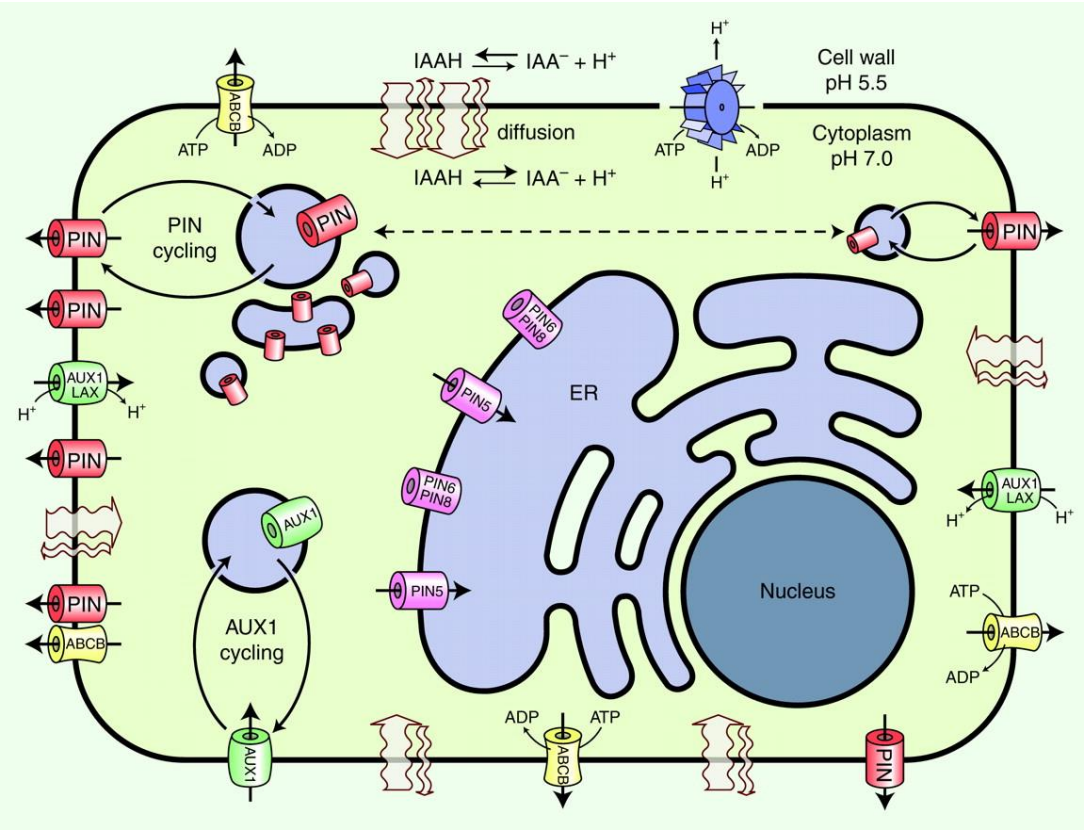


Figure 5: A schematic diagram of the auxin transport mechanisms in the plant cell. This includes proposed and known cellular localisation of transporters. Adapted from Zažímalová et al. (2009)

1.4.4 ATP-binding cassette auxin transporters

Although PIN proteins have been the main focus of research into auxin efflux transporters, there are several other families of auxin transporters. In the *Arabidopsis* genome, there is another family of auxin efflux transporters belonging to the ATP-binding cassette (ABC) superfamily of transporters (Fig. 5). The ABC superfamily is an extremely large family of transports found

throughout all extant prokaryotes and eukaryotes (Jones and George, 2004). ABC transporters have trans-membrane domains and are usually involved in the translocation of molecules across membranes, although some ABC members have roles in DNA translation and DNA repair (Jones and George, 2004, Davidson et al., 2008). The role of ABC family members in DNA repair and translation seems to be limited to prokaryotes, as eukaryotes only possess efflux ABC transporters (Paulsen and Skurray, 1993). In *Arabidopsis* there are 21 ABC 'B' type (multidrug resistance [MDR]/phosphoglycoprotein [PGP]) transporters some of which have been implicated in auxin efflux transport (Terasaka et al., 2005, Mravec et al., 2008). MDR/PGPs have been shown to be involved in the polar distribution of auxin, as well as being important for auxin dependant development. *Arabidopsis mdr1* mutant and double mutant *mdr1;pgp1* showed a decrease in apical dominance and epinastic growth (Geisler et al., 2005). When crossed to *DR5::GUS Arabidopsis mdr1* showed a decreased central elongation zone in the roots when exposed to exogenous 1-naphthylphthalamic acid (NAA), an auxin, compared to wild-type plants treated in the same way (Geisler et al., 2005). The *Arabidopsis* double mutant *mdr1;pgp1* also showed a decreased in lateral root formation and hypersensitive root gravitropic and phototropic responses, further demonstrating their role in polar auxin transport (Geisler et al., 2005). MDR/PGP transporters have also been found to have similar roles in monocots (Yasuno et al., 2009). Another *Arabidopsis* MDR/PGP transporter, *PGP4*, showed a more complex role in auxin regulation (Terasaka et al., 2005). When expressed in *Schizosaccharomyces pombe* under low auxin conditions, *PGP4* acted as an auxin influx pump. Conversely, under high auxin conditions *PGP4* reversed its role and acted as an auxin efflux pump (Terasaka et al., 2005). This dual role of *PGP4* has been confirmed by functional analysis in root hair elongation in *Arabidopsis* (Terasaka et al., 2005). While mutants in the MDR/PGP family of transporters show less dramatic phenotypes than the PIN family, they play a role in the highly sophisticated processes of auxin homeostasis and auxin dependant development.

1.4.5 Auxin influx transporters

Whilst there has been a lot of research focused on the role of efflux transporters for the polar distribution of auxin, auxin influx transporters have an equally important role in auxin distribution. The AUXIN PERMEASE/LIKE AUX (*AUX/LAX*) family was first discovered through gene-tagging approaches, which identified *AUX1* (Bennett et al., 1996). Since the completion of the *Arabidopsis* genome sequencing project, three more *AUX/LAX* (*LAX1, LAX2, LAX3*) family members have been identified. The *AUX/LAX* family is a subgroup of a large family of, amino acid and auxin permease (AAAP) (Fig. 5) (Young et al., 1999). Members of the AAAP family are mainly H^+ symporters and the *AUX/LAX* family are predicted to use this mechanism of influx (Bennett et al., 1996, Swarup et al., 2001). AUX1 was confirmed as an auxin influx transporter using *Xenopus* oocytes. Transcripts of *AUX1* tagged with *Yellow Florescent Protein* (YFP) were injected to *Xenopus* oocytes. When the pH surrounding the oocytes was changed so that most of the IAA was in a protonated form (pH 6.4) and therefore free diffusion was highly inhibited, the oocytes expressing *AUX1-YFP* showed a significant increase in IAA uptake compared to control oocytes (Bennett et al., 1996, Swarup et al., 2001, Yang et al., 2006).

Experimental data on the AUX/LAX family in *Arabidopsis* has shown that it is involved in auxin transport in various auxin dependant developmental processes (Vascular development: protophloem, Root development: columella, lateral root cap and expanding epidermal cells at the root apex, Embryo development: apical hook development and root cell organisation) (Bennett et al., 1996, Swarup et al., 2001, Ugartechea-Chirino et al., 2010, Vandenbussche et al., 2010). Similarly to *AUX1*, *LAX3* has been implicated in lateral root development (Swarup et al., 2008). *AUX1* is present throughout the formation and development of the lateral root until emergence, whereas *LAX3* is only expressed after lateral root emergence (Marchant et al., 2002, Swarup et al., 2008). After lateral root emergence, LAX3 is expressed in the elongating and developing root steele cells (Swarup et al., 2008). Analysis of *lax2* mutants has revealed that *LAX2* has a role in the

vascular pattering of cotyledons (Péret et al., 2012). Two alleles of *lax2* showed a significant increase in the number of vascular breaks in cotyledons, but no change in vascular breaks in leaves when compared to wild type (Péret et al., 2012). Although the *AUX/LAX* family is closely linked with the *PIN* family in developmental processes, the subcellular trafficking of AUX/LAX proteins is regulated by distinct mechanisms (Reinhardt et al., 2003, Swarup et al., 2008, Dharmasiri et al., 2006, Kleine-Vehn et al., 2006). The PIN family is generally thought to be localised through phosphorylation (discussed full in a later section), whereas the AUX/LAX subcellular localisation is thought to be controlled by the chaperone protein AUXIN RESISTANT 4 (AXR4) (Dharmasiri et al., 2006, Kleine-Vehn et al., 2009). This is further supported by experimental evidence using subcellular trafficking inhibitors, in which PIN and AUX/LAX proteins showed different sensitivities to the inhibitors (Kleine-Vehn et al., 2006).

1.4.6 Control of auxin distribution and auxin sensing

The pin-formed inflorescence phenotype has appeared in *Arabidopsis* mutants that have defects in the function or regulation of PIN1 transporters, which suggests that mutants with pin formed inflorescences play a role in the regulation of PIN1 activity (Fig. 3). The other genes that show similar pin-shape inflorescence phenotypes were *PID* (Fig. 3C) and *MONOPTEROS* (*MP*) (Fig. 3D) (Bennett et al., 1995, Przemeck et al., 1996). PID, like PIN1, is thought to be involved in auxin transport, as well as auxin signalling (Geldner et al., 2001, Kleine-Vehn et al., 2009). The most recent evidence on the PIN/PID interaction is that PID is able to control phosphorylation of PIN which in turn leads to PIN transcytosis (Kleine-Vehn et al., 2009). This process is critical for PIN proteins to function as polar auxin transporters. MP is a member of the *AUXIN RESPONSE FACTOR* (*ARF*) transcription factor family. *ARFs* bind to conserved regions of DNA known as auxin response elements (Przemeck et al., 1996, Ulmasov et al., 1995). Auxin response elements are short (25-35bp) regions and an auxin responsive promoter will contain several auxin response elements

(Ulmasov et al., 1995). In the absence of auxin, ARFs are not able to bind to auxin response elements because they form heterodimers with AUX/IAA proteins (Kim, 1997). AUX/IAA proteins are nuclear proteins with four conserved domains (I, II, III and IV) and are active repressors of auxin responsive promoters (Kim, 1997). The presence of auxin causes the AUX/IAA proteins to be targeted for degradation, allowing the ARFs to be free to bind to auxin response elements (Vanneste and Friml, 2009, Kim, 1997). Auxin signalling draws many similarities with the signalling response in *Drosophila* to the morphogen, RA. The main parallel between auxin and RA, as developmental regulators, is the ability of auxin and RA to change transcriptional regulation. In the presence of auxin, ARFs bind to auxin response elements, which is homologous to how RAR/RXR bind to RAREs in the presence of RA (Przemeck et al., 1996, Ulmasov, 1997, Ulmasov et al., 1995).

The degradation of AUX/IAA occurs in a targeted manner; when auxin enters a cell the bond between AUX/IAA and ARFs is broken and ARFs are free to bind to auxin response elements (Dharmasiri et al., 2005). Auxin is then able to act as 'glue' between the II conserved domain and an auxin response F-box protein, TRANSPORT INHIBITOR RESPONSE 1 (TIR1) (Dharmasiri et al., 2005). TIR1 is part of the Skp1-Cullin-F-box protein (SCFTIR1) ubiquitin ligase complex, which targets AUX/IAA for degradation in the proteasome (Gray et al., 2001). Mutations in the II domain of AUX/IAA increase the stability of the ARF AUX/IAA complex, leading to decreased auxin sensitivity (Gray et al., 2001). Recent research has shown added layers of complexity to what otherwise appears as a straight forward system of auxin sensing. Crystallography revealed another co-factor necessary for the function of TIR1, called inositol (1,2,3,4,5,6) hexakisphosphate (InsP6) (Calderon Villalobos et al., 2012). Mutation of the InsP6 binding site in TIR1 disrupted the auxin TIR1-Aux/IAA interaction and downstream regulation. The full role of InsP6 in auxin dependent-TIR1 signalling is unknown.

Another layer of complexity in the auxin TIR1-Aux/IAA signalling pathway is the differences in auxin binding affinities by the different components of the pathway. Experimental evidence has shown that the auxin response F-box family (AFB), which includes TIR1, have different affinities for auxin (Calderon Villalobos et al., 2012). In turn, the auxin-AFB binding domains of the Aux/IAA proteins are not conserved which allows for a difference in AFB – Aux/IAA interactions (Calderon Villalobos et al., 2012). Taken together this builds up a potential pathway for a concentration dependent auxin responses across auxin gradients.

It has been suggested that auxin response F-box family (AFB) may be a candidate as an auxin sensor for plants cells (Parry and Estelle, 2006). However, this is unlikely as AFB proteins are not targeted to the plasma membrane or cytoplasm where sensing is expected to occur (Tao et al., 2005). Also since the *Arabidopsis* quadruple mutant *tir1*;*afb*1;*afb2*;*afb3* is still viable and defects are only seen once the three members of the AFB family are mutated, it is expected that an auxin sensor would have a more severe phenotype (Dharmasiri et al., 2005).

Opposed to AFB proteins, AUXIN-BINDING PROTEINS (ABPs) have been suggested as another candidate for cellular auxin sensors. ABP1 is a glycoprotein and it has been shown to bind auxin at physiological concentrations (Shimomura, 2006, Kramer, 2009). ABP1 is found in the lumen of the ER and occasionally in golgi and extracellular spaces (Shimomura et al., 1993, Kramer, 2009). Interestingly, ABP1 cannot bind auxin at the neutral pH of the ER, but can bind auxin in the acidic pH of golgi and apoplast, suggesting it may have a role in the vesicle trafficking of PIN proteins (Tian et al., 1995). Mutants in *abp1* are embryo lethal, suggesting its critical role in plant development (Kramer, 2009). Recent studies has shown that ABP1 is able to regulate the endocytosis of the PIN family through regulation of Rho-GTPases and that ABP1 is able to regulate auxin response without *de novo* gene transcription (Chen et al., 2012).

1.4.7 Auxin biosynthesis and flower initiation

The phenotypes of *pin1*, *pid* and *mp* suggest that auxin plays a role in floral primordium development. Auxin's role in floral primordium development was demonstrated more directly by studies on the auxin biosynthesis genes. Studies on the *YUCCA* (*YUC*) family of flavin monooxygenases involved in auxin biosynthesis have allowed for scientist to investigate floral development with the loss of auxin biosynthesis (Zhao et al., 2001, Cheng et al., 2006, Cheng and Zhao, 2007, Zhao, 2010). Zhao et al. (2001) first discovered *YUC1* was an auxin overproduction mutants. Cheng et al. (2006) were able to determine that the over-expression of *YUC1* led to the increase of auxin through increased production in a rate limiting step of biosynthesis. However, Cheng et al. noticed that the loss of *yuc1* did not cause any phenotypic changes Genomic searches revealed that there are 11 members of the *YUC* family in *Arabidopsis*. The creation of double, triple and quadruple mutants of *yuc* genes led to pleiotropic phenotypes in the plants, along with the quadruple *yuc1*;*yuc2*; *yuc4*;*yuc6* mutant having pin-like inflorescences (Cheng et al., 2006). The introduction of a bacterial auxin biosynthesis gene *iaaM* was able to rescue floral defects in some of the double and triple mutants. This further confirmed that lack of tissue specific auxin was causing the phenotypes seen in the pin-shaped inflorescences (Cheng et al., 2006). This established unambiguous evidence that auxin is necessary for the correct formation of floral primordia.

1.4.8 Floral organogenesis and pattern formation

Auxin not only plays a role in the initiation of floral meristem but it also is essential in pattern formation and floral organ identity. In *Arabidopsis* many auxin transport/response/biosynthesis genes were mistakenly identified as floral identity and development genes (Sessions and Zambryski, 1995). One such gene is *ETTIN*, which is another *ARF* (ARF3) transcription factor

(Sessions and Zambryski, 1995). The *ettin* mutants affect all four floral organs in *Arabidopsis*, with the strongest defect being in the gynoecium patterning (Sessions and Zambryski, 1995). The pleiotropic effects of *ettin* on the floral meristem, lateral organ initiation and organ boundaries suggest auxin's strongly influential role in all of these processes (Sessions and Zambryski, 1995, Cheng and Zhao, 2007). Both *pin1* and *pid* occasionally give rise to flowers. These flowers always have significant floral defects and are usually sterile. In *pin1*, flowers normally have no stamen and have aberrant shaped (wide and fused) petals, also organ number is variable and most often reduced for stamen and sepals but can be increased for petals (Okada et al., 1991). *pid* flowers share a lot of the same properties as *pin1* flowers, with both petal shape and organ number being affected (Cheng and Zhao, 2007, Vanneste and Friml, 2009). Petals in *pid* plants are usually larger than their wild-type counterparts and more numerous (up to 11 in *pid* compared to four in wild-type flowers), as well as being fused and sometimes heart shaped or tubular (Bennett et al., 1995). In the experiments by Cheng et al. (2006) on *yu*c mutants they created a vast amount of floral mutants. These auxin biosynthesis mutants, along with the auxin transport mutants suggest that either high auxin localisation in certain cells of the floral meristem or an auxin gradient in the meristem may be responsible for induction of genes leading to correct floral patterning and organogenesis (Vanneste and Friml, 2009). The flower phenotypes of auxin transport and biosynthesis mutants do not show any "homeotic" changes of floral organs, instead all of the phenotypes are changes in organ number or shape.

1.4.9 Auxin maxima and minima

The idea of hormone gradients driving expression patterning of target genes is not a new concept in biology. It has been a well-defined concept in animal developmental biology where hormone gradients can drive cell migration and a set of gene expressions, thus tissue morphogenesis (Anderson et al., 2003, Bernal et al., 2003, Roberts et al., 2006, Zoeller, 2010, Driever and

Nüsslein-Volhard, 1988b, Driever and Nüsslein-Volhard, 1988a). Whilst there are many phytohormones, auxin is the most well-known and has been shown to be involved in many developmental processes (Okada et al., 1991, Reinhardt et al., 2000, Friml et al., 2002, Cheng and Zhao, 2007, Koenig et al., 2009, Sorefan et al., 2009). It was, however, only a few decade ago that it was discovered that auxin maxima and minima (auxin depletion in cells) can control developmental processes in plants (Friml et al., 2003). two of the most well established cases of auxin minima and maxima are root growth and gravitropism (Tanaka et al., 2006, Ottenschläger et al., 2003, Morita, 2010). Using reporter genes such as GUS and GFP under an artificial auxin response promoter called DR5 (based on a soy bean promoter called GH3), it is possible to visualize the auxin gradients in different plant tissues at a cellular or tissue level (Ulmasov, 1997, Ulmasov et al., 1997b). Because the DR5 promoter consists of only auxin response elements and lacks other promoter response elements, it can effectively visualize auxin distribution in plant tissues. In addition, the expression levels of the reporter gene under DR5 closely correlate to the auxin levels seen from direct measurements from *Arabidopsis* (Ulmasov et al., 1997b). Using *DR5::GUS* it has been found that there is an auxin maxima in the quiescent centre and columella initial cells. In the mature columella cells and other differentiated root cells, the auxin maxima are dispersed (Ottenschläger et al., 2003). In root gravitropism, auxin transport is crucial for the roots to respond to changes in gravity. When a plant root is turned horizontal to the plane of gravity, organelles containing starch (statoliths) in the columella cells fall to be as close as possible to the pull of gravity (Swarup et al., 2005, Morita, 2010). The change in position of the statoliths causes the translocation of PIN3 to their position within minutes. Auxin is then transported from the maxima in the columella cells to form a new maxima in the lateral root cells, which causes uneven cell elongation until the root is growing vertically to the force of gravity (Swarup et al., 2005, Morita, 2010).

1.4.10 Auxin a morphogen?

Auxin has been suggested to work as a morphogen in *Arabidopsis* for the emergence of lateral roots and in embryogenesis (Friml et al., 2002, Friml et al., 2003, Pagnussat et al., 2009). During the emergence of lateral roots, using the DR5 promoter linked to visual markers, auxin accumulation can be seen at the point a new root is emerging (Friml et al., 2002). Loss of this accumulation, observed through the generation of mutants in polar auxin transport, regulation or auxin biosynthesis, can lead to a decrease or total loss in lateral root formation (Friml et al., 2002). The application of exogenous auxin is also able to induce the formation of lateral roots (Ottenschläger et al., 2003). Whilst this was originally thought to indicate that auxin was acting as a morphogen, it is now considered to show that auxin works as a morphogenetic trigger. A morphogenetic trigger specifies the site of a new organ formation through local increases in its accumulation which causes the cells to gain a new developmental fate (Benková et al., 2009).

In contrast to lateral root emergence, auxin's role in the RAM is much closer to that of a true morphogen. In the RAM, loss of polar auxin transport through inhibition with NPA leads to strong defects in tissue polarity and patterning. Exogenous application of auxin to perturb auxin distribution in the columella cells (RAM cells) causes the re-establishment of correct patterning (Sabatini et al., 1999, Friml et al., 2002). The cells surrounding the new auxin maxima acquired new developmental fates, based on their distance from the auxin maxima.

During embryogenesis, like root development, auxin plays a crucial role in the formation of tissue polarity and patterning, as well as growth direction. Work by Friml et al., (2003) used *pin* mutants in order to investigate the role of polar auxin efflux in establishing apical-basal polarity in developing embryos (Friml et al., 2003). They uncovered that the apical polarity of embryos is caused by PIN7 mediated transport of auxin to the proembryo. Once apical-basal polarity is established the embryo continues to develop until it reaches the globular stage. During the globular stage of development the embryo initiates auxin biosynthesis and auxin transport is

reversed by PIN1 and PIN4 towards the basal end of the embryo (Friml et al., 2003, Pagnussat et al., 2009). Auxin is transported to the hypophysis in order for the RAM to be formed. The hypophysis is the most apical cell of the suspencer, indicating that auxin is acting in a highly specific manner to control cell fates (Pagnussat et al., 2009). Further research has shown that the biosynthesis of auxin in the globular embryo is correlated with the perceived auxin gradient (Pagnussat et al., 2009, Friml et al., 2003).

However, a recent study refutes the fact that auxin is acting in a gradient during gametophytic development. Gametophytic development like embryogenesis, was thought to be controlled by auxin efflux and biosynthesis (Pagnussat et al., 2009). Using a combination of both theoretical and experimental approaches, Lituiev at al. (2013) suggest that auxin acts indirectly to influence auxin production and cell fate. Mathematical modelling could not create an auxin gradient using the previously proposed mechanisms (such as auxin efflux and biosynthesis) to maintain it (Lituiev et al., 2013). When a theoretical gradient was created, it was shown to be shallow and therefore not robust enough for cell determination and patterning. Higher resolution microscopy of the visual markers under the DR5 promoter showed that auxin activity could not be detected in the developing gametophyte where it previously had been (Lituiev et al., 2013). Together, this new evidence reopens the debate on whether auxin truly is a plant morphogen.

Auxin is a precisely transported and biosynthesised phytohormone, that is able to controls almost every aspect of plant development and growth. Evidence from the literature suggests that auxin is able to determine cell fate and organ formation through gradients. Whilst it has been hypothesised that auxin is the first plant 'morphogen' which is necessary to establish developing lateral organs, none of the auxin phenotypes support this hypothesis adequately. All of the auxin phenotypes have shown the formation of ectopic and abnormal organs, instead of showing homeotic changes from one organ to another (both perfectly normal morphology). The Asteraceae flower head offers a unique system in order to test the hypothesis that auxin is a

morphogen. Asteraceae flower heads contain three distinct lateral organs precisely patterned in order to form a single function unit. Perturbations in the native auxin balance of the developing flower head could cause homeotic conversions or phenotypes usually associated with auxin distribution (ectopic or abnormal organs).

1.5 Flower development

Over the last three decades there have been great advances in our understanding of the development and pattern formation of flowers in model species such as *Arabidopsis thaliana* and *Antirrhinum majus*. The molecular genetics of tissue patterning (ABC patterning) and floral symmetry are now well-understood in model species, but very little is known about the molecular genetics of Asteraceae flower heads (Alvarez-Buylla et al., 2010, Carpenter and Coen, 1990). Floral symmetry gene *CYCLOIDEA* (*CYC*), well-known for its roles in *Antirrhinum*, has been shown to be important in the formation of ray florets in the Asteraceae flower head (Kim et al., 2008). *LEAFY* (*LFY*), a well-known plant-specific DNA-binding transcription factor, has been demonstrated to be important in the regulation of ABC floral identity genes throughout flower development (Luo et al., 1996, Weigel et al., 1992). However, nothing is known for its role in flower head development in Asteraceae.

1.5.1 *CYCLOIDEA*

The variety of shapes and patterns seen throughout the Asteraceae family flower heads raises the question: What role do the genetic controls of flower symmetry in model species play in shaping the Asteraceae flower head? One of the most well-known genes in floral symmetry is *CYCLOIDEA* (*CYC*). *CYC* was first isolated from *Antirrhinum majus* (snapdragon) by Luo et al. (1996). A wild-type snapdragon flower is dorsoventrally bilateral. This allows for flowers from the same whorl to have different organ shapes based upon their position in the dorsoventral axis (Luo et al., 1996,

Luo et al., 1999). The mutation of *CYC* led to a dramatic change in phenotype in relation to floral symmetry. Instead of bilateral flowers seen in wild types, semipeloric and peloric flowers were generated in the *cyc* mutant plants (although the peloric mutant was only seen in *cyc*;*dichotoma* (*dich*) double mutants) (Luo et al., 1996). Peloric mutants lose their dorsoventral flower asymmetry completely, while semipeloric mutants have some residual flower asymmetry. Luo et al. (1996) also observed effects of *cyc* on primordium initiation and organ morphology. *CYC* is expressed in the dorsal region of *Antirrhinum* flowers, which coincides with its phenotypes, where it was seen to stunt growth and to reduce the number of primordium. The phenotypes seen in *dich* single mutants are less extreme than the ones seen in the *cyc* mutants but show enough similarity (both affect dorsoventral asymmetry) to suggest that *dich* and *cyc* are partially redundant (Luo et al., 1996).

Since the isolation of *CYC* and *DICH*, there has been significant research into both *CYC-like* genes and their interactions that play critical roles in floral symmetry and organogenesis. *CYC* is a member of the class II TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) transcription factor clade (Martín-Trillo and Cubas, 2010). *TCP* transcription factors are exclusive to plants and are an evolutionary ancient set of proteins that contain the *TCP* binding domain. Whilst they are not found in *Chlamydomonas* (a unicellular alga), they are found in *Chara* (multicellular algae), ferns and mosses (Floyd and Bowman, 2007, Navaud et al., 2007). *TCP* transcription factors bind to DNA using a basic helix-loop-helix (bHLH) motif that has also been shown to have protein-protein interactions (Cubas et al., 1999). The bHLH motif was shown to be necessary (but not sufficient) for DNA binding through the use of yeast one-hybrid studies (Kobayashi et al., 1999, Kosugi and Ohashi, 2002). TCP proteins need to form either homo- or hetrodimers to be able to bind to their targeted DNA sequences (Kosugi and Ohashi, 2002). Whether or not TCP transcription factors are able to activate transcription by themselves is still unknown. There is limited evidence of TCP transcription factors that may be able to self-regulate (Martín-Trillo and Cubas, 2010). However, it

is generally reported in the literatures that TCP transcription factors regulate transcription via protein-protein interactions (Kosugi and Ohashi, 2002, Martín-Trillo and Cubas, 2010).

1.5.2 *CYCLOIDEA-Like* genes in model species

The role of *CYC-like* genes in other model species is highly conserved to the role *CYC* that plays in *Antirrhinum*. In the model legumes *Lotus japonicus and Pisum sativum, CYC*'s role is to establish bilateral symmetry (zygomorphic flowers) (Feng et al., 2006). In contrast to *CYC*, the *Lotus japonicas* ortholog, *LjCYC2* expression is detected at a through the development of the meristem and not just throughout petal formation. Mutation in *ljcyc2* indicates that new functions in inflorescent development have evolved since the divergence with *CYC* (Feng et al., 2006). In *Cadia*, another legume with unusual actinomorphic flowers, *CYC* has an increased expression domain which has led to innovation of radial symmetrical flowers by dorsalisation of the flowers (Citerne et al., 2003, Citerne et al., 2006). Innovation of zygomorphic to actinomorphic flowers through an increased CYC expression domain has also been suggested for Elatinaceae, where expression patterning of *CYC-like* homologues suggest that an increased expression domain of *CYC-like* genes has caused the flower to become dorsalised (Zhang et al., 2010). In the monocot species *Oryza sativa, CYC-Like* genes control the bilateral symmetry along the lemma-palea axis by determining palea identity and development (Yuan et al., 2009).

CYC expression in *Arabidopsis* (actinomorphic flowers) is present at early stages of flower development, however, *CYC* expression is then rapidly lost (Cubas et al., 2001). Over-expression of *CYC* in *Arabidopsis* has been shown to increased petal size, suggesting *CYC* still contributes to flower development through control of cell proliferation but has lost the ability to control symmetry (Cubas, 2004, Cubas et al., 2001). Whilst much is known about the floral symmetry genes and their roles in snapdragon and other model species, there is significantly less known

about their roles in the Asteraceae family. The way that these transcription factors may work in the flower head of the Asteraceae family is extremely interesting, because the flower head is far more complex than in another model species studied. An Asteraceae flower head can contain several flower types; disc florets that are radially symmetrical and ray florets that are bilaterally symmetrical. There are also chiameric flower types that can appear as an intermediate between the ray and disc florets, with bilaterally symmetrical properties.

1.5.3 Pseudanthia and *CYCLOIDEA-Like*

Research into the role of *CYC-Like* genes in non-Asteraceae pseudanthium is limited, but recently there has been an investigation into the role of *CYC-Like* genes in the Myrtaceae family. The pseudanthium of the Myrtaceae, *Actinodium cunninghamii* (*A. cunninghamii*), otherwise known as the swamp daisy, looks superficially like an Asteraceae flower head. The Asteraceae flower head is considered to be an "open II-type" inflorescence meristem, in which the merstematic cells are consumed throughout development allowing for no further growth (Bull-Herenu and Classen-Bockhoff, 2011). In contrast to the Asteraceae flower head, the pseudoanthium of *A. cunninghamii* is an "open I-type" inflorescence, which allows for proliferation after reproductive organ development (Bull-Herenu and Classen-Bockhoff, 2011). The ray florets of *A. cunninghamii* are not ray florets at all; instead they are branched short shoots which mimic petals/ray florets of other flowers. Expressional analysis of *CYC1-Like* and *CYC2-Like* genes in the developing pseudoanthium of *A. cunninghamii*, revealed conserved functions of *CYC-LIKE* genes in development. The *Arabidopsis* member of the *CYC1* clade is *BRANCHED1* (*BRC1*), which has been shown to have a role in controlling branching patterning in *Arabidopsis* inflorescences (Classen-Bockhoff et al., 2013). In *A. cunninghamii* the two paralogs of *BRC1* are highly expressed in the branched short shoots, and as growth returns to the shoots the expression disappears similar to how *BRC1* expression is high at the inflorescence nodes and reduced when growth occurs

(Classen-Bockhoff et al., 2013). One of the functions of *CYC2-Like* genes in the Asteraceae, is to act as an identify gene for the formation of ray florets (*SvRAY2*), whilst another function is to promote the outgrowth of the ray floret petals (*SvRAY1*) (Kim et al., 2008). Although the pseudanthium of *A. cunninghamii* does not have ray florets, a *CYC2-Like* paralog is suggested to help with the extension of the showy bracteoles (Classen-Bockhoff et al., 2013).

1.5.4 *RADIALIS*

The *RADIALIS* (*RAD*) locus was first identified by Luo et al. (1996) but was not fully characterised until 2005 (Corley et al., 2005). *RAD* was once thought to be on the same locus as *CYC* due to the fact they have very similar phenotypes. However, they were later found to be two distinct loci (Carpenter and Coen, 1990, Corley et al., 2005). Like *CYC*, mutation of *RAD* causes defective phenotypes in the dorsoventral symmetry of floral organs. *rad* mutants have ventralised petals with some of the flowers almost being peloric (completely radially symmetric) (Corley et al., 2005). The majority of the *rad* phenotypes are less extreme with only part ventralisation of the dorsal petals (Corley et al., 2005). Also, whilst *cyc* had an effect on organ number, *rad* did not. The dorsal stamen of *rad* plants were longer than the wild type but not functional, this is in contrast to *cyc* which affected stamen length but the stamen were still fully functional (Corley et al., 2005). *RAD* is a member of the Myeloblastosis (MYB) transcription factor family. MYB transcription factors are one of the largest families of transcription factors in plants and they are known to play roles in almost all plant functions, from development to hormone response to disease and light responses (Corley et al., 2005, Jabbour et al., 2010, Martín-Trillo and Cubas, 2010). The unifying feature of all MYB transcription factors is that they contain at least one N-terminus DNA binding repeat (MYB domain) (Ogata et al., 1994, Stevenson et al., 2006). Whilst these repeats are known to be highly variable, they are all around 50 amino acids long and have three regularly spaced tryptophan residues (Ogata et al., 1994, Yanagisawa, 1998).

RAD expression was studied by RNA *in situ* hybridisation using DIG-labelled probes. In wild-type Antirrhinum plants the expression of *RAD* was only observed in the dorsal part of the flowers. This expression is consistent with the expression of *CYC* and *DICH* (Corley et al., 2005). The *rad;cyc* double mutants generated peloric flowers, whereas the single mutants (*rad* or *cyc*) alleles could only generate semipeloric phenotypes. After further research into the interaction between *CYC/DICH* and *RAD* it has been found that *CYC*/*DICH* activates *RAD* (Corley et al., 2005, Costa et al., 2005, Baxter et al., 2007, Preston and Hileman, 2009). It is these three genes that are responsible for setting up the dorsoventral axis of asymmetry in snapdragon. *RAD* has a further role in flower shape and symmetry. *RAD* is able to interact with another MYB transcription factor *DIVARICATA* (*DIV*) (Galego and Almeida, 2002). A recent study has shown that *RAD* directly antagonises *DIV* through direct competition for DNA binding sites (Raimundo et al., 2013). The competition for DNA binding sites is facilitated by two other MYB transcription factors, called *DIV*-and-*RAD*-*interacting-factors* (*DRIF1* and *DRIF2*). DRIF1 and DIV form a heterodimer that is required for the correct transcriptional activity of *DIV* (Raimundo et al., 2013). However, in the presence of RAD the bond between DRIFs and DIV is disrupted and the DRIFs bind to RAD instead (Raimundo et al., 2013). Once bound to RAD, the DRIFs are transported out of the nucleus and into cytoplasm, further inhibiting DIVs activity (Raimundo et al., 2013). It is thought that *RAD* arose from a duplication of *DIV* hence why it is able to bind to the same DNA sites as *DIV* (Howarth and Donoghue, 2009).

1.5.5 *DIVARICATA*

DIV, like *RAD,* is a MYB transcription factor but it has two MYB DNA binding repeats instead of one (Galego and Almeida, 2002, Stevenson et al., 2006). Unlike *CYC/DICH* and *RAD*, *DIV* is responsible for ventral identity in the *Antirrhinum* flower, not dorsal identity. The activity of *DIV* gives a clear distinction between the ventral and lateral petals by elongation (Galego and Almeida, 2002). The

DIV mRNA is expressed throughout the entire region of the flower but it is unable to manifest its effect in the lateral petals due to the antagonistic action of *RAD* (Corley et al., 2005, Galego and Almeida, 2002). It was once thought that the closely related *DIV1* and *DIV* may be redundant but both *div1* single and *div1/div* double mutant studies show no altered phenotype or enhancement of the *div* phenotype respectively (Galego and Almeida, 2002). The lack of phenotypic change in both the *div1* and *div1/div* mutants strongly indicates that *DIV1* has no redundancy with *DIV* (Galego and Almeida, 2002). The characterisation of *CYC*, *DICH*, *RAD*, and *DIV* has resulted in a fairly well defined model of floral asymmetry in *Antirrhinum*, that is best summarised in Figure 6.

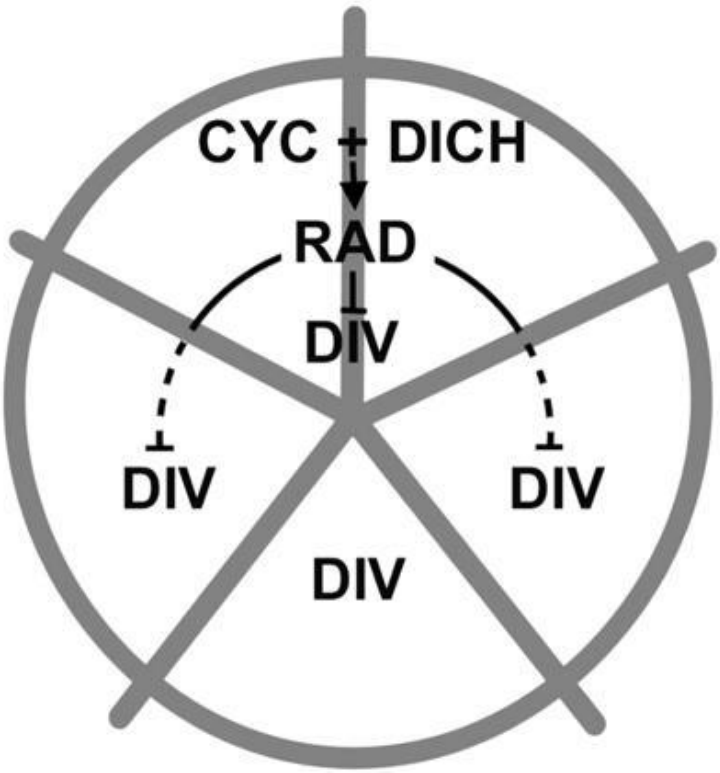


Figure 6: A model of the interactions between the transcription factors that control the dorsoventral axis of floral asymmetry in *Antirrhinum majus*. In brief *CYC* and *DICH* are induced early on in the dorsal region of flower primordia. Then through a direct interaction *RAD* is induced in the dorsal region of the floral primordia by *CYC* and *DICH*. *DIV* induced by an unknown mechanism is expressed in all petals but is only active in the ventral petals due to antagonism by *RAD* through competition for DNA binding sites. Diagram from Corley *et al*. 2005.

1.5.6 *TCP* genes and the flower head

The most prevalent research into the symmetry gene's effects on inflorescent development in Asteraceae has been done on the TCP transcription factors. Coen et al. (1995) made a hypothesis formulated upon the *cyc* mutation seen in *Antirrhinum*, which was that *CYC-like* genes would control the development of bilaterally symmetrical flowers (ray florets) in Asteraceae flower heads (Coen et al., 1995b). Three papers, by Kim et al. (2008), Broholm et al. (2008) and Chapman et al. (2012) have all showed similar effects of *CYC-like* genes on ray florets in three different Asteraceae flower heads.

The work by Kim et al. (2008) is focused on how the key morphological and ecological trait, ray florets, is controlled by two *CYC-like* genes found on the *RAY* locus in *Senecio vulgaris* (*S. vulgaris*, Common groundsel). In *S. vulgaris* there are both radiate and discoid flower head forms. The native form of British *S. vulgaris* is discoid, but soon after the introduction of the Sicilian *Senecio squalidus* (with a radiate form) into Britain a radiate form of *S. vulgaris* appeared (Kim et al., 2008). This conversion from discoid to radiate was thought to have been caused by the transfer of the *RAY* locus from *Senecio squalidus* to *S. vulgaris*. Kim et al. (2008) identified two genes for the RAY locus, termed RAY1 and RAY2, which turned out to be members of the TCP family and *CYC2-Like* genes. *In situ* hybridisations revealed that both *RAY* genes were expressed at the site of ray floret formation in young flower heads, but with the discoid form having a stronger expression of *RAY1* than the radiate form (Kim et al., 2008). Transgenic plants over-expressing *RAY1* further confirmed that the increased expression of *RAY1* is able to alter the dorsal petal length of ray florets in radiate *S. vulgaris*. The phenotypes seen from the transgenic plants ranged from shortened ray florets to a complete loss of floral symmetry in the ray florets and thus conversion back to the discoid form. In contrast to *RAY1*, when *RAY2* was over-expressed in the radiate form

it resulted in a phenotype of tubular ray florets and occasionally extra ray florets (Kim et al., 2008).

The results obtained by Kim et al. (2008) present a distinct role for *CYC-like* genes in the development of the Asteraceae flower head. The *RAY* locus has been instrumental in the formation of radiate flower heads in *Senecio* and most likely in all Asteraceae, with *RAY2* able to alter ray floret morphologies. This conclusion is supported by work on *Gerbera hybrida* (*G. hybrida*, Asteraceae) *CYC-like* gene, *GhCYC2* (Broholm et al., 2008). Phylogenetic analysis from Kim et al. (2008) showed that *GhCYC2* and *RAY2* are orthologous to each other. Results from *in situ* hybridisations on *GhCYC2* showed that it is also localised to developing ray florets. Over-expression of *GhCYC2* showed different phenotypes dependant on the floret type. In ray and hybrid florets over-expression resulted in a decrease in size and retardation of growth, which is consistent with the results from Kim et al. (Broholm et al., 2008). Over-expression of *GhCYC2,* however*,* causes an increase of growth in the disc florets. This phenotype is not reported by Kim et al. (2008) in their over-expression line but is suggested to be caused by the disc florets of *G. hybrida* not being completely radially symmetrical (Broholm et al., 2008). By still having a hint of bilateral symmetry, the *G. hybrida* disc florets may have still been controlled by *GhCYC2*, whereas *S. vulgaris* showed complete radial symmetry thus *RAY2* had no effect (Kim et al., 2008).

Many of the different tribes of Asteraceae have ornamental varieties with vast differences in their flower head forms. The data above suggests that any differences in ray floret number, size and length might be due to differences in the expression or function of *CYC-like* genes (Kim et al., 2008, Luo et al., 1999, Luo et al., 1996, Broholm et al., 2008). In an ornamental species (like *Zinnia*) with an increased number of ray florets one would expect there to be an increased domain of *RAY2* (*GhCYC2*) expression. Also ornamental varieties of *Zinnia* that have tubular ray florets may have very high localised *RAY2* expression, as over-expression of *RAY2* in *S. vulgaris* causes a phenotype of tubular ray florets (Kim et al., 2008).

Recent work by Chapman et al. (2012) has investigated the role of *CYC-Like* genes in the formation of ray florets in *Helianthus annuus* (sunflower) cultivars. The *teddy bear* cultivar of the sunflower investigated appeared to lose the actinomorphic symmetry of the disc florets. This was found to be due to an insertion into the promoter region of a *CYC2-Like* gene (*HaCYC2c*), which caused for its expression to no longer be limited to the developing ray florets but instead was expressed across the whole developing flower head (Chapman et al., 2012). The disc florets in the *teddy bear* cultivar underwent asymmetrical petal elongation that lead to them developing bilateral symmetry. *HaCYC2c* has been mutated in another cultivar *tubular-rayed* (Chapman et al., 2012). Tubular-rayed cultivars have, as the name suggests, ray florets which have lost their bilateral symmetry and become actinomorphic. Interestingly *HaCYC2c* is a different *CYC2* paralog, that has evolved to control bilateral symmetry, than the *CYC2* paralogs in *S. vulgaris* and *G. hybrid*, suggesting that the evolution of ray florets has occured several times using paralogs from the same gene family (Chapman et al., 2012).

The Asteraceae family, being so large, has an extremely large number of natural and horticultural flower heads, which presents a unique opportunity to study the evolution of CYC-like genes and ray florets throughout the Asteraceae evolutionary history. The two most ancient tribes of Asteraceae are the Banardesieae and Mutisieae (Bayer and Starr, 1998). By investigating the ancient functions of *RAY* genes in unusual florets, such as bifurcated florets of these primitive tribes, it would be possible to see how old the *RAY* pathways are and how the ancient *RAY* pathways have evolved to shape floret morphologies in modern Asteraceae tribes (Koch, 1930). Also, a more in-depth look at the Senecio clade will clarify the means by which discoid flower heads arise. This is because the Senecio clade have had several separate evolutionary events in which the radiate form has been lost and it would be very interesting to see if the radiate forms were lost by the same or by different mechanisms (Kim et al., 2008).

Unfortunately research into MYB transcription factors (*RAD* and *DIV*) in the Asteraceae family has been limited. But by assessing their respective roles in the development of floral organs in other species it is possible to speculate about their roles in the development of the Asteraceae flower head. Before the isolation of the Senecio *RAY* genes it had been noticed through the production of genetic crosses that the *RAY* locus was semi-dominate (Comes, 1998). This was evident due to the fact that the radiate form was semi-dominant over the discoid form. The dominance of the radiate form over the discoid could be due to effects of the *CYC-like* genes on *RAD* and *DIV*. In *Antirrhinum*, loss of either *RAD* or *CYC* leads to loss of dorsoventral identity and shifts the flower towards a radially symmetric form (the same form as disc florets) (Corley et al. 2005). Over-expression of *RAY1* in the radiate form of *S. vulgaris* led to shorten or no ray florets, suggesting an expressional balance of *CYC-like* genes regulates disc versus ray floret identity in the flower head. It is possible that the loss of the ray florets in discoid *S. vulgaris* is caused by a change in expression of *CYC-like* genes, which results in a failure to upregulate *RAD* (Fig. 6). This would allow for *DIV* to act uninhibited throughout the flower head thus forming only disc florets. Recent work has shown that when a *DIV-like* gene is knocked down in the radiate *S. vulgaris* flower head, the ray florets form an elongated ventral petal (Personal communication, Minsung Kim). This suggests that *DIV-like* genes still play a role in petal development in the Asteraceae flower head but that a new function of *DIV* has arisen. With such a complex system further research is necessary to determine the possible roles of *RAD* and *DIV* in the development of the Asteraceae flower head.

1.6 *LEAFY*

Of the limited research that has been performed on Asteraceae flower heads, it has mostly focused on the formation of the ray florets. In contrast to the ray florets, phyllaries have largely been neglected even though they play a critical role in the structure of the flower head. Early research on the sunflower indicated that the phyllaries are part of the flower head patterning.

Physical manipulation of the flower head through compression or wounding had profound effects on the flower head patterning (Hernandez and Palmer, 1988, Hernandez and Green, 1993). Physical compression of the developing flower head led to phyllaries being formed in the centre of the flower head. In the wounded flower heads the original pattern reformed, phyllaries and ray florets formed where disc florets would have normally formed. The new phyllaries formed were shown to be involucral and not bracteoles, further confirming that the patterning had been 'reset' (Hernandez and Palmer, 1988). Involucral phyllaries are tough leaf-like structures that protect the developing florets and help provide physical support to the flower head. The number of layers of phyllaries can vary depending on the species. Based on the structure and morphological features of phyllaries the gene *LEAFY* (*LFY*) is a logical candidate gene as a phyllary identity gene (Tapia-Lopez et al., 2008). *LFY's* role in phyllary formation is supported by the *lfy* mutant in *Arabidopsis*. In the *Arabidopsis lfy* mutants cauline leaves are formed on inflorescence instead of flowers, which is analogus to phyllaries forming in the wounded flower heads instead of disc florets.

The hypothesis of *LFY* as a putative identity gene for phyllaries is further supported by the known roles that *LFY* plays in the maintenance and identity of the SAM, along with vegetative and floral growth (Carpenter and Coen, 1990, Moyroud et al., 2009, Weigel and Nilsson, 1995). *LFY*/*FLORICAULA* (*FLO*) were both identified in the early 1990's as floral mutants in *Arabidopsis* and *Antirrhinum* respectively. The *Antirrhinum flo* mutant had no flowers, instead the flowers were replaced by shoots (Carpenter and Coen, 1990). Whilst in the *Arabidopsis lfy* mutant the basal part of flowers was still being replaced with shoots, but phenotypic shoot/floral intermediates and floral organs were formed at the apical side (Weigel and Meyerowitz, 1993). Cloning of the full length of *LFY*/*FLO* identified that they are homologous to each other and thus they are presumed to have very similar functions (Weigel and Nilsson, 1995). *LFY* interacts with a large class of transcription factors called the *MCM1*, *AGAMOUS*, *DEFICIENS*, *SRF* (MADS)-box gene family, which is now known to contain most of the floral homeotic genes (Schwarz-Sommer et al., 1990). MADS-box is a highly conversed DNA binding domain of 50-60 amino acids that binds to

the CArG-box motif in DNA sequences (West et al., 1997). MADS-box genes are found throughout eurkaryotic evolution, but whilst animals and fungi only have a few MADS-box genes plants can have over 100 (Becker and Theissen, 2003, Gramzow et al., 2010). *Arabidopsis* LFY DNA binding domains from *APETALA1* (*AP1*) and *AGAMOUS* (*AG*) identified a potential LFY motif (pseudo-palindromic motif of CCANTGG/T) (Hamès et al., 2008). Unfortunately the validity of this motif is still highly questionable as it appears to be variable in *Arabidopsis* (in *AP3* LFY binding domain CCNNNG) (Lamb et al., 2002). Structural analysis of LFY revealed that it is made of seven alpha helices (a protein fold never seen before at the time) with three of the helices forming a helix-turn-helix (Hamès et al., 2008). Despite the protein fold of LFY being novel, the helix-turn-helix motif showed a similarity to other DNA binding proteins, such as homeodomain transcription factors (Hamès et al., 2008). Evolutionary analysis of plant MADS-box genes revealed that there are orthologous genes in the gymnosperms, suggesting that the family had already diverged in the common ancestor of angiosperms and gymnosperms (Himi et al., 2001). *NEEDLY* (the *Pinus radiata LFY* ortholog) is able to compliment the *lfy* mutant in *Arabidopsis*, suggesting that *LFY* function has remained unchanged over millions of years (Himi et al., 2001).

1.6.1 *LEAFY* and the *ABC* genes

As *lfy* produced floral phenotypes, researchers investigated into whether or not the expression of ABC floral identity genes were regulated by *LFY* (Alvarez-Buylla et al., 2010, Carpenter and Coen, 1990). *LFY* was shown to play a role in the control of flowering time by over-expression leading to an early flowering time (Pena et al., 2001). Whilst *LFY* is expressed in the vegetative growth phase, upon the start of the transition from vegetative growth to flowering there is a significant increase in the amount of *LFY* mRNA found in the SAM (Wagner et al., 2004). In *Arabidopsis* it was found that correct *LFY* expression is necessary for the expression of the A class gene, *AP1,* as well as the correct regulation of B and C class genes (Moyroud et al., 2009). In the 'ABC' model A class genes

are responsible for sepal and petal development, B class for petal and stamen development and C class genes for stamens and carpels. The regulation of *AP1* was shown to be direct by the use of an inducible LFY over-expression construct (Bowman et al., 1993, Weigel and Meyerowitz, 1993). Recent research has shown that *LATE MERISTEM IDENTITY2* (*LMI2*), regulated by *LFY* , works with *LFY* to activate *AP1* during the transition to floral meristem (Pastore et al., 2011). Using an activation domain, tagged *LFY* revealed its ability to co-regulate *AG* a class C gene (Parcy et al., 2002).

Although *LFY* can have a direct effect on target genes (such as *AP1*), *LFY* normally functions with the co-activators *UNUSUAL FLORAL ORGANS* (*UFO*) and *WUSCHEL* (*WUS*) (Samach et al., 1999, Moyroud et al., 2009). *WUS* is expressed throughout the SAM and floral meristems and acts as a meristem identity gene. It has been established that the co-expression of *LFY* and *WUS* is sufficient to induce the activation of *AG* (specifies stamens and carpels), although there is no experimental evidence that *LFY* and *AG* are able to directly dimmerise (Parcy et al., 2002). The interaction between *LFY* and *AG* homologs can be traced to before the divergence of monocot and eudicot plants, further indicating the importance of the interaction for flowering plants. *LFY*'s co-activation with *UFO* leads to the up-regulation of the B gene *APETALA3* (*AP3*) (specification of petals and stamens) (Alvarez-Buylla et al., 2010). Although there is different experimental evidence about the interactions between *LFY* and *UFO*, repression of *UFO* has been experimentally shown to cause a *lfy* loss of function phenotype, demonstrating its importance in floral meristem development (Alvarez-Buylla et al. 2010). *LFY* is not just a positive regulator of meristem and floral identity genes it can also negatively regulate them. The two most investigated examples of negative regulation by *LFY* are on *TERMINAL FLOWER1* (*TFL1*) and *EMBRYONIC FLOWER* (*EMF*) (Alvarez-Buylla et al., 2010, Chen et al., 1997, Moyroud et al., 2009). The negative regulation of *TFL1* by *LFY* is thought to work through the action of negative feedback loops, which are known for their importance in changes between two developmental states (Xi et al., 2010). The *LFY*/*TFL1* interaction is complex and consists of several loops interacting together (Fig. 7); *LFY*

positively up-regulates *AP1* and *TFL1*, in turn *AP1* up-regulates *LFY* and down-regulates *TFL1*. *TFL1*, after being up-regulated by *LFY* down-regulates both *AP1* and *LFY* (Jaeger et al., 2013).

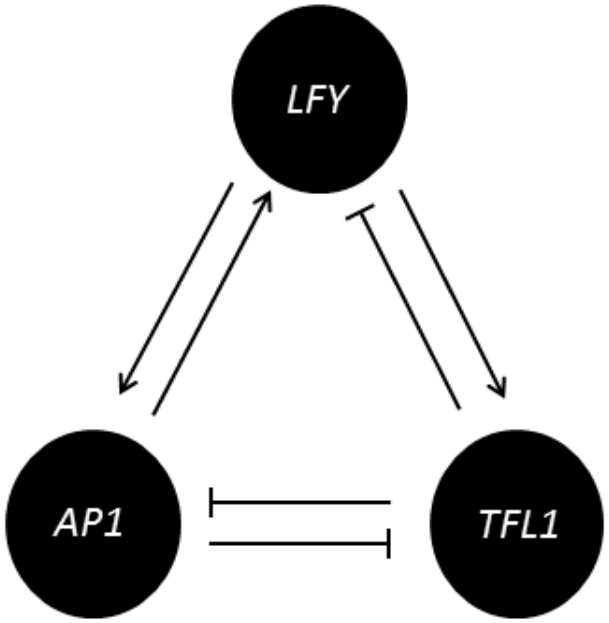


Figure 7: A schematic diagram of the feedback loops between *LFY, AP1*, and *TFL1* in the *Arabidopsis* floral meristem. Diagram adapted from Jaeger et al. 2013.

1.6.2 *LEAFY* and auxin

Until recently, there had been no direct evidence that *LFY* was regulated by auxin, instead there was just the suggestion that auxin and *LFY* may be linked due their roles in flower development (Vernoux et al., 2000). Two research articles have now shown that *LFY* and auxin regulate each other in a series of feedback loops (Li et al., 2013, Yamaguchi et al., 2013). Using live imaging it was possible to see that *LFY* expression occurs just after auxin accumulation, suggesting that *LFY* is activated by auxin (Li et al., 2013). In turn, *LFY* negatively regulates itself and the auxin

biosynthesis pathway through *YUC4*, whilst simultaneously acting as a positive feedback loop on auxin signalling (Li et al., 2013). *LFY* was also found to genetically interact with *PID* and influence auxin efflux (Li et al., 2013). Further investigation has revealed that auxin regulates *LFY* through auxin activated *MP* and is robust enough to prevent floral initiation during vegetative development (Yamaguchi et al., 2013).

1.7 *MADS-box* genes and the flower head

Literatures on the expression of MADS-box genes in the Asteraceae family suggest that they are expressed across the flower head. A microarray of *G. hybrida* using developing floral primordia showed that different MADS-box genes were expressed with specificity to individual flower types (Broholm et al., 2010). Further study into the roles of MADS-box genes in Asteraceae flowers has revealed potential roles for the *AG* homolog (Aida et al., 2008). In chrysanthemum, knockdown *AG* transgenic plants were created and produced modified ray florets. These modified ray florets had extra secondary corollas, had lost their pistils and instead had a modified pistil/corolla hybrid (Aida et al., 2008). Other work on different B class genes in *G. hybrida* showed that down regulation of *AP3* and *PISTILLATA* (*PI*) led to defects in the petal and stamen, suggesting that the ABC patterning MADS-box genes play a conserved role in floret organogenesis rather than floret identity (Broholm et al., 2010).

1.8 *TERMINAL FLOWER1*

Several other inflorescence genes have been implicated in flower development. One of these genes is *TFL1*, which was identified around the same time as *LFY*/*FLO* were implicated in floral development (Alvarez et al., 1992). TFL is a member of the phosphatidylethanolamine-binding proteins (PEBPs) that have diverse roles in animals, plants, yeast, and bacteria (Kikuchi et al., 2009). PEBPs are generally involved in signalling pathways involved in growth and differentiation,

and act in a variety of ways from protease and kinase inhibitors, stoichiometric inhibitors to Ras-signaling modulators (Hanzawa et al., 2005, Kikuchi et al., 2009). In plants, TFL generally acts as a SAM homeostasis gene maintaining indeterminate growth (Conti and Bradley, 2007, Liljegren et al., 1999). TFL also acts as a floral transition repressor (Liljegren et al., 1999). The *tfl* mutants have their SAM converted into terminal flowers, while transgenic plants over-expressing TFL have extended growth phases for all phases, which in turn leads to large plants with highly branched inflorescences (Tahery, 2009). The roles of TFL and LFY and their respective orthologues suggested that they must antagonise each other in feedback loops to control growth phases and meristem identity (Conti and Bradley, 2007, Liljegren et al., 1999).

In *Arabidopsis* there are 5 other proteins that encode PEBPs and one of these is a homolog of TFL, called *FLOWERING LOCUS T* (*FT*). Like TFL, FT is a critical regulator of flowering but it antagonises flowering TFL and is an activator of flower rather than a repressor (Tahery, 2009). In 2005 there was a striking find in the TFL/FT relationship, although the proteins are <60% identical it was revealed that the swapping of a single amino acid could change their function. Experiments by Hanzawa et al. (2005) showed that by swapping histidine for tyrosine, TFL function could be changed into FT and that this process was reversible. This amino acid swap can be achieved by altering a single nucleic acid and this allows these two proteins to be able to swap signalling pathways (Hanzawa et al., 2005). The *ft* mutant is similar to TFL over-expresser plants and FT over-expresser plants which produce terminal flowers, like *tfl* mutants. *FT* is regulated by a photoperiod controlled floral initiation gene *CONSTAN*S (*CO*) (Tiwari et al., 2010). *CO* then regulates both *FT* and *LFY,* which in turn repress *TFL* causing the switch from vegetative growth to reproductive growth (Tiwari et al., 2010).

1.9 Aims

This thesis aims to investigate the fundamentals underpinning the development of the Asteraceae flower head. Previous studies using physical manipulation (compression and wounding) of the flower head, indicate the possibility of a morphogen determining flower head development through the restriction of the morphogen style signal causing homeotic conversions. We hypothesise that this aforementioned morphogen is the phytohormone auxin and that this is the first true example of auxin producing homeotic coversions. Auxin's role as a morphogen-like signal in flower head development will be evaluated using the manipulation of endogenous auxin levels, visualisation through the DR5 promoter, molecular expression analysis and histological techniques. Furthermore, known and putative lateral organ identity genes, *RAY2* and *LFY* respectively, will be examined for concentration dependant responses to auxin. This research will be able to confirm whether or not auxin is truly able to act in a morphogen-like manner and is responsible for pattern formation and organogenesis in the Asteraceae flower head. The significance of these findings is not confined within the Asteraceae family. This research will provide a framework around which all flower development can be based. The mechanisms behind flower head development will also be of evolutionary interest as it will provide the framework behind which the convergent evolution of pseudanthium and species radiation can be studied.

Chapter 2

General Methods

2.1 Plant material and plant transformation

2.1.1 Plant growth conditions

All *S. vulgaris* and *M. inodora* plants were grown in a growth chamber under long day conditions (150 μ mol $m^{-2}S^{-1}$, 16 hr light, 24 ˚C day temperature) in 4 inch square pots, on Sinclair compost (William Sinclair Holdings plc, UK) until flowering. Photos of flower heads were taken using a Nikon D3100 camera with a 105 mm Nikkor macro lens (Nikon, UK). Plants for tissue culture were grown in Magenta boxes containing culture media; full strength Murashige and Skoog (MS) medium (DUCHEFA BIOCHEMIE B.V., NL), 3% sucrose (w/v) and 0.8% (w/v) plant agar (Melford, UK) pH balanced to 5.8 with 1M NaOH. *S. vulgaris* seeds were sterilised with 20% (v/v) sodium hypochlorite (domestic grade) for 10 minutes then washed 3 times for 15 min per wash with sterile water prior to transfer to petri dishes (Scientific Laboratory Supplies Limited, UK) containing culture media. Seeds had 0.1% Gibberellic Acid A3 (Melford, UK) pipetted upon them to increase the rate of germination (Personal communication with Professor Richard John Abbott). The plates were then transferred to a Percival© tissue culture (Percival Scientific, Inc., USA) cabinet (100 µ mol $m^{-2}S^{-1}$, 16 hours light, 22˚C) for one week of incubation. After incubation the germinated seedlings were transferred to magenta boxes containing culture media and left to grow for one month in a Percival© tissue culture cabinet (100 µ mol $m^{-2}S^{-1}$, 16 hours light, 22˚C) before being used as leaf explants for tissue culture transformation.

2.1.2 Exogenous auxin application

M. inodora and *S. vulgaris* plants were grown on soil as previously described (Section 2.1) until the first flower heads opened. After initial flower head opening, the developing flower heads were sprayed with an aqueous solution containing 1% methanol, 0.5% Tween-20 and one of the following additives; 1% DMSO (control) or different auxin concentrations 3 µM, 5 µM, 10 µM Indole-3-Acetic Acid (IAA), 3 µM, 5 µM, 10 µM, 50 µM naphthalene-1-acetic acid (NAA) or the auxin transport inhibitor 10 µM 1-Naphthylpthalamic Acid (NPA). Once sprayed the plants were then covered overnight with a black polyethylene bag (16 hours). Phenotypic flower heads were observed for three to four weeks post treatment and different phenotypes continued to develop for up to two months. Localised auxin application was performed by dissecting away a portion of the *M. inodora* phyllaries using forceps to expose the developing flower head meristem. Applying lanolin wax mixed with either 10 µM IAA, 10 µM NPA, or DMSO (control) mixed with lanolin wax as previously described (Reinhardt et al., 2000).

2.1.3 Floral dipping method

Floral dipping of *S. vulgaris* plants was performed on plants that were six weeks old and just starting to flower (first flower had opened). At this stage of floral development the plants were dipped in a solution of 5% sucrose (w/v) and 0.005% (v/v) silwet L-77 (LEHLE SEEDS, USA) containing a resuspended culture of *Agrobacterium tumefaciens* (*A. tumefaciens GV3101*). *A. tumefaciens* containing the binary vector of interest, was grown to an optical density (600 nm) of between 0.6-1.0. Floral dipping was performed for 6 minutes whilst under a vacuum, following dipping plants were placed on their side and covered with an opaque plastic lid inside the growth chamber (150 μ mol $m^{-2}S^{-1}$, 16hr light, 24˚C day temperature) . This was to ensure that the *A. tumefaciens* did not dry out during the incubation period. After 16 hours of incubation the plants were up righted and the cover was removed. The plants were allowed to grow as normal and the seeds were collected when available, approximately one month after dipping. The seeds were

then plated onto half strength MS plates containing kanamycin (140mg/L; (Melford, UK) for selection (if the binary vector contained the kanamycin resistance gene) and treated with 0.1% Gibberellic Acid A3 (Melford, UK) to synchronise germination time. Kanamycin resistant plants were transferred to soil and confirmed for the presence of a transgene by PCR. For binary vectors with the glufosinate (Basta) resistance gene, seeds were sown onto a thin layer of soil and allowed to germinate normally. Once the first true leaf emerged, plants were sprayed with 120mg/L glufosinate (Bayer CropScience, UK). Plants were treated 2-3 times in total, with one week in-between treatments. Resistant plants were confirmed for the presence of a transgene using PCR.

2.1.4 Virus induced gene silencing (VIGS)

Virus induced gene silencing (VIGS) was performed on week old seedlings of both *S. vulgaris* and *M. inodora*, as well as 2 week old seedlings of *S. vulgaris*. *Phytoene desaturase* (*PDS*) gene fragments were cloned from both *S. vulgaris* and *M. inodora* using degenerate primers. Once it was confirmed that the *PDS* gene was successfully inserted into pTRV2, the construct was transformed in *A. tumefaciens* (GV3101). A further round of confirmation was performed using colony PCR and sequencing. pTRV2-*PDS* constructs were grown overnight (optical density 1.0 at 600 nm), pelleted and then suspended in an equal volume of infiltration media (10 mM MES, 200 µM acetosyringone, and 10 mm $MgCl_2$) and left to incubate for 3 hours in the dark at room temperature. pTRV1 plasmid, also transformed in *A. tumefaciens*, was grown, pelleted and suspended as pTRV2-*PDS*, the added to pTRV2-*PDS* in a 1:1 ratio (Liu et al., 2002). Seedlings were added to the infiltration mix after incubation and vacuum infiltrated for 5-10 minutes (or until bubbles were no longer coming off the cotyledons). Seedlings were then removed from the infiltration mix and dried off on paper towels before transplanting to 40 cell tray inserts (Gardman, UK) and covered for 3 days. After 40 hours of incubation the cover was removed

slowly to allow for the seedling to adjust. Seedlings typically developed a phenotype 1-2 weeks after the inoculation with *A. tumefaciens*. Two week old *S. vulgaris* and two month old *M. inodora* plants underwent syringe infiltration of *A. tumefaciens* in order to try and induce VIGS. *A. tumefaciens* cultures for pTRV1, pTRV2, pTRV2-*PDS*, pTRV2-*LFY* and pTRV2-*PIN1* were prepared as described previously. Approximately 1 ml of the 1:1 *A. tumefaciens* mix was infiltrated into each plant (multiple leaves for *S. vulgaris* or directly into the developing inflorescence for *M. inodora*). After infiltration, plants were grown till flowering and observed for distinct phenotypes based on the construct infiltrated.

2.1.5 Plant transformation techniques-tissue culture method

Leaf explant transformation was performed using *A. tumefaciens* (GV3101) and sterile tissue culture techniques. One month old Magenta-grown plants were harvested and roughly 2 cm^2 explants cut from the leaves. *A. tumefaciens* cultures containing the binary vector of interest were grown overnight at 30˚C to an optical density (600nm) of 0.6-1.0. Overnight cultures were pelleted at 4000g in a SIGMA 3-16KL centrifuge (Sigma, UK) and the resulting pellet was resuspended to an optical density (600nm) of 0.6-0.8 in a solution of 3% (w/v) sucrose, MS and 100 µM acetosyringone (Sigma, UK). Explants were incubated for twenty minutes in the resuspeneded *A. tumefaciens* solution in the dark at room temperature, as *A. tumefaciens* is light sensitive. Following incubation, the explants were dried to remove excess *A. tumefaciens* on a paper towel and were transferred to co-culture media (3% sucrose (w/v), MS, TDZ (1 mg/L) and NAA (0.1 mg/L), pH to 5.8 with 1M NaOH) for three days of incubation at 22°C in the dark. Post incubation, the explants were transferred to fresh co-culture plates that contained the antibiotics kanamycin (40 mg/L) and cefotaxamine (250 mg/L) and left for two weeks in a Percival© tissue culture cabinet (100 µ mol $m^{-2}S^{-1}$, 16 hours light, 22˚C). The explants were transferred onto fresh co-culture plates containing the antibiotics, kanamycin (100mg/L) and cefotaxamine (250 mg/L).

Transfers occurred at two week intervals until the appearance of calli with shoots. Once the calli formed shoots they were transferred to Magentas containing root induction media (3% (w/v) sucrose, half strength MS) and the antibiotics (kanamycin 100 mg/L and cefotaxamine 250 mg/L). Rooting shoots were transferred to new Magentas every month or sooner if required. Once the new transgenic plants had rooted sufficiently, they were transferred to soil and covered with a plastic lid for 2 days to allow the plants to adapt to the new humidity. The cover was slowly removed over the period of a week. The whole process from explant to transgenic plant took from six to ten months. The presence of a transgene was confirmed using PCR (Primers used in Table 2) and visual markers (Beta-glucuronidase staining) if applicable.

2.2 Bacterial growth conditions and transformation

Agrobacterium tumefaciens (*A. tumefaciens*, GV3101) and *Escherichia coli* (*E. coli*, DH5α) were grown in Luria-Bertani (LB) broth (Per litre: 5 g yeast extract, 10 g tryptone and 5 g NaCl) with low NaCl, as higher NaCl concentrations can cause *A. tumefaciens* to clump together during growth. *E. coli* was grown at 37˚C in a MaxQ 5000 shaking incubator at 180 rpm (ThermoFisher, UK). *A. tumefaciens* was grown at 30˚C in a shaking incubator (180 rpm). For both species incubation time was between 16-20 hours depending on the optical density (600nm) required (normally between 0.6-2.0).

2.2.1 *E. coli* transformation

Competent *E. coli* cells were transformed with plasmid DNAs using heat shock. In brief, 100µL aliquots of *E. coli* (stored at -80˚C) competent cells were defrosted on ice. Once defrosted the cells were gently mixed with plasmid (1µL for plasmid DNA or 10µL for ligated sample) and left to incubate for 30 minutes on ice. After the incubation, the cells were transferred to a 42˚C water bath for 1 minute (heat shock). Immediately following the heat shock, cells were placed on ice for 5 minutes. 900µL of LB broth was added and the cells were then placed in a 37˚C incubator for 1 hour. After one hour, all the cells were plated out on LB agar plates (LB broth plus 0.8% agar) containing the appropriated selection antibiotic for the inserted plasmid (kanamycin 100mg/L, ampicillin 50mg/L and spectinomycin 50mg/L). Plates were allowed to dry for 5 minutes before the incubation at 37˚C for 16 hours. After the incubation resistant colonies growing on the plate were screened using colony PCR before sequencing.

2.2.2 *A. tumefaciens* transformation

Competent *A. tumefaciens* (GV3101) cells were transformed with binary vectors using electroporation. 50µL aliquots of competent cells (stored at -80˚C) were defrosted on ice. Once defrosted the cells were gently mixed with plasmid (1 µL) and left to incubate for 30 minutes on ice. After the incubation, cells were transferred to a 0.2 cm gap Gene Pulser electroporation cuvette (Bio-Rad, UK) and electroporated using a GenePulser® II (Bio-Rad, UK) at 2.4 kV, 25 µF and 600 Ω. Immediately after electroporation, 950 µL of LB broth was added to the transformed *A. tumefaciens*, and cells were transferred to a 30˚C incubator for 2 hours. Following the incubation, 100 µl of the cells were plated out onto LB agar plates containing vector and strain specific antibiotic (vector: kanamycin 100mg/L or spectinomycin 50mg/L strain: rifampicin 25mg/L and gentamicin 50mg/L) and left to incubation for 2 days at 30˚C. After the incubation, resistant colonies growing on the plate were screened using colony PCR before sequencing.

2.3 RNA, DNA and Cloning

2.3.1 RNA and DNA extractions and cDNA synthesis

DNA was extracted from *M. inodora* and *S. vulgaris* using 2-3 g of leaf tissue. The tissue was frozen in liquid N_2 then ground into a fine white powder in a liquid N_2 chilled pestle and mortar. 5 ml of urea buffer (7M Urea, 0.35M NaCl, 0.05M Tris-HCl pH 8, 0.04M EDTA pH 8, 1% Sodium lauroyl sarcosinate) was added to the ground tissue and homogenised. Then 5 ml of pH 8 Phenol:chloroform:isoamylacohol, 25:24:1 respectively (Fisher Scientific, UK) was added to the mixture and this was homogenised for 15 min at 4ºC before centrifugation at 2500 xg using a Mistral 3000i centrifuge (MSE Ltd, UK). The supernatant was decanted and added to a 1/10 volume of NH_4Ac and an equal volume of isopropanol. Precipitated DNA was removed and dissolved in 500 µL of H_20 and precipitated once more using an equal volume of 100% ethanol and a 1/10 volume of 3M sodium acetate. DNA was resuspended in H_2O and stored at -20 ˚C until needed. RNA extractions were performed on 100mg of young flower head tissue (expect for dissected lateral organs in which case 50 mg of tissue was used) using RNeasy mini kit (Qiagen, UK) according to the manufacturer's specifications. Extracted RNA was then treated with RQ DNase I (Promega, UK) at 37 ˚C for 30 minutes before quantification using a Nanodrop™ 1000 (ThermoFisher, UK). Following manufacturer's instructions, cDNA was synthesised from 1 µg of RNA with polydT_{18} primer using Superscript II reverse transcriptase (Invitrogen, UK).

2.3.2 Degenerate Cloning of *PIN1*, *LFY* and *PDS*

Degenerate primers for *PIN1* and *LFY* were designed by creating alignments of homologs for each respective gene in Clustal X2.1. Homologs for each gene were obtained by BLAST searches on the NCBI, U.S. National Library of Medicine databases. Only mRNA sequences were used to create alignments, as introns are less well conserved than exons. Highly conserved regions were selected to create degenerate primers from. With *PIN1*, due to the large number of gene family members, a second alignment was created with *PIN2-8* along with PIN1 homologs to design *PIN1* specific degenerate primers. Degenerate primers for *PDS* were based on those used in a previous study by Wege et al, 2007. Degenerate PCR was performed on PTC-200 Gradient Thermal Cycler (MJ Research, Waltham) using cDNA templates made from young flowers of *S. vulgaris* and *M. inodora*. The conditions were as follows: an initial denaturing of 98ºC for 2 minutes, 98°C for 30 seconds, one of 12 annealing temperatures (44°C - 64 °C) for 30 seconds, and 72°C for 30 seconds, for 39 cycles with a final annealing of 72°C for 5 minutes. A mixture of Taq (Roche, UK) and Phire® HotStart (Thermo-Fisher, UK) DNA polymerases were used. PCR bands of the correct size were then excised and DNA was eluted using a NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL, Germany) before being ligated into pGEM® T Easy plasmid kit (Promega, UK) for sequencing. Once sequences for *LFY* and *PIN1* were obtained from *S. vulgaris*, primers (with SacI and XbaI enzyme sites) were designed based on *S. vulgaris LFY* and *PIN1* sequences. These *PIN1* and *LFY* PCR products were digested with SacI and XbaI (New England biolabs, UK) and then ligated with a T4 DNA ligase (New England Biolabs, UK) into a binary vector pBI121 (GenBank Accession, AF485783). Cloned *PDS* fragments along with the VIGS vector pTRV2 were digested with SacI and BamHI (New England biolabs, UK) (Liu et al., 2002). T4 DNA ligase was used according to manufacturer's specification, to ligate the sticky ends of the *PDS* fragment and pTRV2 plasmid in a 3:1 ratio respectively. Plasmid specific primers (Table 1) were used for colony PCR in order to confirm a successful ligation and also for sequencing of *PDS* in pTRV2. All constructs were transformed in to *E. coli* and *A. tumefaciens* respectively.

2.3.3 *DR5::GUS* Construct

The *DR5::GUS* construct was made by PCR amplification of the DR5 promoter from pUC19 (Ulmasov et al., 1997) with primers (Table 1) that contained the ClaI and XbaI restriction sites. The conditions for PCR were as follows: an initial denaturing of 98°C for 2 minutes, 98°C for 30 seconds, followed by 58˚C for 30 seconds, and 72°C for 30 seconds, for 40 cycles with a final extension of 72°C for 5 minutes. Phire® HotStart (Thermo-Fisher, UK) DNA polymerase was used following manufacturer's directions. Following digestion with ClaI and XbaI (New England Biolabs, UK), the fragment was ligated into pBI121 (Clontech, UK) using T4 DNA ligase (New England Biolabs, UK). The construct was then transformed into *E. coli* using the heat shock method (42˚C for 1 minute) before sequencing. Once sequencing confirmed a successful ligation, the DR5::GUS construct was transformed into *A. tumefaciens* for plant transformation.

2.3.4 Colony PCR

Colony PCR was used to identify bacterial colonies of *E. coli* and *A. tumefaciens* that contained the inserted plasmid of interest. Colonies were picked off the LB agar plates with autoclaved toothpicks and streaked onto fresh LB agar plates (containing appropriate antibiotics) before being used to inoculate the PCR mix. The PCR mix consisted of BIOTAQ™ polymerase (Bioline, UK) used according to manufacturer's instructions, with 1 mM of both forward and reverse primers (Table 1). After the mix was inoculated, it was denatured at 94˚C for 5 minutes then cycled at 94˚C for 30 seconds, 55˚C for 30 seconds and 72˚C for 1 minute for 40 cycles before a final extension at 72˚ for 5 minutes. PCR product was run on a 1% agarose gel (SeaKem® LE Agarose, Lonza, UK) in 1x Tris-acetate-EDTA buffer (40 mM Tris acetate and 1 mM EDTA) containing 50 µL/L of SafeView (NBS Biologicals Ltd., UK). PCR product was run against Hyperladder I (Bioline, UK) for size comparison at 100 Volts for 30 minutes. Colonies containing the correct plasmid were grown in 15

ml LB with a selection antibiotic for 16 hours and plasmid DNA was prepped with a QIAprep Spin Miniprep Kit (Qiagen, UK) prior to sequencing.

2.3.5 DNA sequencing

Plasmid DNA was extracted with the QIAprep Spin Miniprep Kit (Qiagen, UK) from *E. coli* containing the pGEM® T Easy vector with the gene of interest. Terminator cycle sequencing reactions and DNA precipitations were performed according to the BigDye® Terminator v.1.1 Cycle Sequencing Kit manufacturer's instructions (Applied Biosystems, USA), using a T7 forward primer. Precipitates were sent to The University of Manchester DNA sequencing facility for analysis. Blast searching (NCBI) of the sequenced fragment was used for the initial gene identification.

2.4 Expressional analysis

2.4.1 Quantitiative RT-PCR

Total RNA was extracted from *M. inodora* flower heads at various developmental stages. Developmental stages were determined based upon differences in morphology observed through scanning electron microscopy (SEM) data of flower heads. Flower heads were grouped into 6 different stages, with stage 1 as the youngest and 6 the oldest. Flower heads at stages 2 and 3 were used for the majority of the qRT-PCR experiments, except for when looking at the change in gene expression throughout all of the developmental stages in which cases stages 1-6 were used. For qRT-PCR on individual lateral organs, young developing organs (leaf, phyllary, ray floret and disc floret) were dissected and harvested using a Leica MZ6 stereomicroscope (Leica, Germany). qRT-PCR was used to determine gene expression in auxin sprayed flower heads that has been treated as previously described and collected at 0, 6 and 18 hours after auxin application. All

tissue was extracted, treated and cDNA synthesised as previously described. Primers for qRT-PCR were designed using Primer3 software (Untergasser et al., 2007) on previously cloned sequences of target genes, as well as sequences obtained from NCBI. Annealing temperatures were kept to ±1˚C of 60˚C with a target GC content of 50-60%. qRT-PCR was performed on a ABI PRISM® 7000 using SensiFAST™ SYBR Hi-ROX Kit (Bioline, UK). Reactions were performed according to manufacturer's specifications with final concentrations of 500 nM for forward and reverse primers and 10 ng of RNA per reaction. Master mixes were always made to minimise the effect of pipetting error. All samples were run as triplicates biologically and quadruplicates technically. A melting curve analysis was performed on each run to ensure only single products were made. Samples were normalised to RPS9 and 18s rRNA and their expression determined using the comparative threshold cycle (Schmittgen and Livak, 2008), The PCR efficiency of each target was calculated using LinReg (Hårdstedt et al., 2005).

2.4.2 Immunolocalisation

Protein Immunolocalisation was performed on *S. vulgaris* and *M. inodora* to detect the localisation of PIN1- protein according to Jackson, D. et al. (1994). In brief, young flower heads of S*. vulgaris* and *M. inodora*, were fixed with paraformaldehyde and embedded in paraffin. The tissue was then sectioned (10 µm thickness) using a microtome (Leica Microsystems Ltd, UK). The tissue was then deparaffinised by incubation in Histoclear II (National Diagnostics U.S.A.). This was followed by hydration of the tissue using a graded series of ethanol from 100% down to 0% in water followed by incubation in 1X PBS solution (10X PBS: 1.3M NaCl, 0.07M Na_2HPO_4, 0.03M NaH_2PO_4). Slides were then treated with a 0.1 mg/mL of Proteinase K (Sigma, UK) solution for 20 minutes in a laboratory made humid chamber to stop the slides from drying out. The removal of Proteinase K was followed by three washes in 1X PBS for five minutes per wash. Slides were then incubated overnight in a 10% Bovine Serum Albumin (BSA, Sigma, UK)/1XPBS solution at 4 ˚C. 100

µl of the primary antibody (PIN1 antibodies, Proteintech group, UK) was applied to each slide for 2 hours at a dilution of 1:500 (in 0.1XBSA/1XPBS). The antibody incubation occurred at room temperature in a humid chamber. The primary antibody was then removed and the slide was washed in 0.1X BSA/PBS three times for 15 minutes per wash. At the end of the wash steps, the slides were stored at 4 ˚C overnight in 0.1X BSA/PBS. Next 100µl of anti-goat alkaline phosphatase conjugated secondary antibody, (anti-rabbit, Sigma, UK) was applied to the slides at a concentration of 1:500 (in 0.1XBSA/1XPBS). After a two hour incubation at room temperature in a humid chamber, the secondary antibody was removed and the slides were rinsed twice in 0.1% BSA/1XPBS solution for 15 minutes per rinse. The slides were then stored at 4 ˚C overnight in 1X PBS. Before use the slides were washed in fresh TNM solution (100mM Tris pH 9.7, 100mM NaCl, and 50mM MgCl2) then 400 µl of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT, Promega, UK), prepared to manufacturers specifications, was applied to each slide. Slides were developed in a humid chamber in the dark. Once Slides were developed, they were placed in dsH2O to stop the reaction. Photographs of the slides were taken using a dissection microscope and a camera (Nikon, UK).

2.4.3 *In situ* Hybridisation

In situ hybridisation was performed on wild-type and auxin treated developing flower heads of *M. inodora*. *In situ* hybridisations were performed using RNA probe of *MiLFY, MiRAY1 and MiRAY2,* according to the protocol by Coen, E. *et al*. (1991). In brief, young flower heads were treated, fixed, embedded and sectioned as previously described. Fixation was performed 6 hours post treatment as this was the time point that showed the most change in gene expression using qRT-PCR. Sectioned tissue was deparaffinised with 2 x 10 minute treatments with Histoclear II. Tissue was then rehydrated in a decreasing EtOH series (100%, 95%, 90%, 80%, 60%, 30%, H_2O). Following rehydration, slides were treated for 25 minutes at 37˚C with 0.065 mg/mL of proteinase K (Sigma, UK). Proteinase K digestion was stopped with a 0.2 % solution of glycine before samples

were fixed in 4 % paraformaldehyde. Post fixation tissue was acetylated with acetic anhydride, dehydrated back through the EtOH series and left at 4˚C until hybridisation had occured.

RNA probes were synthesis prior to *in situ* hybridisation. Previously cloned fragments of *MiLFY* and *MiRAY1/2* genes were cloned into pDRIVE (Qiagen, UK) and pGEM easy-T (Promega, UK) respectively. M13 forward and reverse primers were used to amplify both the T7 and SP6 promoters contained within the plasmid, as well as the gene fragment contained between the promoters. Using T7 and SP6 RNA polymerases in the DIG labelling kit (Roche Applied Science, UK), according to manufacturer's specification, both sense and antisense single stranded RNA probes were transcribed with digoxigenin-UTP. Probe concentration was roughly estimated by electrophoresis, 1 µL of the transcribed product on a 1 % agarose gel at 135 Volts with Hyperladder IV. Probes were prepared for hybridisation by heating to 100˚C with 50% formamide (2-5µL of probe with formamide up to a final volume of 20 µL) and were placed on ice until needed. Hybridisation solution (40% formamide, 1x Denhardt's reagent, 9 x 10^{-5} mg/ml T RNA, 10% dextrane sulphate, 1x *in situ* salt solution [0.3M NaCl, 10mM Tris-HCl, 5 mM EDTA, 5mM Na_2HPO_4, $NaH_2PO_42H_2O$]) at 85˚C was mixed with probes before being applied to the tissue and left to hybridise overnight at 50˚C.

After overnight incubation, the slides were washed twice in 0.2X saline-sodium citrate (SSC) before having excess probe removed with a 30 minute RNase A (20mg/L, Sigma, UK) treatment at 37˚C. Tissue was then blocked twice for 45 minutes with BM blocking solution (Roche Applied Science, UK) before having an anti-digoxigenin alkaline phosphatase linked antibody (Roche, UK) applied at a 1:1250 ratio to the slide for two hours. The antibody was removed and the tissue was blocked three more times in a 1% BSA block solution (1% BSA, 25mM NaCl, 0.003% triton x-100, 100mM tris-HCl pH 7.5), with the final blocking step occuring overnight. After the overnight blocking, slides were washed in a substrate buffer (25mM NaCl, 100mM tris-HCl pH 9.7, 50mM $MgCl_2$) for 15 minutes. The alkaline phosphaste substrate, 5-bromo-4-chloro-3-indolyl-

phosphate/nitro blue tetrazolium (BCIP/NBT, Promega, UK) was prepared according to manufacturer's specification before being applied to the tissue. Tissue was incubated at room temperature for 3-6 hours with BCIP/NBT before development was complete. Development was stopped in 1x TE buffer (10mM Tris-HCl pH 7, 1mM EDTA pH 8) before slides were dehydrated and mounted as previously described. Slides were imaged on Lecia DMR fitted with SPOT Insight 4.0 Mp Color F-Mount (SPOT Imaging Solutions, USA) using SPOT advanced software (SPOT Imaging Solutions, USA).

2.5 Histology and Scanning Electron Microscopy

2.5.1 Beta-glucuronidase staining

Transgenic plants transformed with the Beta-glucuronidase (GUS) reported system were visualised using well-established methods (Sessions et al., 1998). In brief, transgenic and wild-type *S. vulgaris* flower heads of different developmental stages (from phyllary formation to fully developed flower heads) were collected and fixed in 90% acetone for 20 minutes. Flower heads were then washed for 10 min in GUS staining buffer (100mM potassium phosphate buffer pH 7.0, 10 mM Ethylenediaminetetraacetic acid, 0.1% triton x-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide). Following washing, fresh GUS staining buffer containing the GUS substrate, 5-Bromo-4-chloro-3-indolyl-ß-D-glucuronic acid, cyclohexyl ammonium salt (2mM) was applied to the flower heads and vacuum infiltrated for 10 minutes. Tissue was left in the dark incubating at 37˚C overnight for a maximum of 24 hours. The flower heads were then dehydrated with 30% ethanol (EtOH) and then fixed with an acidic formaldehyde solution (3.7% formaldehyde, 50% EtOH and 5% acetic acid) for 30 minutes respectively. Tissue was continued to be dehydrated with 70%, 85%, 90% and 100% EtOH (30 minutes each step). The stained flower

heads were then photographed using a Leica MZ6 stereomicroscope with a Nikon D3100 camera attached. Flower heads that were stained were then infiltrated with a solution of Histoclear II/100% EtOH (1:1) and Histoclear II (National Diagnostics U.S.A.) for 30 minutes per flower head. Flower heads were then added to 100% liquid paraffin (Sigma, UK) at 58˚C overnight before being embedded and sectioned (14 µm thick microtome sections). Sectioned material had the paraffin removed with 2x 10 minutes washes in Histoclear II before having coverslips mounted with Roti®-Histokitt II (Carl Roth, Germany). Sectioned material was imaged on a Lecia DMR fitted with SPOT Insight 4.0 Mp Color F-Mount (SPOT Imaging Solutions, USA) using SPOT advanced software (SPOT Imaging Solutions, USA). Phase contrast and Nomarski Interference contrast settings were used in order to enhance contrast.

2.5.2 Scanning Electron Microscopy

SEM was performed upon *M. inodora* flower heads of different developmental stages and auxin treated flower heads. Tissue was fixed in a paraformaldehyde solution (4% paraformaldehyde, 0.01% Dimethyl sulfoxide, 1xPBS, 0.001% tween-20 and 0.001% triton x-100) overnight at 4˚C. Fixed tissues were dehydrated in an ethanol series (30%, 50%, 70%, 85%, 95%, 100% for 30 minutes per step). Dehydrated tissue then underwent critical point drying (CPD) using a Polaron critical point dryer (Quorum Technologies, UK) before being mounted onto SEM stubs (Agar Scientific, UK) using carbon tape (Agar Scientific, UK). Mounted stubs were then sputter coated with gold for 2 minutes using a Polaron E5100 sputter coater (Quorum Technologies, UK). Samples were imaged on a Quanta 250 FEG (FEI UK Limited, UK) using the secondary detector.

2.6 Software

Image colour balancing and cropping was performed using Adobe Photoshop CS6 (Adobe, USA) and figures put together on Canvas X (ADC systems, USA). Tables and data analysis was performed on Excel (Microsoft, USA) and graphs made on GraphPad 6 (Prism, USA).

Primer Name	5'-3' sequence	Use
SP6	TATTTAGGTGACACTATAG	Sequencing and colony PCR
T7	TAATACGACTCACTATAGGG	Sequencing and colony PCR
M13 forward	CGCCAGGGTTTTCCCAGTCACGAC	Sequencing and colony PCR
M13 reverse	TCACACAGGAAACAGCTATGAC	Sequencing and colony PCR
pTRV1 forward	CTTGAAGAAGAAGACTTTCGAAGTCTC	Sequencing and colony PCR
pTRV1 reverse	GTAAAATCATTGATAACAACACAGACAAAC	Sequencing and colony PCR
pTRV2 forward	GGTCAAGGTACGTAGTAGAG	Sequencing and colony PCR
pTRV2 reverse	CGAGAATGTCAATCTCGTAGG	Sequencing and colony PCR
DeLFY forward	CARAGRGAGCAYCCSTTYATYGT	Degenerate primers
DeLFY reverse	GACGMAGCTTKGTKGGRACATACCA	Degenerate primers
DeChryCYC forward	AGCAAAACCCTWGATTGGCT	Degenerate primers
DeChryCYC reverse	YCTTTCYCKAGCTCTTGCTC	Degenerate primers
DePIN1 forward	CCNAAYACBYTNGTNATGGG	Degenerate primers
DePIN1 reverse	CTKGARCTCCAVACRAACAT	Degenerate primers
DePDS forward	GAGGTGTTCATCGCAATGTCAAAGGC	Degenerate primers
DePDS reverse	GTGTTGTTGAGCTTTCGGTCAAACCATATATG	Degenerate primers
DR5 forward	CCATCGATGGTTCATTAATGCAGCTGGCAC	Construct primers
DR5 reverse	CCATCGATGGTGAGCGCAACGCAATTAATG	Construct primers
QPCR MiLFY forward	CTTGATGAGGAGGGGTCAAA	qRT-PCR
QPCR MiLFY reverse	TAGACAAACGCGGATGTGAG	qRT-PCR
QPCR MiRAY2 forward	CCCAACACAAAGGGGTTAAA	qRT-PCR
QPCR MiRAY2 reverse	GATTGGCAGGAGATCCAAAA	qRT-PCR
QPCR MiRAY1 forward	TGGCTCTTTACCAAATCGAA	qRT-PCR
QPCR MiRAY1 reverse	GTTGCTGACTTCTTCCTTTGG	qRT-PCR
QPCR MiRPS9 forward	GCGTTTGGATGCTGAGTTGAAG	qRT-PCR
QPCR MiRPS9 reverse	GGCGCTCAAGGAAGTTCTCTAC	qRT-PCR
QPCR Mi18S forward	CACGTAAAAACAACCGAGTGTCG	qRT-PCR
QPCR Mi18S reverse	CAAAGCATCGAGAGGATCAAAC	qRT-PCR

Table 1: A list of all the different primers used throughout all experiments.

Chapter 3

A Novel Morphogen-like Role of Auxin in Determining Capitulum Pattern Formation in Asteraceae

3.1 Abstract

As multicellular organisms, plants and animals have independently evolved patterning mechanisms to form different lateral organs from a group of homogenous cells. Gradients of morphogens and hormones play a pivotal role in determining the lateral organ identity and position in animal development (Driever and Nüsslein-Volhard, 1988a, Driever and Nüsslein-Volhard, 1988b, Shimozono et al., 2013, Rogers and Schier, 2011) and yet, no example of such mechanisms has been reported in plants. Here we show that a gradient of the plant hormone auxin provides a developmental cue for capitulum pattern formation by regulating organ identity genes such as *MiRAY2* and *MiLFY* in a concentration dependant manner. A spatio-temporal auxin gradient occurs in the developing capitula regulating the successive formation of phyllaries, ray florets and disc florets. Disruption to the endogenous auxin gradient led to homeotic conversions of lateral organs in the capitulum. This is the first report that revealed the mechanism controlling capitulum pattern formation, providing compelling evidence for a novel morphogen-like role of auxin in determining lateral organ identities. This further highlights how developmental tools such as hormone gradients have been reinvented in plants to meet the unique aspect of post-embryonic developmental processes, in which lateral organs are asynchronously formed.

3.2 Nature Letter

The pseudanthium (false flower) is one of the most successful traits that has recurred throughout the evolution of angiosperms (Harris, 1999). In a pseudanthium, a group of flowers and bracts have evolved to mimic and function as a single flower. The most common pseudanthium is the capitulum of the Asteraceae family. Although the capitulum closely resembles a solitary flower, it consists of many flowers (florets) and phyllaries (modified bracts) compressed into a single structure (Fig. 1a-c). Adoption of this characteristic capitulum is believed to be the key to the evolutionary success of Asteraceae as one of the largest plant families (Cronquist, 1981). The pattern formation of the capitulum is precisely controlled, with lateral organs always positioned in the following order; phyllaries (P), ray florets (R) and disc florets (D), which mimic sepals, petals and anthers, respectively (Fig. 1b, c).

However, little is known about the mechanisms determining the order and identities of these lateral organs. The plant hormone, auxin plays important roles in plant development. In particular, some evidence that auxin provides positional cues in cell and tissue specification has been reported (Uggla et al., 1996, Pagnussat et al., 2009). We hypothesized that auxin provided a developmental cue for capitulum pattern formation. To test this, we manipulated the endogenous auxin distribution by applying 1-N-Naphthylphthalamic acid (NPA), a polar auxin transport inhibitor (Geldner et al., 2001), or Indole-3-acetic acid (IAA) auxin onto developing stage 2 (Fig. 2j) and 3 (Fig. 2k) *Matricaria inodora* (scentless camomile, Anthemideae) capitula. Application of NPA on *M. inodora* repressed ray floret formation [5 µM (n=12/20); 50 µM (n=5/12)] but did not affect the formation or morphologies of phyllaries or disc florets (Fig. 1d), indicating that polar auxin transport and local auxin accumulation play a critical role in ray floret formation. Localised inhibition of auxin transport with NPA showed confined repression of ray floret formation [local induction, 10 µM (n=10/59), Fig. 1e, bracket]. In auxin treated capitula, disc florets were converted into either phyllaries or ray florets (Fig. 1f-p, Supplementary Table 1). These conversions were

homeotic and the converted phyllaries and ray florets had normal morphology and colour. The position of the converted ray florets and phyllaries could be manipulated, depending on the developmental stage of the capitulum (Fig. 1f-n) and the site of auxin application (local induction, Fig. 1o arrows). Treatments to very young capitula (stage 2) led to conversions near the margin of the capitulum dome (Fig. 1i, j, n), while treatments to older capitula (stage 3) placed conversions towards the centre of the capitulum dome (Fig. 1k-m). This indicates that the meristematic zone where lateral organs emerge and their organ identities are determined (thus where applied auxin can alter the identity of lateral organs), shifts from the peripheral to central region of the developing capitulum. In all cases, converted organs were positioned in the order of phyllary, ray floret and disc floret (Fig. 1f-l), mirroring the patterning in a non-treated capitulum (Fig. 1a, b). This hints that auxin concentration plays a role in determining the identity of lateral organs. The concentration/effect of applied auxin is likely to decrease in the capitulum as it develops further. The order of converted lateral organs may reflect the gradual decrease in auxin concentration in the meristematic zone of capitulum post auxin application. To determine the effect of auxin concentration on lateral organ conversion, *M. inodora* plants were sprayed with 3 µM or 10 µM IAA. Capitula treated with 3 µM IAA showed a significantly ($P=0.042$) higher rate of ray floret conversion, while 10 µM IAA showed a significantly ($P=0.0205$) higher rate of conversion to phyllaries (Fig. 1q, Supplementary Table 1). Taken together, these findings suggest that different auxin concentrations determine the identity of different lateral organs and that an auxin gradient exists in the developing capitula.

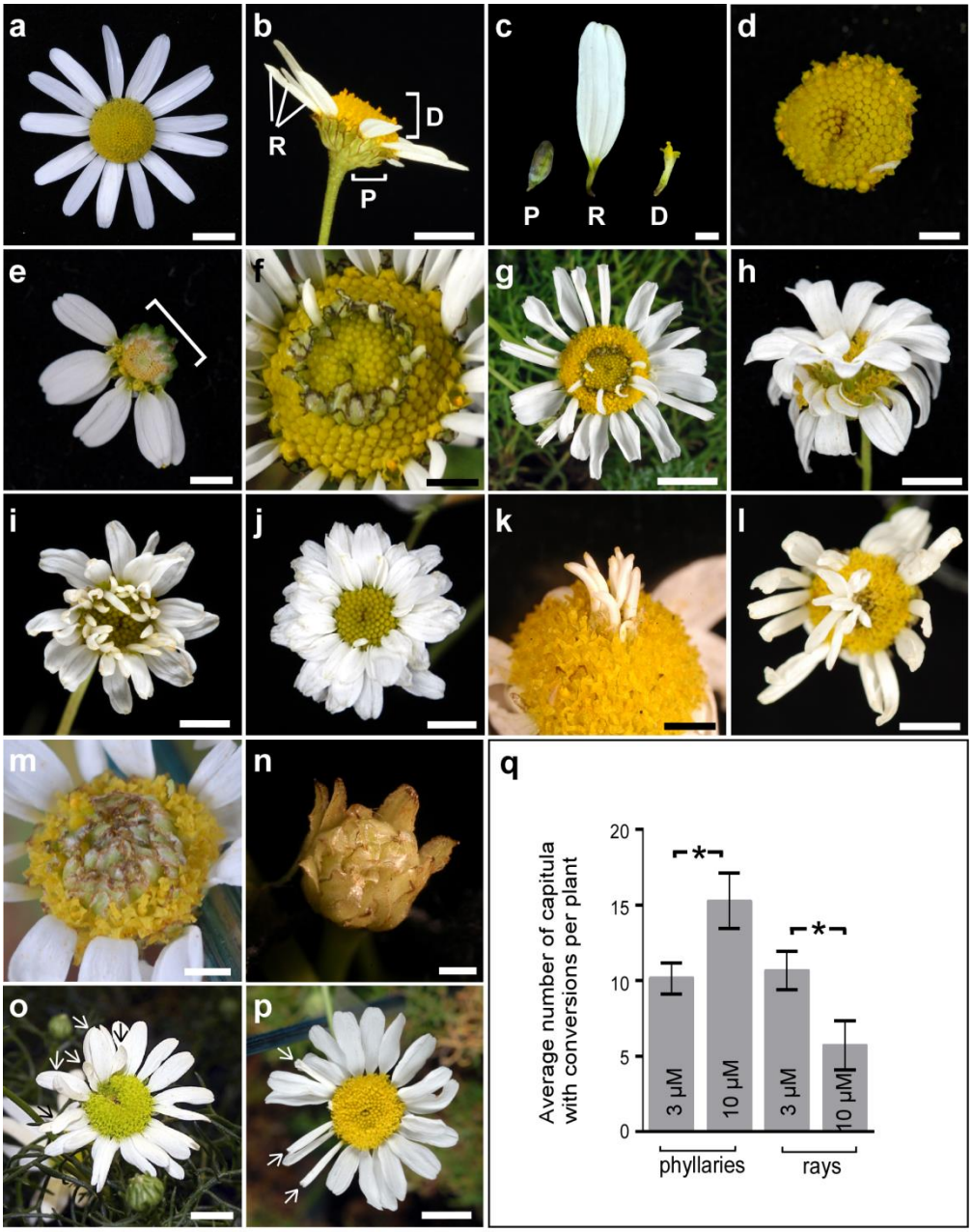


Figure 1: Auxin application induced homeotic conversions in the capitulum. **a**-**c**, Non-treated *M. inodora* capitula (**a**, **b**) and lateral organs (**c**). **d**-**p**, Capitula treated with NPA (**d**, **e**) or auxin (**f**-**o**). Earlier (**e**, **f**, **h**, **j**) and later (**g**, **i**, **k**) stages of capitula with ray floret and phyllary conversions, solely phyllary conversions (**m**, **n**) or with tubular ray florets (**p**, arrows). **e**, **o**, Capitula locally treated with NPA (**e**) or auxin (**o**). Converted (**o**, arrows) or aborted (**e**, bracket) ray florets. **q**, Quantification of lateral organ conversions post auxin treatments (*:P =<0.05). Scale

bars, 5 mm (**a**, **b**, **g**-**j**, **l**, **p**), 2 mm (**d**, **g**, **k**, **m**, **n**, **o**), 200 µm (**c**). P; phyllaries, R; ray florets, D; disc florets. Error bars represent s.e.m.

To investigate the presence of such an auxin gradient in the developing capitula, a visual auxin marker, *DR5*::*GUS* (β-glucoronidase) (Ulmasov et al., 1997a), was transformed into an Asteraceae model species *Senecio vulgaris*. GUS staining of the transgenic *S. vulgaris* capitula revealed that auxin concentration decreases as the capitulum develops; the auxin concentration was high (dark blue) in younger (stage 1 and 2) capitula (Fig. 2a, b, e, f, corresponding stages in *M. inodora* shown in i and j), in which phyllaries (P) and ray florets (R) were formed. In contrast, auxin concentration was low (pale blue) in older (stage 4) capitula (Fig. 2b, c) where disc florets (D) were formed (Fig. 2g, k). No auxin was detected in lateral organs as the capitulum (stage 5 and 6) matured (Fig. 2c, h, l). These results were corroborated by quantification of GUS activity (Fig. 2m). In a capitulum, particularly at the stage 3, auxin concentration was higher in the periphery (Fig. 2d). Sections of *DR5::GUS* capitula further showed that higher auxin concentration in the periphery of the capitulum where lateral organ incipients were formed (Fig. 2e-g). Furthermore, auxin concentration differed in the incipients (Fig 2e-g), being the highest in phyllary incipient (Pi), followed by ray floret incipient (Ri) and the lowest in disc floret incipient (Di). Taken together, *DR5::GUS* transgenic plants showed that a spatio-temporal auxin gradient occurs in developing capitula. The auxin concentration in the meristematic region of the capitulum (where lateral organs are generated) decreases as the capitulum forms phyllaries, ray florets and disc florets consecutively. This suggests that auxin acts as a morphogen, determining different lateral organ identities in a concentration dependent manner.

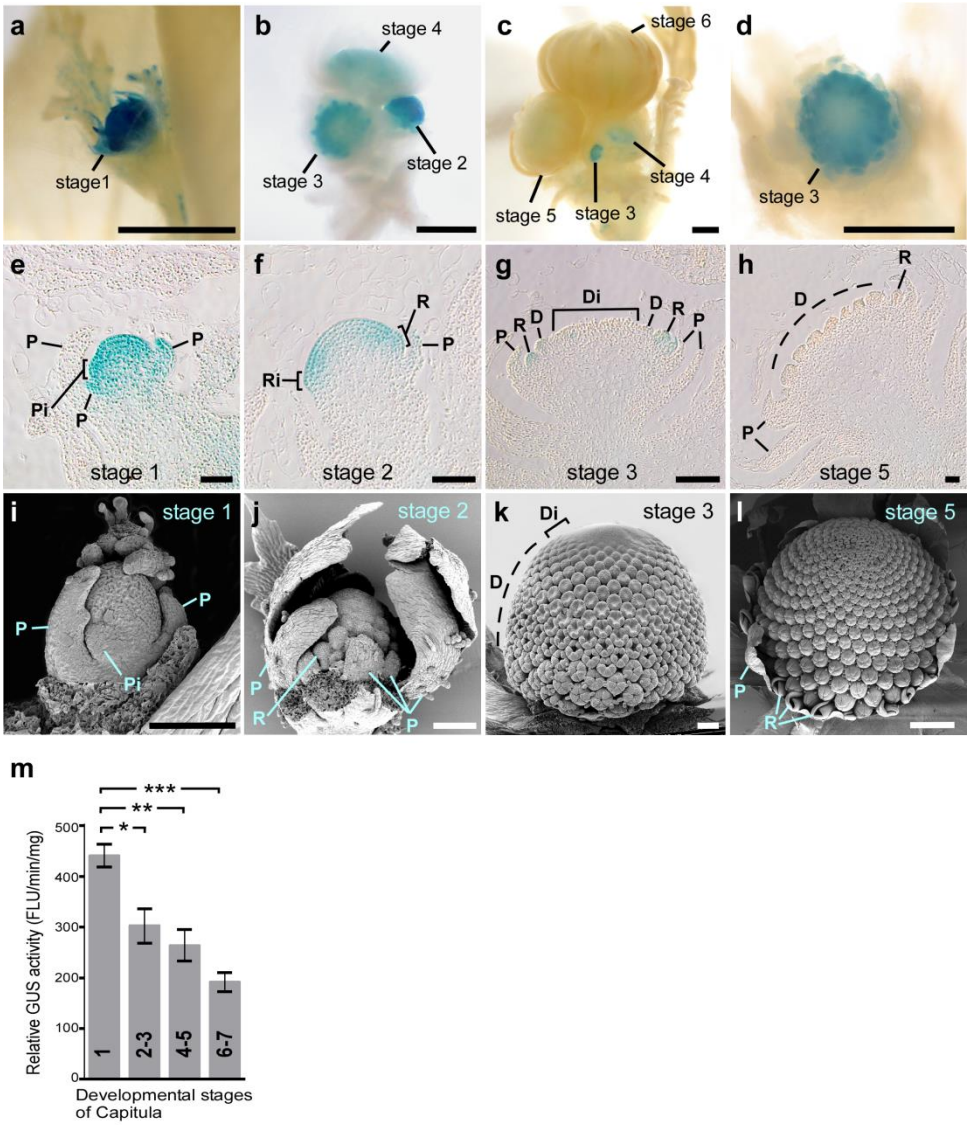


Figure 2: Auxin concentration decreases in a developing capitulum. a-h, *DR5::GUS* capitula of *S. vulgaris* stained for GUS expression from young to mature developmental stages (1-6). Side (a) and top (d) views, or a cluster (b, c) of capitula. Capitulum meristem produces phyllaries (P: e), ray (R: f) and disc florets (D: g) successively, and terminates as florets differentiate and mature (h). i-l, SEM images of *M. inodora* capitula equivalent to *S. vulgaris* developmental stages (e-h). (m) Quantification of GUS activity by MUG (4-methylumbilleferyl glucuronide) assay (*:P=0.0289, **:P=0.0077, ***:P=0.0009). P; phyllaries, Pi; incipient phyllary primordium, R; ray florets, Ri; incipient ray floret primordium, D; disc florets, Di; incipient disc floret primordium. Scale bars, 0.5 mm (a, b, c, d, l), 100 µm (e-k).

To further understand the mechanisms by which an auxin gradient regulates lateral organ identity, we investigated whether auxin regulates target genes such as lateral organ identity genes in a concentration dependent manner. A *CYCLOIDEA* homolog, *RAY2*, expressed in the developing ray florets, determines ray floret identity in Asteraceae species including *S. vulgaris* (Broholm et al., 2008, Chapman et al., 2012, Kim et al., 2008). In *S. vulgaris*, auxin treatments led to ray floret conversion or tubular ray florets formation (Supplementary Fig. 1b, d), which phenocopied *RAY2* over-expressing plants (Supplementary Fig. 1c, e). These *RAY2* over-expresser phenotypes were also observed in some auxin-treated *M. inodora* capitula (Fig. 1f-l, p), suggesting that *M. inodora RAY2* (*MiRAY2*) may be up-regulated post auxin treatment. qRT-PCR analysis showed that *MiRAY2* was strongly expressed in ray florets in non-treated young *M. inodora* capitula (Fig. 3a). qRT-PCR also showed that auxin regulates the expression levels of *MiRAY2* in a concentration dependent manner; the expression level of *MiRAY2* was up-regulated in the stage 2 (Fig. 2j) *M. inodora* capitula 6 hours post 3 µM IAA treatment (Fig. 3b), while it stayed the same to that of the mock control post 10 µM IAA treatment (Fig. 3c). *in situ* hybridisations confirmed that the expression of *MiRAY2* in the capitulum was no longer confined to the developing ray florets (Fig. 3g); rather it was extended towards the meristem dome post 3 µM IAA treatment (Fig. 3i). In contrast, capitula treated with 10 µM IAA showed very little change in *MiRAY2* expression pattern (data not shown). *leafy* (*lfy*) mutants make leaves instead of flowers in *Arabidopsis* and *Antirrhinum* (Weigel et al., 1992, Coen et al., 1990). As phyllaries resemble leaves, we hypothesised that down-regulation of *LFY* is essential for the formation of phyllaires. qRT-PCR analysis showed that the expression level of *M. inodora LFY* (*MiLFY*) was strongly down-regulated in phyllaries, compared to ray or disc florets in non-treated young *M. inodora* capitula (Fig. 3d). The expression level of *MiLFY* in the capitula was down-regulated in response to 10 µM IAA application (Fig. 3f) but not to 3 µM IAA (Fig. 3e), post 6 hours after treatments. *in situ* hybridisation results confirmed that the *MiLFY* expression in the ray and disc floret primordia seen in the mock treated capitulum (Fig. 3h) was

down-regulated in capitulum treated with 10 µM IAA (Fig. 3j). Consistent with the qRT-PCR data, capitula treated with 3 µM IAA showed no significant change in the *MiLFY* expression pattern (data not shown). Both *MiRAY2* and *MiLFY* expression analyses were performed on stage 2 capitula because their innate expression levels were constant in this stage (Supplementary Fig 2). Thus, alteration in their expression levels post auxin treatments was solely in response to the applied auxin. Taken together, these expressional data (Fig. 3b and f) combined with the phenotypic data (Fig. 1e-m and Supplementary Table 1) showed that auxin can up or down-regulate lateral organ identity genes such as *MiRAY2* and *MiLFY* in a concentration dependant manner, which in turn determine lateral organ identities in the capitulum.

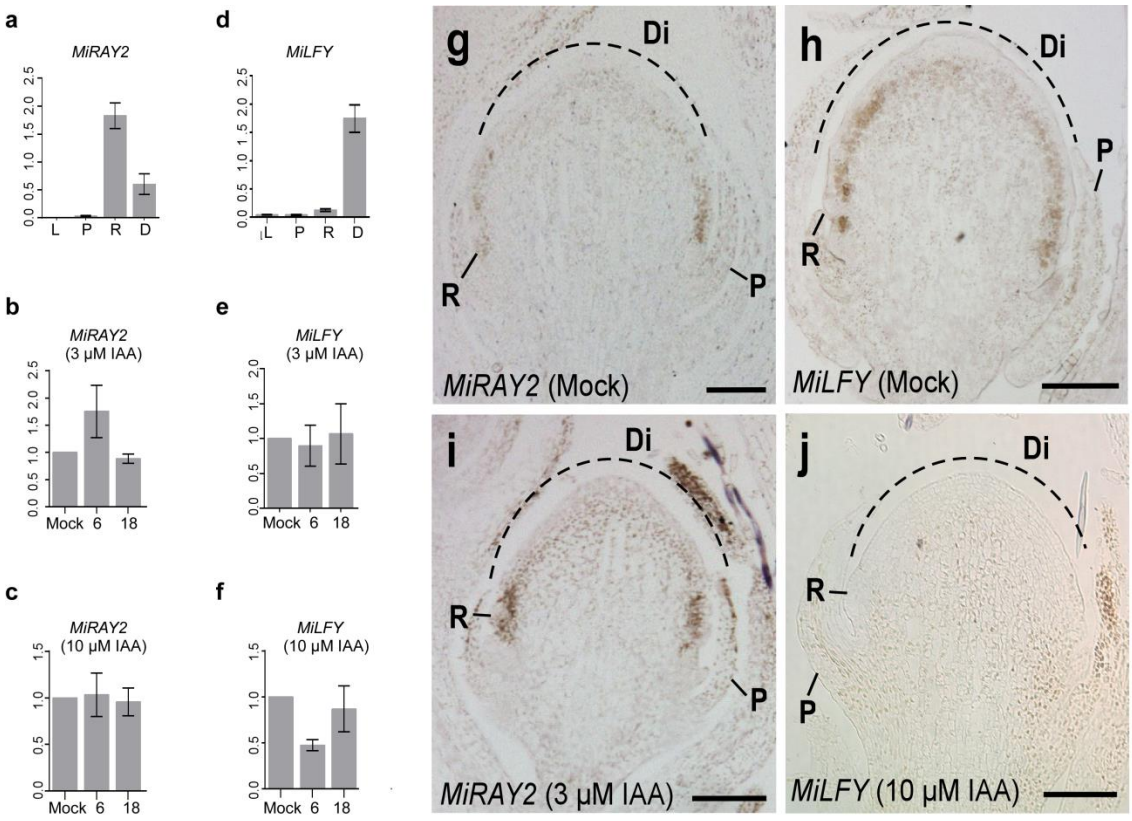


Figure 3: Auxin regulates lateral organ identity genes in *M. inodora* capitula. a-j, qPCR (**a**-**f**) and *in situ* hybridisation (**g**-**j**) analyses of *MiRAY2* (**a**-**c**, **g**, **i**) and *MiLFY* (**d**-**f**, **h**, **j**) on non-treated lateral organs (**a**, **d**) and on whole capitula non- treated (**g**, **h**), treated with 3 µM (**b**, **e**, **i**) or 10 µM (**c**, **f**, **j**)

IAA. X axes: lateral organs (**a**, **d**) or hours post auxin application (**b**, **c**, **e**, **f**). Y axes: the relative expression of *MiRAY2* (**a**-**c**) or *MiLFY* (**d**-**f**). Scale bars: 50 μm (**g**-**j**). L; leaves, P; phyllaries, R; ray florets, D; disc florets, Di, disc floret incipients. Error bars represent s.e.m.

Our results consistently suggest that an endogenous auxin gradient provides a developmental cue for lateral organ identity and thus determines patterning of the capitulum in a concentration dependent manner (Fig. 4). This provides a compelling evidence that auxin acts as a morphogen, determining the identities of lateral organs. This contrasts to the previous studies showing auxin determines the lateral organ position (Friml et al., 2003, Koenig et al., 2009, Reinhardt et al., 2000, Sabatini et al., 1999) or patterning of cell types or tissues (Uggla et al., 1996, Pagnussat et al., 2009, Sorefan et al., 2009). While the classical concept of morphogen gradients is based on the French flag model (Wolpert, 1969), evidence has been accumulated to demonstrate the equal importance of temporal and spatial distributions of gradients in many developmental processes (Jaeger et al., 2008). The dynamic nature of the spatio-temporal auxin gradient is essential for the capitulum pattern formation in which distinct lateral organs are asynchronously generated. This new role of auxin further sheds light on understanding how animals and plants have evolved similar principles in their developmental processes, including the same components such as gradient cues and morphogen responsive target genes which determine the pattern formation. Moreover, for the first time, our results uncover the mechanism controlling pattern formation of the capitulum, the most common pseudanthium form seen in nature (Cronquist, 1981). This highlights how convergent evolution can reinvent a complex structure such as capitulum by introducing a new developmental machinery (auxin gradient cue) to re-govern the pre-existing target genes such as *RAY2* and *LFY* with conserved functions.

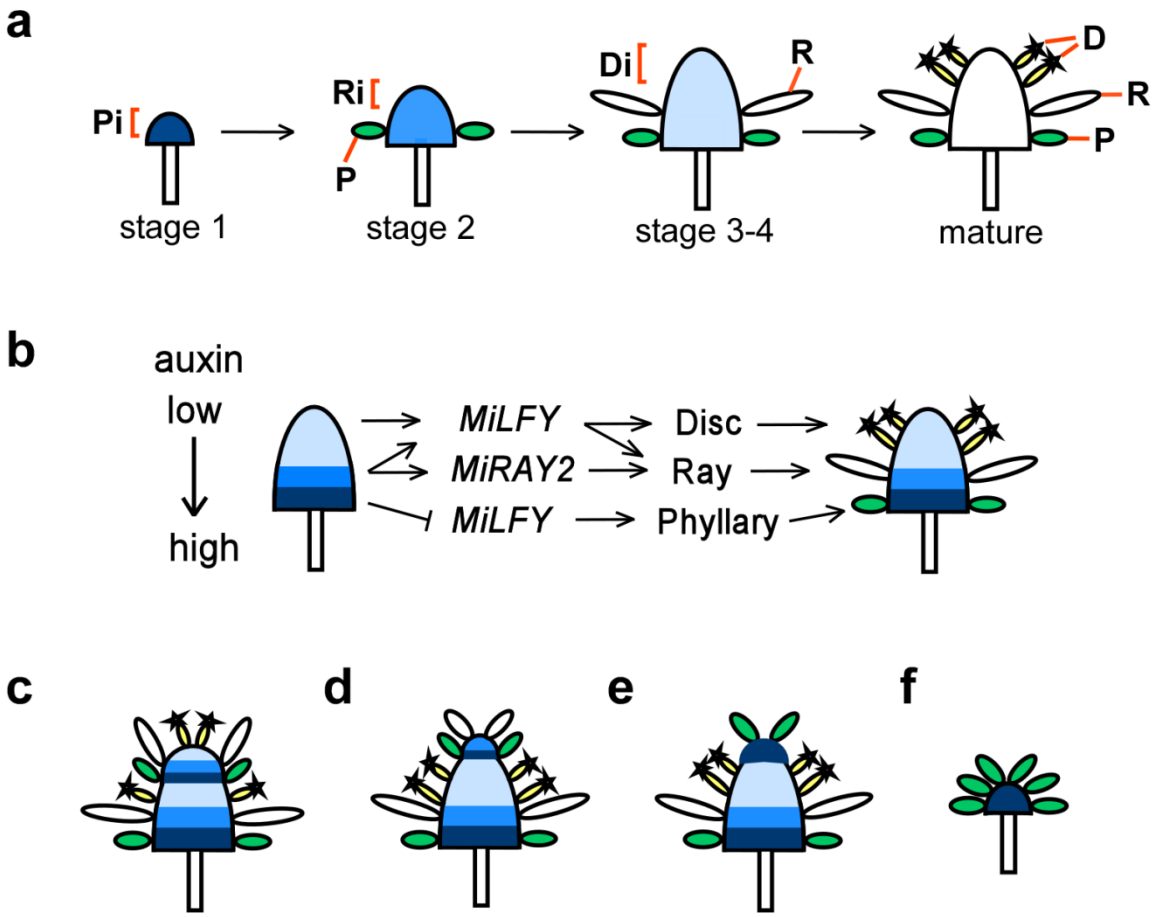


Figure 4: Model for the role of auxin gradients on capitulum pattern formation. a, A schematic representation showing wild-type *M. inodora* capitula at different developmental stages and their respective auxin concentrations. Auxin concentration changes from high (dark blue) to low (pale blue) in the region of lateral organs initiation, as the capitulum forms phyllaries (P), ray florets (R) and disc florets (D) consecutively. The brackets represent the meristematic regions where lateral organs will be initiated. **b**, Capitulum showing superimposed auxin gradients at different developmental stages and downstream gene regulation. High auxin levels at the early developmental stages repress *MiLFY*, leading to the formation of phyllaries. As the capitulum develops auxin levels decrease. Consequently, the repression of *MiLFY* diminishes, allowing for ray and disc floret formation. Once the auxin levels reach the threshold, it up-regulates the *MiRAY2* expression in the region where ray florets will be formed. Further decreases in the auxin levels result the disc floret formation. **c-f**, Homeotic conversion phenotypes and their respective

superimposed auxin gradients. **c**, A capitulum with prolonged high auxin levels forms phyllaries exclusively (Fig. 1n). **d**, Exongenous auxin applications induce an ectopic auxin gradient that promotes the reappearance of phyllaires, ray florets and disc florets in the centre of the capitulum (Fig. 1f-h). **e** and **f**, Auxin applications at the later developmental stages terminates the capitulum with phyllaires and ray florets (Fig. 1**k**, **l**) or just phyllaries (Fig. 1**m**).

3.3 Method Summary

Plant Material and GUS staining

Plants were grown in a greenhouse (24 °C 16 hour light). Capitula of *S. vulgaris* and *M. inodora* were sprayed once with an aqueous solution containing 1% Methanol, 0.5% Tween-20 and various concentrations of the different auxins and an auxin transport inhibitor; 3 µM, 5 µM, 10 µM, Indole-3-acetic Acid (IAA), 3 µM, 5 µM, 10 µM, 50 µM naphthalene-1-acetic acid (NAA) and 10 µM 1-Naphthylpthalamic Acid (NPA). Local auxin induction experiments were performed with 10 µM IAA as described previously (Reinhardt et al., 2000). The DR5 promoter was amplified (Supplementary Table 2) from a pUC19plasmid (Ulmasov et al., 1997a) and cloned into pBI121(Clontech), and transformed in *S. vulgaris (Kim et al., 2008)*. Capitula were GUS stained and SEM imaged using methods previously described (Sessions et al., 1998, Kim et al., 2003).

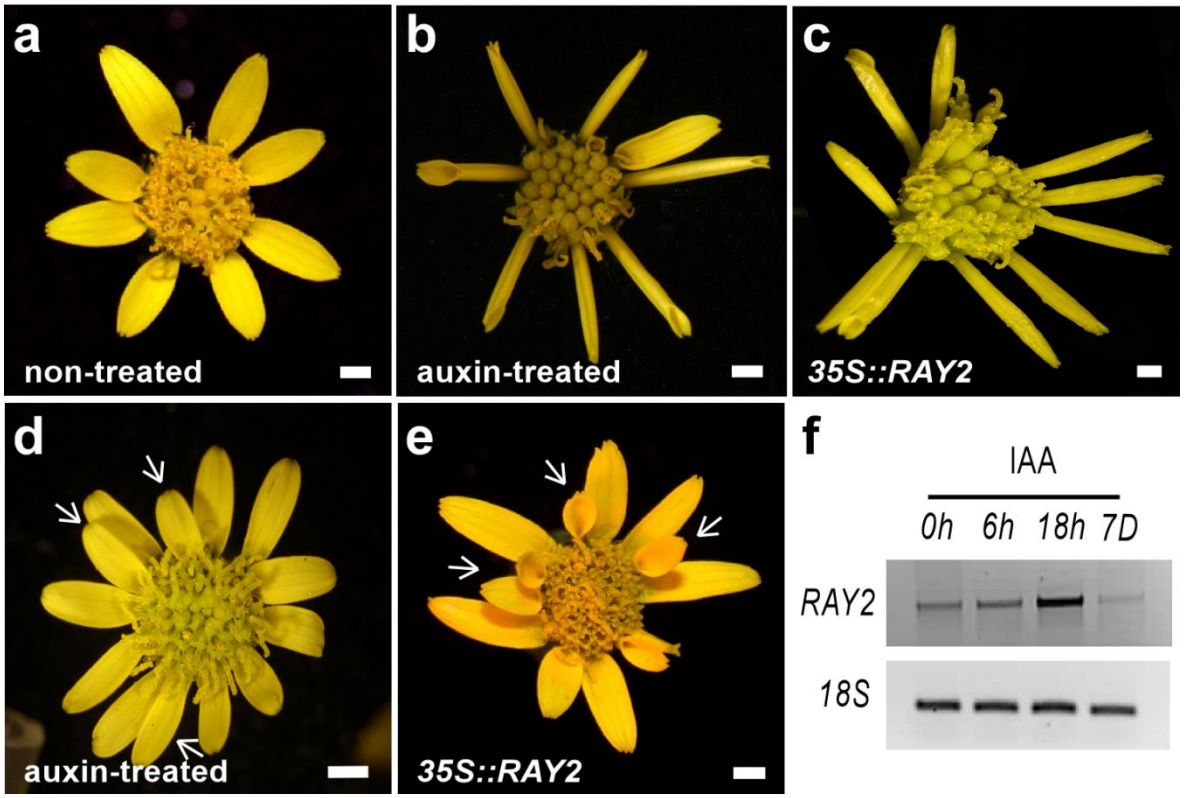
Expressional analysis

For quantitative RT-PCR analysis (qRT-PCR) and *in situ* hybridisations, stage 2 whole capitula from *M. inodora* were treated with an aqueous solution containing 1% Methanol, 0.5% Tween-20 and 3 µM or 10 µM IAA. Capitula were sprayed at time point 0h, and tissues were harvested 0h, 6h and 18h after the treatment. Total RNA was extracted using RNeasy Mini Kit (Qiagen). Following DNase I (Promega) treatment cDNAs were synthesised using Superscript II (Invitrogen). qRT-PCR was performed using FastStart Universal SYBR Green Master (Roche) on ABI Prism 7000 machine

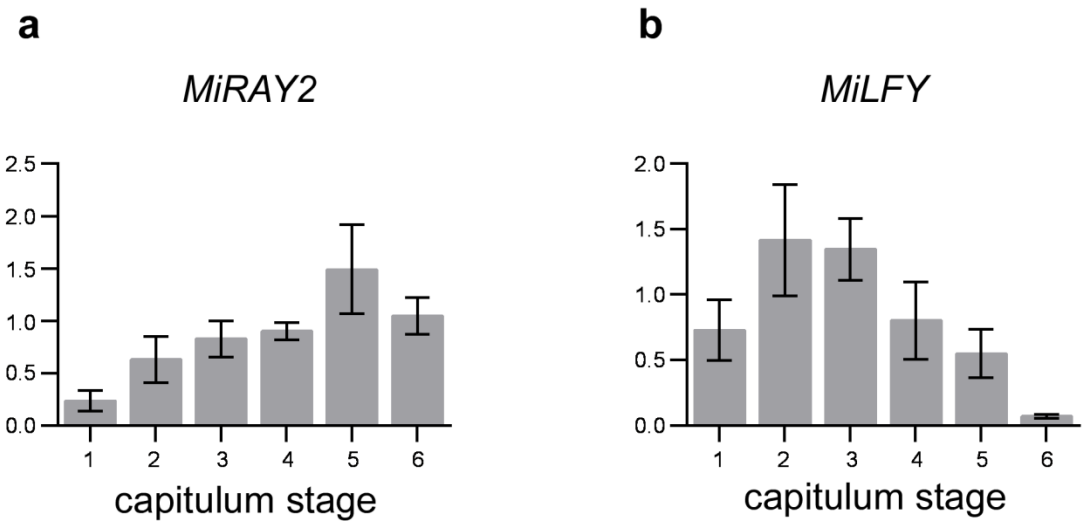
(Applied Biosystems) with the gene specific primers and *RPS9* and *18s rRNA* as control genes. All qRT-PCR were performed in technical triplicates on biological triplicates, as described previously (Etchells et al., 2012). *in situ* hybridisations were performed according to Coen *et al*., 1990 (Coen et al., 1990), using antisense and sense probes of *MiRAY2* and *MiLFY*. MUG assay on *DR5::GUS S. vulgaris* capitula of different developmental stages was performed as described previously (Hull and Devic, 1995).

3.4 Supplementary Data and Methods

Supplementary Figures



Supplementary Figure 1: Auxin treatment of *S. vulgaris* phenocopied *RAY2* overexpression phenotype. **a**, wild-type *S. vulgaris* capitulum. **b** and **d**, auxin treated capitula. **c** and **e**, *S. vulgaris* capitula overexpressing *RAY2*. **f**, semi-quantitative RT-PCR of *RAY2* expression post IAA treatments.



Supplementary Figure 2. qRT-PCR expression analyse of *MiRAY2* (**a**) and *MiLFY* (**b**) on non-treated *M. inodora* capitula. X axes show capitulum developmental stages shown in Figure 2i-l Y axes show the relative expression of *MiRAY2* (**a**) or *MiLFY* (**b**). Note that their expression levels were constant in the stage 2 and 3 capitula.

Supplementary Tables

Batch	IAA concentration	Average total number of flower heads	Percentage of phenotypic flower heads	
			Disc to phyllary conversion	Disc to ray conversion
1	Mock	115	0.00	0.00
	3 µM	101	0.28	0.53
	10 µM	140	0.72	0.32
2	3 µM	77	0.37	0.37
	10 µM	59	0.75	0.11
3	3 µM	87	0.31	0.38
	10 µM	58	0.47	0.26

Supplementary Table 1: *M. inodora* capitulum phenotypes of various auxin treatments.

Primer Name	5'-3' sequence
M13 forward	CGCCAGGGTTTTCCCAGTCACGAC
M13 reverse	TCACACAGGAAACAGCTATGAC
LFY degenerate forward	CARAGRGAGCAYCCSTTYATYGT
LFY degenerate reverse	GACGMAGCTTKGTKGGRACATACCA
DeChryCYC forward	AGCAAAACCCTWGATTGGCT
DeChryCYC reverse	YCTTTCYCKAGCTCTTGCTC
DR5 forward	CCATCGATGGTTCATTAATGCAGCTGGCAC
DR5 reverse	CCATCGATGGTGAGCGCAACGCAATTAATG
QPCR MiLFY forward	CTTGATGAGGAGGGGTCAAA
QPCR MiLFY reverse	TAGACAAACGCGGATGTGAG
QPCR MiRAY2 forward	CCCAACACAAAGGGGTTAAA
QPCR MiRAY2 reverse	GATTGGCAGGAGATCCAAAA
QPCR MiRPS9 forward	GCGTTTGGATGCTGAGTTGAAG
QPCR MiRPS9 reverse	GGCGCTCAAGGAAGTTCTCTAC
QPCR Mi18S forward	CACGTAAAAACAACCGAGTGTCG
QPCR Mi18S reverse	CAAAGCATCGAGAGGATCAAAC

Supplementary Table 2: Primer sequences.

Supplementary Methods

Plant growth conditions

All plant material was grown in a growth chamber under long day conditions (150 µmol $m^{-2}S^{-1}$, 16 hr light, 24˚C day temperature) in 4 inch square pots, on Sinclair compost until flowering. Photos of capitula were taking using a Nikon D3100 camera with a 105 mm Nikkor macro lens (Nikon). Plants for tissue culture were grown from seeds, in Magenta boxes containing culture media; MS 3% sucrose (w/v) and 0.8% (w/v) plant agar (Melford, UK) pH balanced to 5.8. Seeds were sterilised with 20% (v/v) sodium hypochlorite (domestic grade) for 10 minutes then washed for three 15 minute washes with sterile water prior to transfer to petri dishes with culture media. Seeds then had 0.1% Gibberellic Acid A3 (Melford) pipetted upon them to increase the rate of germination. The plates were then transferred to a Percival© tissue culture cabinet (22 ˚C, 16 hours light, 100 µ mol $m^{-2}S^{-1}$) for one week. After one week the germinated seedlings were transferred to magenta boxes and left to grow for one month before being used as leaf explants for tissue culture transformation.

Plant treatments

Plants were grown as previously described until the first capitula opened. At which point the developing capitula were sprayed with an aqueous solution containing 1% Methanol, 0.5% Tween-20 with one of the following additives; 1% DMSO as control; various auxin concentrations 3 µM, 5 µM, 10 µM, Indole-3-acetic Acid (IAA), 3 µM, 5 µM, 10 µM, 50 µM naphthalene-1-acetic acid (NAA) and the auxin transport inhibitor 10 µM 1-Naphthylpthalamic Acid (NPA). Once sprayed the plants were then covered overnight (16 hours). Phenotypic capitula were seen three to four weeks post treatment and phenotypes continued for up to two months. Local induction experiments were performed by applying lanolin wax mixed with either 10 µM IAA, 10 µM NPA,

or DMSO on *M. inodora* capitula with phyllaries dissected away to expose the developing capitulum meristem as previously described (Reinhardt et al., 2000).

Plant transformation

Leaf explant transformation was performed using *A. tumefaciens* (GV3101) pBI121 modified with DR5 promoter and sterile tissue culture techniques. One month old Magenta grown plants were harvested and roughly 2 cm^2 explants cut from the leaves. Explants were incubated for twenty minutes with solution of *A. tumefaciens* resuspended to an optical density 600nm 0.6-0.8 in a 3% (w/v) Sucrose containing full strength MS and with 100 µM acetosyringone in the dark at room temperature. Following incubation, the explants were dried to remove excess agrobacterium and incubated on co-culture media [3% sucrose (w/v), MS salts, TDZ (1 mg/L), and NAA (0.1 mg/L) pH to 5.8 with NaOH] for three days at 22°C in the dark. Post dark incubation the explants with transferred to fresh co-culture plates that contained antibiotics (kanamycin 40 mg/L and cefotaxamine 250mg/L) and left for two weeks in a Percival© tissue culture cabinet (22˚C, 16 hours light, 100 µ mol $m^{-2}S^{-1}$). The explants were then continuously transferred onto fresh co-culture media with antibiotics [Kanamycin (100mg/L) and cefotaxamine (250 mg/L)] every two weeks until the appearance of calli with shoots. Once the calli formed shoots they were transferred to Magenta boxes containing root induction media [3% (w/v) sucrose, half strength MS salts] and antibiotics [kanamycin 100 mg/L and cefotaxamine 250 mg/L)]. Rooting shoots were transferred to new Magenta every month or sooner if needed. Once the new transgenic plants had rooted sufficiently, they were transferred to soil and covered with a humidity lid for 2 days, the cover was slowly removed over the period of a week. The whole process from explant to transgenic plant took from six to ten months. The presence of a transgene was confirmed using PCR (Supplementary Table 2) and visual markers (Beta-glucuronidase staining) if applicable.

Constructs

All PCRs were performed using Phire Hot Start DNA Polymerase (Thermofisher, UK), dNTPs from Bioline (UK) and primers were synthesised by Eurofins (Germany). The *DR5::GUS* construct was made by PCR ampfilcation of the DR5 promoter from pUC19 plasmid (Ulmasov et al., 1997a) with primers that contained the ClaI and XbaI sites. Following digestion the fragment was ligated into pBI121 (Clontech, UK).

Beta-glucuronidase staining

Transgenic plants transformed with Beta-glucuronidase (GUS) reported system were visualised using well established methods (Sessions et al., 1998). In brief, transgenic and wild-type capitula of different developmental stages were collected and fixed in 90% acetone for 20 minutes. Capitula were then washed for 10 minutes in GUS staining buffer (100mM potassium phosphate buffer pH 7.0, 10mM Ethylenediaminetetraacetic acid, 0.1% triton x-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide). Following the wash, fresh GUS staining buffer that had the GUS substrate, 5-Bromo-4-chloro-3-indolyl-ß-D-glucuronic acid, cyclohexyl ammonium salt (2mM), added to it was applied to the capitula and vacuum infiltrated for 10 minutes. Tissue was left in the dark incubating at 37˚C overnight for a maximum of 24 hours (average time was 20 hours). The capitula were then dehydrated with 30% ethanol (EtOH) then fixed with an acidic formaldehyde solution (3.7% formaldehyde, 50% EtOH, 5% acetic acid) for 30 minutes respectively. Tissue was continued to be dehydrated with 70%, 85%, 90% and 100% EtOH (30 minutes each step). The stained capitula were then photographed using a Leica MZ6 stereomicroscope with a Nikon D3100 camera attached. Capitula that were well stained were then infiltrated with a solution of Histoclear II/100% EtOH (1:1) and Histoclear II for 30 minutes each. Capitula were then added to 100% liquid paraffin (Sigma, UK) at 58˚C overnight before being embedded and sectioned (14 µm thickness). Sectioned material had the paraffin removed with 2x 10 minutes washes in Histoclear II before having coverslips mounted with Histomount. Sectioned material was imagined on a Lecia DMR fitted with SPOT Insight 4.0 Mp Color F-Mount

(SPOT Imaging Solutions, USA) using SPOT advanced software (SPOT Imaging Solutions, USA). Phase contrast and Nomarski Interference Contrast settings were used in order to obtain some pictures.

Quantitiative RT-PCR

RNA was extracted from *M. inodora* capitula at various developmental stages. Based upon scanning electron microscopy (SEM) data of capitula at different developmental stages, capitula were grouped into 6 different stages. Capitula of stages 2 and 3 were used for most qRT-PCR experiments, except when looking at the change of gene expression throughout all of the developmental stages in which cases stages 1-6 were used. For individual lateral organ qRT-PCR, young developing organs (leaf, phyllary, ray floret and disc floret) were dissected and harvested under a stereo microscope (Nikon, UK). In qRT-PCR experiments of auxin sprayed capitula, capitula were treated as previously described and collected at 0, 6 and 18 hours. Total RNA was extracted using RNeasy Mini Kit (Qiagen, UK). Following DNase I (Promega, UK) treatment cDNAs were synthesised using Superscript II (Invitrogen, UK) according to manufacturers' description. Primers for qRT-PCR were designed using Primer3 software (Untergasser et al., 2007) on previously cloned sequences of target genes as well as sequences obtained from NCBI. Annealing temperatures were kept to ±1˚C of 60˚C with target GC content of 50-60%. qRT-PCR was performed on a ABI PRISM® 7000 using SensiFAST™ SYBR Hi-ROX Kit (Bioline, UK). Reactions were performed according to manufacturer's specifications with final concentrations of 500 nM for forward and reverse primers, and 10 ng of RNA per reaction. Master mixes were always made to minimise the effect of pipetting error. All samples were run as triplicates biologically and quadruplicate technically. A melting curve analysis was performed each run to ensure only single products were made. Samples were normalised to *Ribosomal Protein Subunit 9* (*RPS9*) and *18s rRNA* and their expression determined using the comparative threshold cycle method (Schmittgen

and Livak, 2008). The PCR efficiency of each target was calculated using LinReg (Hårdstedt et al., 2005).

In situ Hybridisation

In situ hybridisation was performed on wild-type and auxin treated developing capitula of *M. inodora*. *In situ* hybridisations on *MiLFY* and *MiRAY2* were performed using the protocol by Coen et al. (Coen et al., 1990). In brief, young capitula were treated, fixed (6 hours post treatment), embedded and sectioned as previously described (Carpenter and Coen, 1990). Sectioned tissue was deparaffinised with 2 x 10 minute treatments with Histoclear II. Tissue was then rehydrated in a decreasing EtOH series (100%, 95%, 90%, 80%, 60%, 30%, H2O). Following rehydration, slides were treated for 25 minutes at 37˚C with 0.065 mg/mL of proteinase K (Sigma, UK). Proteinase K digestion was stopped with a 0.2% solution of glycine (Sigma, UK) before being fixed in 4% paraformaldehyde. Post fixation tissue was acetylated with acetic anhydride then dehydrated back through the EtOH series and left at 4˚C until hybridisation.

RNA probes were synthesis prior to the start of *in situ* hybridisation. Ampflied fragments of *MiLFY* and *MiRAY2* genes were ligated into pDRIVE (Qiagen, UK) and pGEM easy-T (Promega, UK) respectively. M13 forward and reverse primers (Supplementary Table 2) were used to amplify both the T7 and SP6 promoters contained on the plasmid as well as the gene fragment contained between the promoters. Using T7 and SP6 RNA polymerases in the DIG labelling kit (Roche Applied Science, UK) according to manufacturer's specification, both sense and antisense single stranded RNA probes were transcribed with digoxigenin-UTP. Probe concentration was roughly estimated by running 1 µL of the transcribed product on a 1% agarose gel at 135v with hyperladder IV. Probes were prepared for hybridisation by being heated to 100˚C with 50% formamide (2-5 µL of probe with formamide up to a final volume of 20 µL), then being placed on ice until needed. Hybridisation solution (40% formamide, 1x Denhardt's reagent, 9 x 10-5 mg/ml T RNA, 10% dextrane sulphate, 1x *in situ* salt solution (0.3 M NaCl, 10 mM Tris-HCl, 5 mM EDTA, 5

mM Na_2HPO_4, $NaH_2PO_4 \bullet 2H_2O$) at 85 ˚C was then mixed with probes before being applied to the tissue and left to hybridise overnight at 50 ˚C.

The next day slides were washed twice in 0.2X SSC before having excess probe removed with a 30 minute RNase A (20mg/L, Sigma, UK)) treatment at 37˚C. Tissue was then blocked twice for 45 minutes with BM blocking solution (Roche Applied Science, UK) before having an anti-digoxigenin alkaline phosphatase linked antibody applied at a 1:1250 ratio to the slide for two hours. The antibody was removed and the tissue was block thrice more in a 1% BSA block solution (1% BSA, 25 mM NaCl, 0.003% triton x-100, 100 mM tris-HCl pH 7.5), with the final blocking step being overnight. After the overnight blocking, slides were washed in a substrate buffer (25 mM NaCl, 100 mM tris-HCl pH 9.7, 50mM $MgCl_2$). The alkaline phosphaste substrate, 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT, Promega, UK), was prepared according to manufacturer's specification before being applied to the tissue. Tissue was averagely incubated from 3-6 hours with BCIP/NBT before development was complete. Development was stopped in 1x TE buffer (10 mM Tris-HCl pH 7, 1 mM EDTA pH 8) before slides were dehydrated and mounted as previously described. Slides were imaged on Lecia DMR fitted with SPOT Insight 4.0 Mp Color F-Mount (SPOT Imaging Solutions, USA) using SPOT advanced software (SPOT Imaging Solutions, USA).

Scanning Electron Microscopy

SEM was performed upon *M. inodora* capitula of different developmental stages and auxin treated capitula. Tissue was fixed in a paraformaldehyde solution (4% paraformaldehyde, 0.01% Dimethyl sulfoxide, 1xPBS, 0.001% tween-20, 0.001% triton x-100) overnight before being dehydrated in an ethanol series (30%, 50%, 70%, 85%, 95%, 100% for 30 minutes a step). Dehydrated tissue then underwent critical point drying using a Polaron critical point dryer

(Quorum Technologies, UK) before being mounted onto SEM stubs (Agar Scientific, UK) using carbon tape (Agar Scientific, UK). Mounted stubs were then sputter coated with gold for 2 minutes using a Polaron E5100 sputter coater (Quorum Technologies, UK). Samples were then imaged on a Quanta 250 FEG (FEI UK Limited, UK) using the secondary detector.

Software

Image colour balancing and cropping was performed using Adobe Photoshop CS6 (Adobe, USA) and figures put together on Canvas X (ADC systems, USA). Tables and data analysis was performed on Excel (Microsoft, USA) and graphs made on GraphPad 6 (Prism, USA).

Chapter 4

A Morphological Study of Flower Head Development in the Asteraceae

4.1 Abstract

Introduction

The Asteraceae family is characterised by its iconic flower head, the most well-known pseudanthium (false flower) in the plant kingdom. The wide range of forms seen in the pattern formation of the Asteraceae flower head has led it to become a novel model for developmental studies. Although pattern formation in the flower head has been investigated for three centuries, there is very little known about flower head pattern formation and development when compared to model species.

Methods

Scanning electron microscopy (SEM) was used in order to produce high resolution images of the different developmental stages of the lateral organs that comprise the *Matricaria inodora* flower head. Transgenic *Senecio vulgaris*, containing *DR5::GUS* were used to study the auxin distribution during floret development.

Results and conclusion

SEM imaging showed that soon after initiation of ray florets, development of the petals is arrested. Once the meristem has terminated, the ray floret ventral petals appear to undergo rapid cell division in order to reach their fully elongated state that appears to coincide with auxin accumulation in the ray florets. Both ray and disc florets have the same initial floral symmetry (asymmetric) and later differentiate. This suggests that initial primordia for both florets are established by a shared common mechanism and also indicates that this primordium form is the ancestral.

4.2 Introduction

Pseudanthium (false flowers) are one of the most interesting examples of convergent evolution in plants. A pseudanthium is a collection of flowers that has evolved into a single structure that mimics a single flower (Weberling, 1989). Pseudoanthia are found throughout angiosperms and are important both agriculturally and horticulturally. The most well-known pseudanthium is the flower head of the Asteraceae family. The Asteraceae family is one of the largest families of flowering plants with over 23,000 species (Bremer, 1994). Asteraceae flower heads vary greatly in their morphology and can range from containing a small number of flowers to containing thousands. Although there are many different types of pseudanthia, very little is known about their growth and development, when compared to model plant species such as *Arabidopsis thaliana* (*Arabidopsis*) and *Antirrhinum majus* (*Antirrhinum*).

The Asteraceae flower head normally consists of three different lateral organs; phyllaries (involucral bracts), ray florets and disc florets, which are normally patterned in this respective order. Phyllaries are green leaf like structures which subtend the florets in a flower head (Classen-bockhoff, 1990). In contrast to the phyllaries which are not used for reproduction, both the ray and disc florets are involved in the reproductive process in most species. The ray florets are zygomorphic flowers with five petals that generally consist of three elongated ventral petals and two very short dorsal petals, which are thought to play a very important role in pollinator attraction (Funk et al., 2009). Disc florets are actinomorphoic florets that have all five petals at the same length, which generally form the majority of florets in the flower head. In both species used in the study, *Matricaria inodora* (*M.inodora*) and *Senecio vulgaris* (*S.vulgaris*), the flower heads consist of several layers of phyllaries followed by a single layer of ray florets, then numerous layers of disc florets.

There is very little known about the molecular genetics involved in the formation of the flower head, and almost all studies have focused on a single gene family, *CYCLOIDEA* (*CYC*)(Coen et al., 1995a, Lou et al., 1995, Luo et al., 1999). *CYC* is a member of the TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) family of transcription factors, which play an important role in flower asymmetry in *Antirrhinum* (Corley et al., 2005, Cubas et al., 1999, Lou et al., 1995, Luo et al., 1999). The underlying genetics of ray floret formation in flower heads has been previously shown to be under the control of a single gene locus dubbed the *RAY* locus (Trow, 1912). Two *CYC-like* genes were cloned from *S. vulgaris* and were termed *RAY1* and *RAY2 (Kim et al., 2008)*. *RAY1* and *RAY2* have been shown to be sufficient for the formation of ray florets in *S. vulgaris* (Kim et al., 2008). Homologs of *RAY1* and *RAY2* in other Asteraceae species have also been shown to be involved in the control of the formation of ray florets (Chapman et al., 2012, Broholm et al., 2008). *CYC* and other TCP transcription factors are thought to play a role in the regulation of cell division (Gaudin et al., 2000, Nath et al., 2003, Li et al., 2005). There is limited evidence that *CYC-like* TCP genes represses the transcription of cell division activator *CYCLIN D3*. Unfortunately, there is no direct evidence of repression of cell division and the expression of *CYC-like* genes might coincide with the discontinuation of cell division (Gaudin et al., 2000, Martín-Trillo and Cubas, 2010). In contrast to *CYC-like* genes, *AtTCP24* has been shown to up-regulate pre-replication control factor genes, which assist in the transition from cell growth to division (Li et al., 2005). Unlike cell division control genes, which do not affect the final organ shape, mutations in TCP genes can dramatically change the final organ shape (Crawford et al., 2004, Nag et al., 2009). In *Antirrhinum*, mutants in the TCP gene *cincinnata* lead to the formation of crinkled leaves due to uncontrolled cell growth in the margins of the leaves (Crawford et al., 2004). Unfortunately there is no information on the molecular genetics of the generation or patterning of either phyllaries or disc florets.

The phytohormone auxin, is well known for its roles in development and growth throughout the plant (Okada et al., 1991, Uggla et al., 1996, Reinhardt et al., 2000, Friml et al., 2003, Reinhardt et

al., 2003). Auxin's role in development has been extensively researched in *Arabidosis thaliana* and has been found to be necessary for the formation and growth of new lateral organs (Okada et al., 1991, Reinhardt et al., 2000, Reinhardt et al., 2003). Manipulation of auxin localisation and transport in *Arabidopsis* led to the formation of ectopic lateral organs or loss of organ (Okada et al., 1991, Reinhardt et al., 2000). A recent study using the *DR5* auxin reporter gene and exogenous auxin application has shown that auxin plays a critical role in flower head formation and patterning, with auxin application able to produce homeotic conversions of lateral organs in the centre of the flower head (Ulmasov et al., 1997b, Zoulias et al., 2014). The homeotic changes caused by auxin in the Asteraceae flower head suggest it could be acting as a morphogen, whereas the formation of ectopic lateral organs in *Arabidopsis* auxin manipulation experiments indicate that it may not act a morphogen (Reinhardt et al., 2000). Auxin is also well known for its roles in cell elongation and division. Phototropism is one of the most well-known roles of auxin in cell elongation (Tao et al., 2008). During phototropism auxin is rapidly synthesised and transported to the side of the stem where less light is being detect (Tao et al., 2008). Cells are then elongated through two mechanisms; the first mechanism of cell elongation is through cell wall acidification caused by the influx of auxin, which in turn activates α-expansins and other wall loosening factors (Schenck et al., 2010, Yamagami et al., 2004). The second mechanism by which auxin causes cell elongation is through the activation of transcription factors through the auxin response pathways, which leads to a sustained period of cell elongation (Yamagami et al., 2004). Auxin is also know to regulate cell division through the activation of cyclin-dependent kinases and G-protein-mediated signal transduction (Campanoni et al., 2003, Campanoni and Nick, 2005).

Whilst there have been studies on the developmental stages of flower heads in the past, the resolution of scanning electron microscopy (SEM) has recently increased, allowing a more detailed insight into the development of the Asteraceae flower head. This study aims to relate high resolution SEM imaging and auxin visualisation to known molecular genetics from model species in order to investigate flower head development in the Asteraceae family.

4.3 Methods

Plant Growth

All *M. inodora* plants used throughout the study were grown in four inch square pots containing Sinclair compost (William Sinclair Horticulture Ltd., UK) under controlled environmental conditions (150 µ mol $m^{-2}S^{-1}$, 24˚C, 16 hours light,). Plants were grown until flowering (2-4 months), at which point developing flower heads at different stages were collected for SEM.

DR5::GUS

Transgenic *S. vulgaris* plants were generated during previous studies (Zoulias et al., 2014). In brief, the DR5 promoter (Ulmasov et al., 1997b, Ulmasov et al., 1997a) was cloned from pUC19 into the binary vector pBI21 (Clontech, UK) containing the GUS reporter gene. The *DR5::GUS* vector was then transformed into *Agrobacterium tumefaciens* before being transformed into *S. vulgaris* using leaf explant tissue culture (Kim et al., 2008)in a Percival© tissue culture (Percival Scientific, Inc., USA) cabinet (22˚C, 16 hours light, 100 µ mol $m^{-2}S^{-1}$).

Beta-glucuronidase

Flower heads of transgenic *DR5::GUS* plants were harvested at different developmental stages (from phyllary formation to fully developed flower head) and were fixed in 90% acetone for 20 minutes. Flower heads were treated and stained as previously described (Sessions et al., 1998). Flower heads were incubated for 24 hours in staining buffer before with 30% ethanol (EtOH) then fixed with an acidic formaldehyde solution. Tissue was dehydrated through an ethanol series (70%, 85%, 90% and 100%) at 30 minutes for each step. Flower heads were then infiltrated with a solution of Histoclear II/100% EtOH (1:1) and Histoclear II for 30 minutes each. Flower heads were added to 100% liquid paraffin (Sigma, UK) at 58˚C overnight before being embedded and sectioned (14 µm thick microtome sections). Sectioned material had the paraffin removed through 2 x 10 minutes washes in Histoclear II before having coverslips mounted with

Histomount. Sectioned material was imaged on a Lecia DMR fitted with SPOT Insight 4.0 Mp Color F-Mount (SPOT Imaging Solutions, USA) using SPOT advanced software (SPOT Imaging Solutions, USA).

Scanning electron microscopy

SEM was performed upon *M. inodora* flower heads of different developmental stages. Tissue was fixed in a 4% paraformaldehyde solution overnight at 4˚C before being dehydrated in an ethanol series (ending at 100% EtOH). Dehydrated tissue underwent critical point drying using a Polaron critical point dryer (Quorum Technologies, UK) before being mounted onto SEM stubs (Agar Scientific, UK) using carbon tape (Agar Scientific, UK). Mounted stubs were sputter coated with gold for 2 minutes using a Polaron E5100 sputter coater (Quorum Technologies, UK). Samples were then imaged on a Quanta 250 FEG (FEI UK Limited, UK) using the secondary detector.

Software

Image colour balancing and cropping was performed using Adobe Photoshop CS6 (Adobe, USA) and figures were constructed on Canvas X (ADC systems, USA).

4.4 Results

The complex Asteraceae flower head is comprised of several different lateral organs which are precisely patterned. Both the ray and disc florets of the Asteraceae flower head have precisely controlled floral symmetry. SEM was used in order to document the developmental stages and growth of the three types of lateral organs that the Asteraceae flower head contains: the phyllaries, ray florets and disc florets. The flower head is converted from the vegetative meristems (Fig. 1A) into an inflorescent meristem and allows for the reproductive stage of development (Fig. 1B). The vegetative meristem is a higher and more elongated dome, whereas the inflorescent meristem is shorter and more rounded (Fig. 1B). The first lateral organ to develop following the transition to the reproductive stage is the phyllary. The phyllaries are clearly distinguishable from the emerging leaf primordia (Fig. 1A), as the leaf primordia are highly dissected, whereas the phyllaries emerge as a simple sheet (Fig. 1B). The phyllaries take over the role of protecting the developing flower head from the leaf primordia as they expand tightly around the developing flower head (Fig. 1C). The meristematic zone of the flower head is maintained in the centre whilst the florets are produced and the size of the flower head increases (Fig. 1C-E). Lateral organs emerge in a centripetal pattern (Fig. 1E, F), with new lateral organs forming in the gap between the two lateral organs from the previous layer. Ray florets are clearly seen emerging from between the junction of two phyllaries before their development is arrested (Fig. 1C). As the flower head begins to reach maturity (Fig. 1D, E), the disc florets are visible at all different developmental stages whereas the ray florets are still arrested in development and are therefore barely visible. Once the meristem has terminated lateral organ formation (Fig. 1F), the disc florets continue to mature, whilst the ray florets rapidly elongate their ventral petals to become visible from underneath the disc florets.

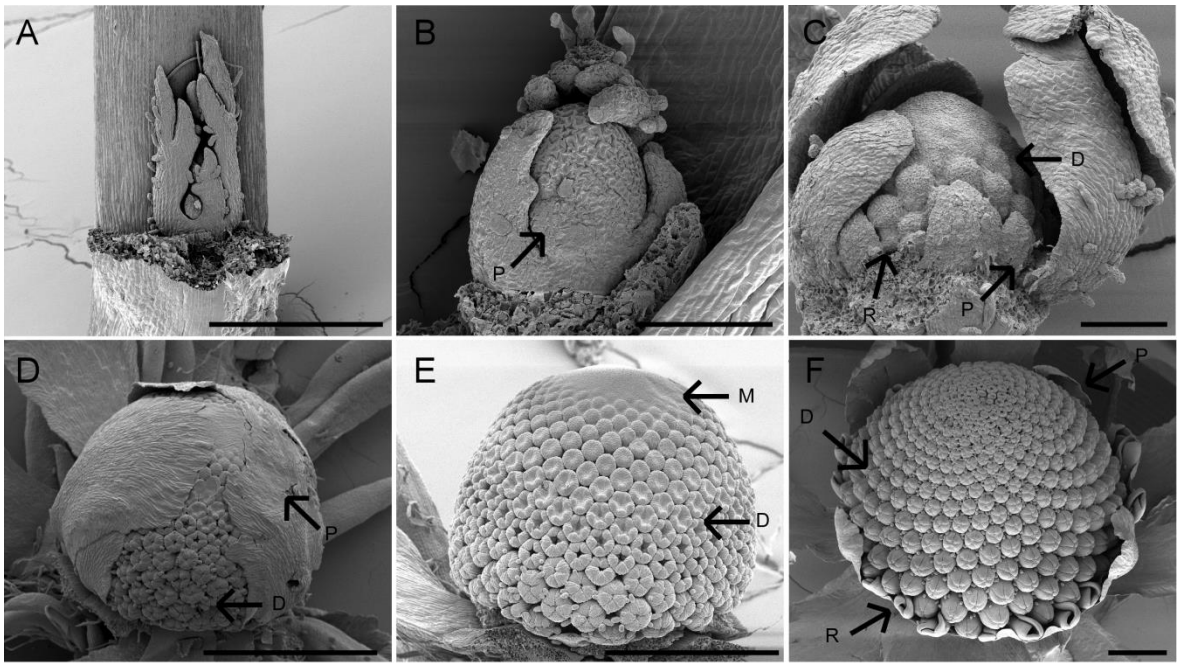


Figure 1. SEM images of developing *M. inodora* meristems. (A) Vegetative meristem. (B-F) Developing inflorescent meristems. D = Disc floret, M = Meristematic zone, P = Phyllary and R= Ray floret. Scale bars are either 100 µm (B, C) or 500 µm (A, D-F).

The ray florets are extremely important functional component of the flower head that attract pollinators; as such they are protected throughout the flower head development until synchronisation of the florets for maximum pollination (Fig. 2A-G) (Funk et al., 2009). Their development is initiated from the meristematic tissue and they quickly form a raised ring of tissue (Fig. 2A, B). Although ray florets are zygomorphic, in the early stages of their development their petals are symmetrical (Fig. 2A, B). As the flower head develops towards formations of the terminal floret (Fig. 1E), the ray florets have barely grown when compared to the disc florets that where formed in the latter in flower head development (Fig. 2B-E). Although the ray florets are developmentally delayed compared to the disc florets, the ray florets have now clearly become zygomorphic (Fig. 2C, D). The three ventral petals of the ray florets that form the distinct out growths are clearly visible early on in the development of the ray florets and continue to expand

whilst the two dorsal petals are inhibited (Fig. 2C-G). A fully developed ray floret was dissected and imaged using SEM, the result of which showed that the floret contained small cells in the three petal dorsal petals (Fig. 2H) suggesting that the elongation is caused by cell division.

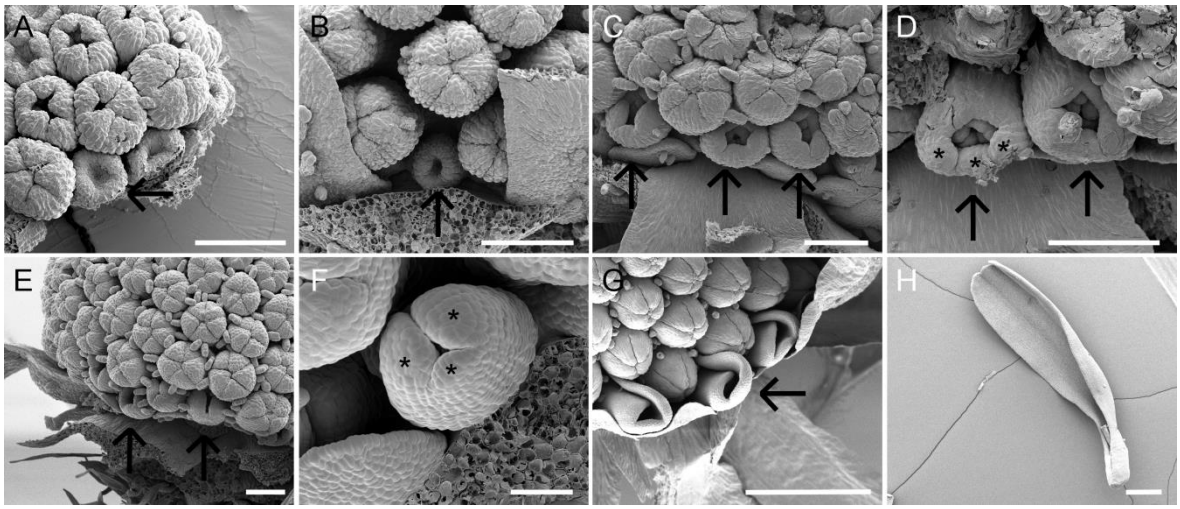


Figure 2. SEM images of developing ray florets in *M. inodora*. (A, B) The images show very young primordia of ray florets (arrows), through to the asymmetrical stages of development (C-G) to finally a developed ray floret (H). Ventral petals are marked with asterisks (D, F) and arrows indicate the position of ray florets (C-E, G). Scales bars are 50 µm (F) 100 µm (A-E) or 500 µm (G, H).

Whilst the ray florets are important in order to attract pollinators, the disc florets of both *M. inodora* and *S. vulgaris* are the main reproductive florets in the respective flower heads. Disc florets, like all lateral organs on the developing flower heads, form in centripetal pattern (Fig. 3A). The disc florets initially form as primordia on the periphery of the meristematic tissue (Fig. 3A, B). After disc floret initiation the five petals that make up the actinomorphic flowers begin to emerge (Fig. 3C). Although the disc floret petals are completely symmetrical at the end of their development, during the early developmental stages they show similar dorsal-ventral petal asymmetry to the developing ray florets (Fig. 2C, Fig. 3C). Figure 3A shows a flower head with several stages of disc floret development, including the development and sequential loss of petal

asymmetry. The five petals eventually merge over the centre of the floret to protect the developing reproductive organs (Fig. 3D).

The asymmetry seen in the developing disc florets raises some very interesting points about their formation (Fig 3A, C). At the earliest stages of disc floret development, they appear as a simple mass of cells (Fig. 3A, B) with no distinguishing characteristics. Previous studies have suggested that the floret primordia formed are identical, and later become differentiated into either ray or disc florets (Yu et al., 1999). The common floret primordia is supported by the single floret ancestor seen in the fossil records as well as genetic experiments showing the difference between discoid and radiate flower heads is only two genes (Barreda et al., 2010, Kim et al., 2008). Both ray and disc florets appear to have a common asymmetrical stage (Fig 2B, C, Fig. 3A, C) but whereas the ray florets maintain that asymmetry, it is lost in the disc florets (Fig. 3D).

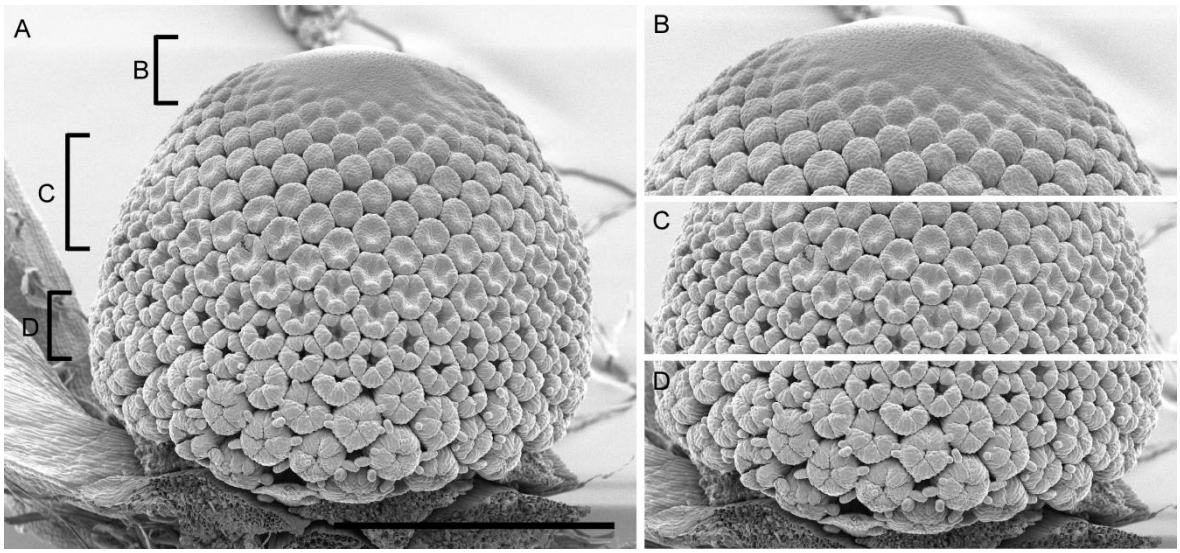


Figure 3. SEM images of a developing *M. inodora* flower head showing several stages of disc floret development. (A) Flower head showing different disc floret developmental stages; From the youngest disc floret primordial (B) through to asymmetrical disc floret petal development (C) to fully developed (D). Brackets indicate area that is focused up. Scale bar is 300 µm (A).

S. vulgaris was previously transformed with the auxin responsive promoter, *DR5,* upstream of the GUS reporter gene. The transgenic *DR5::GUS* plants were used to investigate the role of auxin in the development of the flower head (Zoulias et al., 2014). As previously reported, auxin is accumulated during the formation of phyllaries and ray florets primordia (Fig. 4A-D). During the formation of the phyllary primordia, auxin is accumulated to a maximum (Fig. 4A), as the flower head transitions to the formation of ray florets the auxin concentration decreases (Fig. 4B). Auxin concentrations reach a minimum during the formation of disc floret primordia (Fig. 4B, C). Auxin appears to re-accumulate in the developing ray florets even though the disc florets primordia show very little accumulation of auxin (Fig. 4C). After the meristematic tissue has terminated in the flower head (Fig. 1F), auxin accumulates in the tips of the petals of disc florets (Fig. 4D).

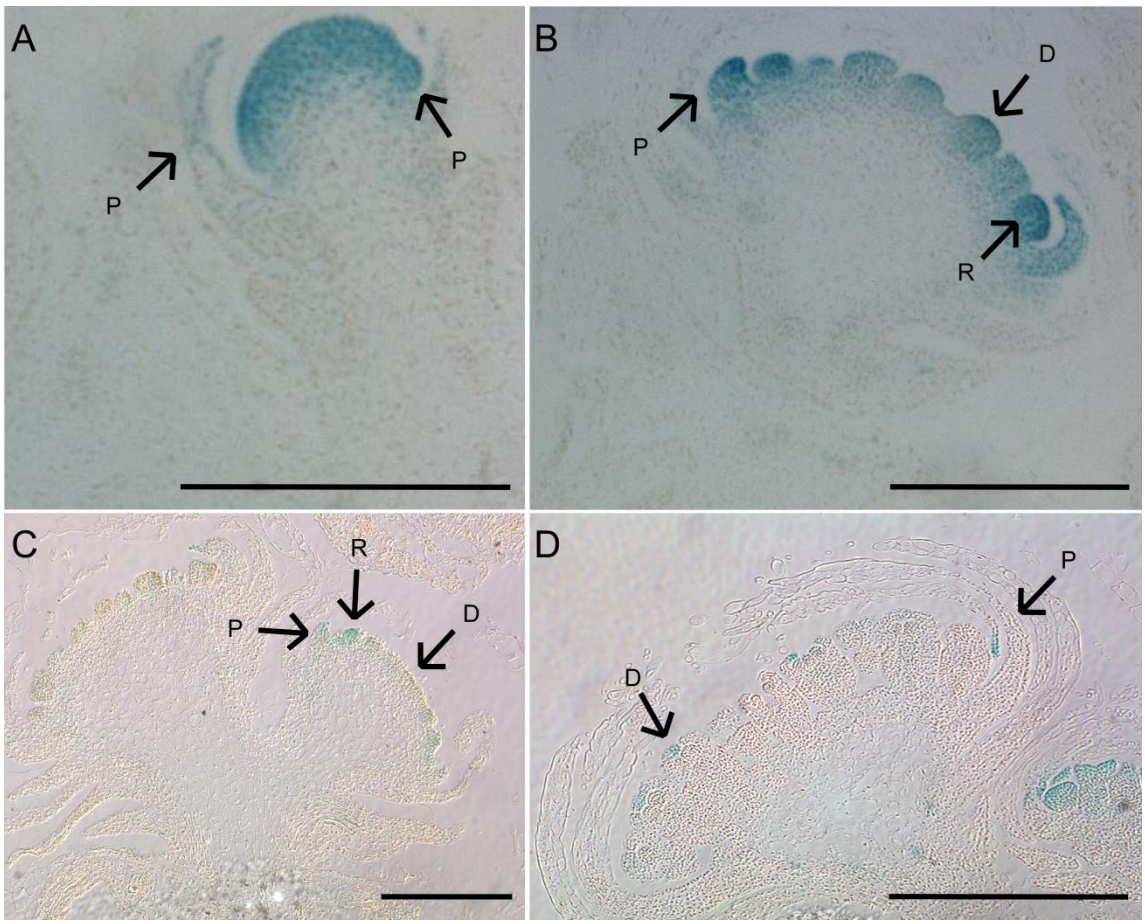


Figure 4. Sections of *DR5::GUS* developing flower heads of *S. vulgaris*. Sections are through various flower heads at different developmental stages of flower head growth from the youngest

(A) through to the oldest (D). Arrows indicate positions of lateral organs with D= Disc floret, P= Phyllary and R= Ray floret. Scales bars are either 50 µm (A, B) or 100 µm (C, D).

4.5 Discussion

The Asteraceae flower head is one of the most common flower forms, yet little is known about its growth and development. Using SEM, detailed analysis of the different developmental stages of both the whole flower head and the different lateral organs was possible. One of the more notable details of the developing flower head is the level of protection given to the ray florets (Chapman and Abbott, 2010). The ray florets are a key trait used to attract pollinators, however, flower heads that are discoid (no ray florets) are usually self-pollinating (Chapman and Abbott, 2010). Before the ray florets are even formed there are already layers of phyllaries to protect them from damage (Fig. 1B). The ray florets maintain an undeveloped state for a long period during flower head development (Fig. 1B-F). This most likely serves two purposes; the first is to protect the ray florets from damage whilst the rest of the flower head develops, the second purpose is to arrest the development of the ray florets to allow the disc florets to fully develop, as it is pointless to attract pollinators if the main reproductive florets are not ready for reproduction. The SEM image of the developing flower head meristem clearly shows that it is a typical "open II-type" meristem that is consumed to end development (Fig. 1E, F) (Bull-Herenu and Classen-Bockhoff, 2011).

The generation of phyllaries is the clearest visible sign that a meristem has transitioned from the vegetative to reproductive state (Fig. 1B). It has been previously reported that the flattening of the meristem is the first sign of the formation of a reproductive meristem. *M. inodora* appears to contradict this observation, as it is initially highly domed (Fig. 1C) until the apex of the flower head begins to form florets by which point it has flattened (Fig. 1E). This difference in observation of

the earliest stage of reproductive development may be due to the fact that previous studies have used *Helianthus annuus*, which in contrast to *M. inodora* have very large flat flower heads. The shape of the phyllaries is similar to a simple leaf form, which has led to the hypothesis that *LFY* is involved in their formation. The suggestion that a loss of *LFY* expression in the earlier flower head meristem allowing for the formation of phyllaries is consistent with the role of *LFY* in model species.

The ability to monitor auxin expression in developing flower heads and detail the development of florets during development facilitates new understanding of the Asteraceae flower head development. Ray florets have the greatest level of understanding about the molecular genetics behind their development. It has been shown that *CYC-like* genes play key roles in the formation of ray florets, but less is known about the development of the ray floret asymmetry (Chapman et al., 2012, Broholm et al., 2008, Kim et al., 2008). During the earlier stages of ray floret development, the developing petal primordium appears to be more symmetrical than asymmetrical (Fig. 2A, B). The symmetry of the developing ray florets suggests that the two dorsral petals are inhibited rather than the ventral petals being elongated. This is consistent with current theories of floret evolution that both ray and disc florets originate from one large tubular floret type, such as seen in some species of the most basal tribe Barnadesieae (Barreda et al., 2010). The limited fossil evidence of Asteraceae flower heads indicates that the original floret type was tubular and actinomorphic, further suggesting that ray florets formed from the inhibition of the ventral petals (Barreda et al., 2010). The well-known interactions between *CYC* and *RADIALIS* (*RAD*) with *DIVARICATA* (*DIV*) to control flower asymmetry in *Antirrhinum,* suggests that they could play a similar role in the Asteraceae floret development (Stevenson et al., 2006, Corley et al., 2005, Costa et al., 2005, Luo et al., 1999, Lou et al., 1995). However, the interactions will be more complex than those in the *Antirrhinum* flower due to the numerous duplications in *CYC, RAD and DIV* gene families in the Asteraceae. The *Arabidopsis* orthologue of *CYC*, TB1,CYC,PCF1 (TCP1), has been shown to have a role in the longitudinal elongation of lateral

organs, which suggests that the *CYC-like* genes during ray floret formation may be contributing to petal elongation causing floret asymmetry (Koyama et al., 2010). Only further research will be able to deduce the true role of *CYC-like* genes in ray floret development.

The elongation of the ventral petals of the ray florets (Fig. 2C-G) also corresponds with the accumulation of auxin in the ray florets. Previous work has shown that different auxin concentrations are able to change floret identity in the flower head, but auxin also appears to have a secondary role in the formation of ray florets (Zoulias et al., 2014). Auxin concentration appears to be at a maxima when ray florets are first formed in *S. vulgaris* flower heads (Fig. 4B), but the concentration drops as disc florets are formed (Fig. 4C). When ray floret development is ready to synchronise with the disc florets, auxin accumulates once more in the developing ray florets (Fig. 4C, D). It is well-known that auxin is involved in both cell division and cell expansion by softening the cell wall (Nakayama et al., 2012, Rayle and Cleland, 1992). In the case of ray floret petal growth it is more likely to be cell division that is affected, due to the lack of elongated cells visible on the fully developed ray florets (Fig. 2H). The cells seen on Figure 2H are small and rounded, suggesting they have not fully expanded laterally and therefore have undergone rapid cell division to expand. The small cells seen in the ray florets are in contrast with the auxin accumulation seen in the tips of the developing disc floret petals (Fig. 4D). In disc floret petals, auxin is seen at the apical tip of the petals and because cell division occurs at the basal end of developing organs it is assumed that auxin is likely be elongating the petals. The cell division seen in the ventral petals of the ray florets could be controlled by several genetic mechanisms. One such mechanism could be through TCP transcription factors. The TCP transcription factor, *CINCINNATA* is known to control cell division in the margin of leaves and petal lobes in *Antirrhinum majus*, and loss of correct *CINCINNATA* expression leads to uncontrolled cell division in the leaves (Crawford et al., 2004). Auxin is also known to play a role in the control of cell division through the activation of the *CYCLIN*-dependent kinases and G-protein-mediated signal transduction (Campanoni et al., 2003, Campanoni and Nick, 2005).

The development of the florets of *M. inodora* seems to vary significantly from the development of florets in *Gerbera hybrida* (Laitinen et al., 2006). Work by Laitinen et al., (2006) indicated that there were no morphological differences in ray and disc primordial development until fairly late in their development. Also, in *Gerbera hybrida* there appears to be no asymmetrical stage of disc floret petal development (Laitinen et al., 2006), whereas in *M. inodora* both disc and ray florets show petal asymmetry at different stages (Fig. 2, 3). The differences in petal symmetry in the florets of *M. inodora* and *Gerbera hybrida* could be down to differences in molecular patterning as the *Gerbera* flower contains a third 'transition' floret type which *M. inodora* does not (Laitinen et al., 2006).

In conclusion, the study of the morphology of the Asteraceae flower heads in precise detail using SEM and combining the visual information with known molecular genetics from both Asteraceae and model species allows for several hypothesis to be formed about the control of flower head development. Further research into the reverse and forward genetics in the Asteraceae family will greatly further our understanding of the development of this fascinating and complex flower form.

Chapter 5

Virus induced gene silencing as a genetic tool in the Asteraceae family

5.1 Abstract

The Asteraceae family is one of the largest families of flowering plants. Members of the Asteraceae family are globally important to agriculture and horticulture. Despite this importance there has been little research to untangle the molecular mechanisms behind their most important traits. The lack of molecular research is in part due to the difficulty to transform species from the Asteraceae family. Here we assessed the promise of virus induced gene silencing as a method for reverse genetics in *Senecio vulgaris* and *Matricaria inodora*. Results indicate that virus induced gene silencing is functional and has great potential as a genetic tool.

5.2 Introduction

To qualify as a successful model plant species, the ability to undergo successful genetic screens is crucial. Whilst forward genetic screens are very successful at identifying new phenotypes of interest, the isolation of the gene(s) responsible for a particular phenotype can be a long and frustrating process typically. Reverse genetics has allowed for the precise manipulation of gene(s) of interest, but relies on having a method of stable transformation. The *Agrobacterium* mediated floral dip method of transformation revolutionised reverse genetics in *Arabidopsis thaliana* and allowed it to flourish as a model species (Clough and Bent, 1998). In contrast to the floral dip method, traditional tissue culture techniques are both time and labour-intensive (Clough and Bent, 1998). The time consuming process of tissue culture transformation limits the potential of using new model species in genetic studies.

The use of virus induced gene silencing (VIGS) has helped non-model species transition into model species (Benedito et al., 2004). VIGS relies on the plant's ability to recognise and degrade double-stranded RNA (dsRNA) as an evolutionary defence mechanism against viral infection (Lu et al., 2003, Ratcliff et al., 1999, Fire et al., 1998, Klahre et al., 2002). The process of dsRNA recognition, leading to sequence specific RNA degradation, is known as RNA silencing (Ratcliff et al., 1999, Hamilton and Baulcombe, 1999, Martinez et al., 2002). In brief, when a plant cell detects dsRNA it is cleaved into small fragments of dsRNA, known as small interfering RNA (siRNA), by the DICER endoribonuclease (Hamilton and Baulcombe, 1999, Hammond et al., 2000). The siRNA is then incorporated into the RNA-induced silencing complex (RISC) and is used as a template to locate complimentary mRNA (Hammond et al., 2000, Elbashir et al., 2001, Martinez et al., 2002). Once the complimentary mRNA is bound to the RISC/siRNA complex the RNase activity of RISC is activated cleaving the mRNA strand (Hamilton and Baulcombe, 1999, Hammond et al., 2000, Elbashir et al., 2001). VIGS takes advantage of this process by introducing a fragment of a gene of interest into a plasmid containing the viral replication machinery (Lu et al., 2003). When

transfected into the native plant, the viral replication machinery expresses the fragment of the gene of interest along with viral dsRNA that is then targeted for degradation (Lu et al., 2003). The sequence of the gene of interest is now recognised as viral and leads to the targeting of the native mRNA to post-transcriptional silencing. As the process of RNA silencing as a defence against viral infection is well conserved, the potential of VIGS to be a cross taxa tool for gene specific silencing was quickly realised. Recent research has shown VIGS to be functional in both monocots and eudicots including economically important species such as cotton, barley and soybean (Zhang et al., 2009, Gao et al., 2011, Lee et al., 2012). In monocot crops (*e.g.,* wheat and barley) that struggle with traditional transformation techniques due to polyploidy, VIGS has been demonstrated to be an effective tool to perform functional gene analysis (Lawrence and Pikaard, 2003, Shah et al., 2009, Holzberg et al., 2002). VIGS also allows for the quicker translation from candidate genes in model species (*e.g., Arabidopsis*) to the application of the genes in a crop species (Manmathan et al., 2013). In tomato and strawberry, VIGS has been used on fruit that has been removed from the parent plant (Romero et al., 2011, Chai et al., 2011). Using fruit that has been removed from the parent allows functional gene analysis at specific developmental stages without interfering with earlier stages of growth and development (Romero et al., 2011). Analysis of gene expression post VIGS has shown that VIGS is inefficient at knocking down gene expression (Liu et al., 2002). Whilst this can be perceived as a negative attribute of VIGS, when performing developmental studies on master regulators having a spectrum of knock down phenotypes is advantageous, especially if they are embryo lethal.

The main viral machinery used for eudicot VIGS is that of the tobacco rattle virus (TRV), although this is not ubiquitous (Liu et al., 2002). TRV is a highly successful plant pathogen that has been found to infect many species outside of the Solanaceae family (Burch-Smith et al., 2006, Wege et al., 2007, Gould and Kramer, 2007). TRV is transmitted to plants when they are parasitized by nematodes which host the virus. In Solanaceae species such as potato and tomato, TRV can cause major agricultural problems such as corky ringspot on the growing tubers (Riga et al., 2007). TRV

has a single stranded RNA genome that is separated into two strands with separate functions. The two different strands, RNA1 and RNA2, were cloned to form the VIGS vectors pTRV1 and pTRV2 respectively (Bergh et al., 1985, Hamilton et al., 1987, Liu et al., 2002).

The Asteraceae family is one of the largest species of flowering plants, it contains many agriculturally (sunflower, lettuce) and ornamentally (chrysanthemum and zinnia) important species. The success of the Asteraceae family is thought to be due to the evolution of its pseudanthium (False flower) (Harris, 1999). Whilst the pseudanthium of the Asteraceae family appears to look like a singular flower it is in fact a collection of florets which mimic the appearance of a singular flower. Although the Asteraceae pseudanthium is an important and interesting biological structure to study, there has been sparse research into the molecular genetics of the pseudanthia development. The lack of research can be attributed to two main factors; Lack of sequence information and technical difficulty in transformation. Sequencing technology has greatly improved in recent years and there are now sequencing projects focused on several Asteraceae species (Bowers et al., 2012). Unfortunately the ability to make transgenic Asteraceae plants is still a long and hard process. Here we investigate the use of VIGS on two up and coming Asteraceae model species, *Senecio vulgaris* (*S. vulgaris*) and *Matricaria inodora* (*M. inodora*), and compare this novel methodology to more traditional tissue culture methods.

5.3 Method

Plant growth and conditions

Seedlings of both *S. vulgaris* and *M. inodora* were sown onto damp vermiculite in propagator trays, covered with plastic lids and left to germinate in a growth room under long day conditions (150 µmol $m^{-2}S^{-1}$, 16hr light, 24˚C day temperature). After one week, seedlings were either used for VIGS, or transferred to in 4 inch square pots on Sinclair compost (William Sinclair Holdings plc, UK) until ready for VIGS (*S. vulgaris* one week, *M. inodora* one month).

Cloning and Vector construction for VIGS

PHYTOENE DESATURASE (*PDS*) gene fragments from both *S. vulgaris* and *M. inodora*, *LEAFY* (*LFY*) from *S. vulgaris* and *PIN-FORMED1* (*PIN1*) from *M. inodora* were cloned into pDrive (Qiagen, UK) using degenerate primers (Table 1) (Wege et al., 2007). Gene fragments along with the VIGS vector pTRV2 were digested with SacI and BamHI (New England Biolabs, UK) (Liu et al., 2002). T4 DNA ligase was used according to manufacturer's specifications, to ligate the sticky ends of the gene fragments and pTRV2 in a 3:1 ratio respectively. Plasmid specific primers (Table 1) were used for colony PCR in order to confirm a successful ligation and also for the sequencing of gene fragments in pTRV2. Once it was confirmed that the correct gene fragment was successfully inserted into pTRV2, the construct was transformed (electroporation) in *Agrobacterium tumefaciens* (*A. tumefaciens*) (GV3101). Transformed *A. tumefaciens* was screened using colony PCR and sequenced to ensure the correct gene fragment was inserted.

Virus induced gene silencing (VIGS)

Virus induced gene silencing was performed on week old seedlings from both *S. vulgaris* and *M. inodora*, as well as two week old seedlings from *S. vulgaris*. Flowering (two months) *M. inodora* was also used for VIGS, in order to manipulate the developing flower head. pTRV2-PDS constructs

were grown overnight (optical density 1.0 at 600 nm), pelleted and then suspended in an equal volume of infiltration media (10 mM MES, 200 µM acetosyringone and 10 mM $MgCl_2$) and left to incubate for 3 hours in the dark at room temperature. pTRV1 plasmid, also transformed in *A. tumefaciens*, was grown, pelleted and resuspended as pTRV2-PDS, with the added to pTRV2-PDS in a 1:1 ratio. pTRV2 with no insert was used as a negative control (Liu et al., 2002). Seedlings were added to the infiltration mix after incubation and vacuum infiltrated for 5-10 minutes (or until bubbles were no longer dispersing from the cotyledons). Seedlings were removed from the infiltration mix and dried on paper towels before transplanting into 40 cell tray inserts (Gardman, UK) and covered for 48 hours. After 48 hours of incubation the cover was removed slowly to allow for the seedling to adjust. Seedlings typically developed a phenotype 1-2 weeks after inoculation with *A. tumefaciens*.

Two week old *S. vulgaris* and two month old *M. inodora* plants underwent syringe infiltration of *A. tumefaciens* in order to try and induce VIGS. *A. tumefaciens* cultures for pTRV1, pTRV2, pTRV2-*PDS*, pTRV2-*LFY* and pTRV2-*PIN1* were prepared as described previously. Approximately 1 ml of the 1:1 *A. tumefaciens* mix was infiltrated into each plant (multiple leaves for *S. vulgaris* or directly into the developing inflorescence for *M. inodora*). After infiltration, plants were grown till flowering and observed for distinct phenotypes.

pTRV1 forward	CTTGAAGAAGAAGACTTTCGAAGTCTC	Sequencing and colony PCR
pTRV1 reverse	GTAAAATCATTGATAACAACACAGACAAAC	Sequencing and colony PCR
pTRV2 forward	GGTCAAGGTACGTAGTAGAG	Sequencing and colony PCR
pTRV2 reverse	CGAGAATGTCAATCTCGTAGG	Sequencing and colony PCR
DeLFY forward	CARAGRGAGCAYCCSTTYATYGT	Degenerate primers
DeLFY reverse	GACGMAGCTTKGTKGGRACATACCA	Degenerate primers
DePIN1 forward	CCNAAYACBYTNGTNATGGG	Degenerate primers
DePIN1 reverse	CTKGARCTCCAVACRAACAT	Degenerate primers
DePDS forward	GAGGTGTTCATCGCAATGTCAAAGGC	Degenerate primers
DePDS reverse	GTGTTGTTGAGCTTTCGGTCAAACCATATATG	Degenerate primers

Table 1. Primers used for the study, in 5’ to 3’ orientation.

5.4 Results

In order to assess whether VIGS is an appropriate methodology for studying forward genetics in *S. vulgaris* and *M. inodora*, many different constructs were need. Degenerate PCR was employed to clone out fragments of the genes of interest for VIGS. From *S. vulgaris PHYTOENE DESATURASE* (*PDS*) and *LEAFY* (*LFY*) fragments and from *M. inodora PDS* and *PIN1* fragments were cloned into pDrive. *PDS* was selected for both species because of its clear phenotype of white leaves when knocked down. This is because *PDS* encodes an enzyme in the carotenoid biosynthesis pathway, and when knocked down/out carotenoid biosynthesis is disrupted leading to the photobleaching of chlorophyll and the production of white leaves (Fig. 1D, Fig. 2C-E) (Kumagai et al., 1995). Constructs were made using traditional restriction and ligation techniques, the gene of interest was digested out of pDrive and ligated into the multiple cloning sites of pTRV2 (Fig. 1A). pTRV1 contains the viral polymerase needed to create dsRNA of the gene of interest and is therefore needed in every VIGS experiment. pTRV1 in *A. tumefaciens* was mixed with pTRV2 in a 1:1 ratio before being infiltrated into the plant. The bacterial inoculant was allowed to activate for three hours by the addition of acetosyringone, a phenolic compound produced in wounded dicotoydens, which is recognised by receptors in Ti plasmid of *A. tumefaciens*.

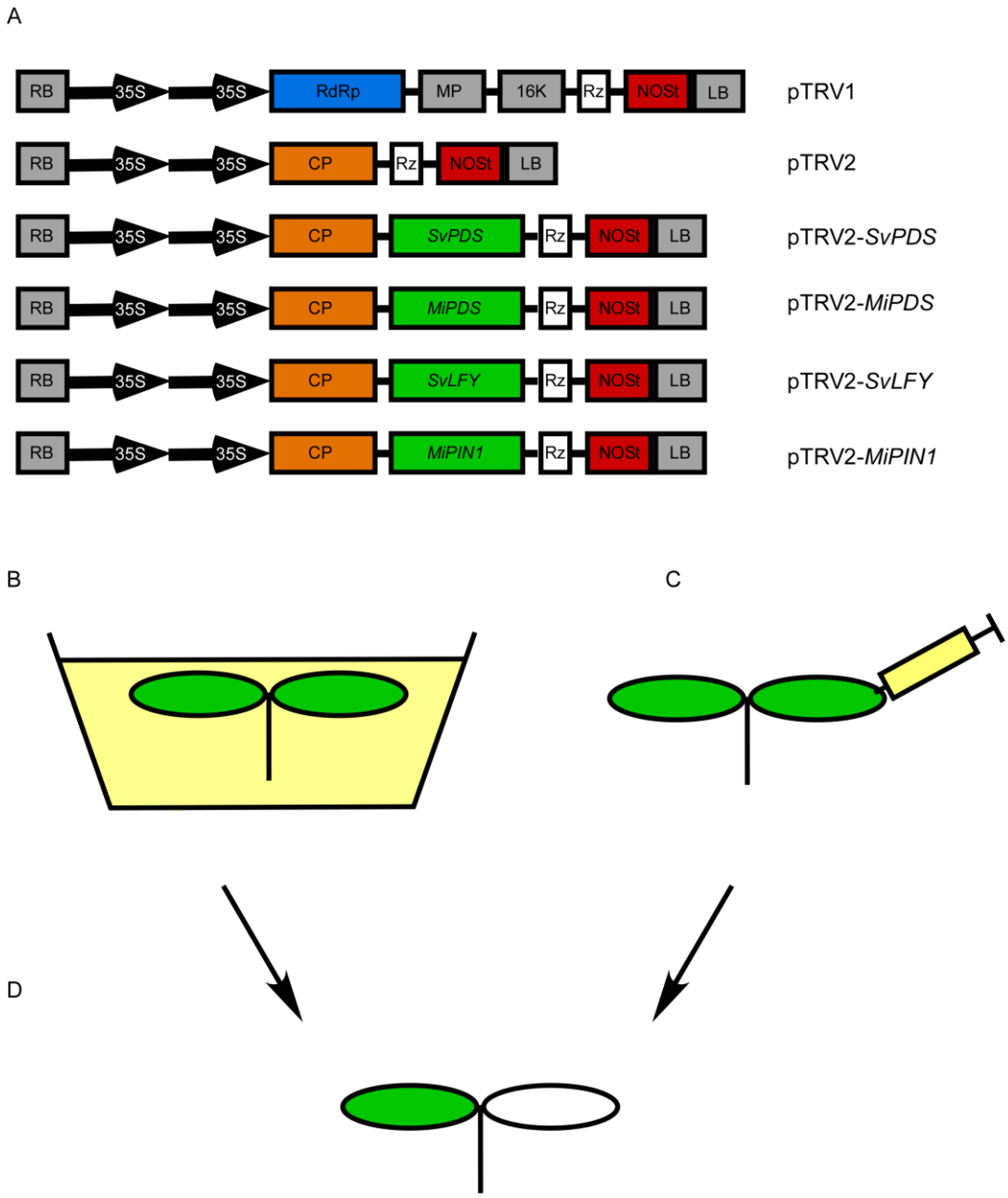


Figure 1. Schematic diagrams of vectors and methods used throughout VIGS experiments. (A) Linearised sections of the pTRV1 and pTRV2 vectors between the right boarder (RB) and left boarder (LB) used for VIGS. (B) The first VIGS method used for both *S. vulgaris* and *M. inodora* was vacuum infiltration on seedlings. Seedlings were submerged into infiltration media containing *A. tumefaciens* with both pTRV1 and pTRV2 and placed into a vacuum. (C) Leaf infiltration of *S. vulgaris* and *M. inodora* was performed by using a syringe to force infiltration media containing *A. tumefaciens* with both pTRV1 and pTRV2 into both species. (D) After one week to a month, plants

were screened visually for a particular phenotype. For the positive control gene, *PDS*, photobleached leaves were screened (D).

Two different infiltration methods were tested for both *S. vulgaris* and *M. inodora*: The first method involved whole seedling infiltration, which consisted of submerging week old seedlings into vessels containing the infiltration mixture (pTRV1:pTRV2 or pTRV1:pTRV2-*PDS*) inside a vacuum and infiltrating until bubbles were no longer dispersing from the cotyledons (Fig. 1B). Infiltrated seedlings were then planted onto soil, incubated in a growth room and allowed to recover. One to two weeks after infiltration, the visual phenotype of *PDS* (photobleached leaves) was screened for (Fig. 1D). The second method of infiltration used for VIGS was syringe infiltration, in which the infiltration mixture is introduced to the plants through a syringe (Fig. 1C). Phenotypes related to the particular gene of interest were screened for a one month period starting a week after infiltration (Fig. 1D).

Species	infiltration method	Gene	Starting number	Survival	Phenotypes observed
S. vulgaris	Vacuum	pTRV2	60	25%	0.00%
S. vulgaris	Vacuum	*PDS*	60	18%	5.00%
S. vulgaris	Syringe	pTRV2	60	93%	0.00%
S. vulgaris	Syringe	*PDS*	60	97%	1.67%
S. vulgaris	Syringe	*LFY*	60	100.00%	0.00%
M. inodora	Vacuum	pTRV2	30	20%	0.00%
M. inodora	Vacuum	*PDS*	30	27%	3.33%
M. inodora	Syringe	pTRV2	2	100%	0.00%
M. inodora	Syringe	*PIN1*	2	100%	50.00%

Table 2. Number of phenotypic plants observed throughout the different VIGS studies on both *S. vulgaris* and *M. inodora*.

For *S. vulgaris* the vacuum infiltration method had the highest rate of success (5%) although this also corresponded with a low survival rate (25%; Table 2, Fig. 2D). There was limited success with the syringe infiltration method in *S. vulgaris* with one plant showing the *PDS* phenotype after infiltration but displaying a very high survival rate (96.67%; Table 2, Fig. 2C). *S. vulgaris* plants infiltrated with the pTRV2- PDS construct using both methods showed typical *PDS* knock- down characteristics of photobleached leaves (Fig. 2 C, D) when compared to wildtype or control leaves that have no photobleaching (Fig. 2A, B, E).

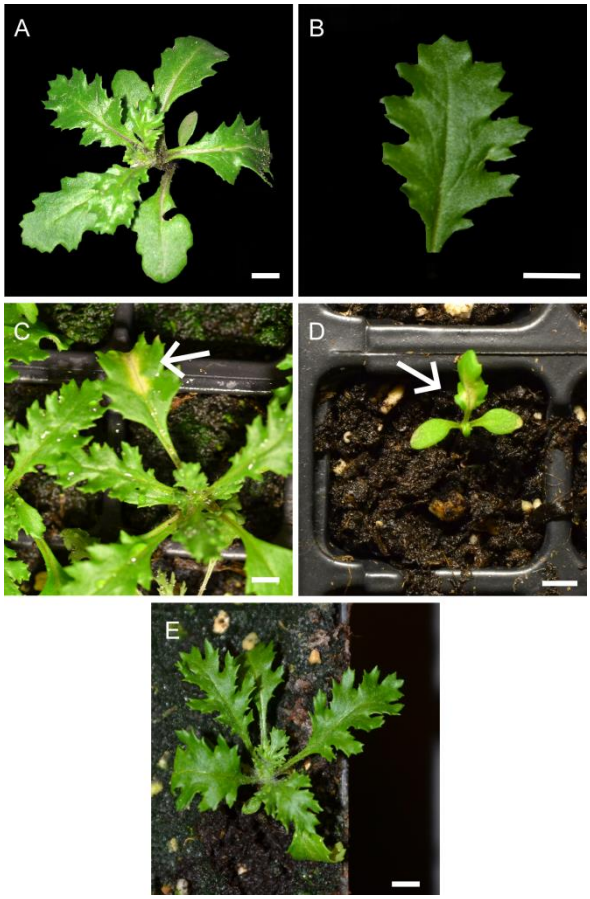


Figure 2. The effects of VIGS on the *PDS* gene in *S. vulgaris*. (A, B) Wild-type *S. vulgaris* plants display healthy green leaves with no signs of photobleacing. (C) *S. vulgaris* syringe infiltrated with pTRV2-*PDS*, display typical photobleaching phenotypes associated with *PDS* knockdown. (D) *S. vulgaris* vacuum infiltrated with pTRV2-*PDS*, display typical photobleaching phenotypes associated with *PDS* knockdown. (E) Control *S. vulgaris* syringe infiltrated with pTRV1 and pTRV2 with no insert. Scale bars are all 5 mm.

VIGS also appeared to be successful in *M. inodora*, showing phenotype for both vacuum (*PDS*) and syringe (*PIN1*) infiltration methods (Table 2). Like *S. vulgaris*, *M. inodora* had a low survival rate for vacuum infiltration of one week old seedlings, but showed very promising results using syringe infiltration on flowering plants (Fig. 3C). The pseudanthium of *M. inodora* is typical of the Asteraceae family, it consists of several rows of phyllaries, followed by a single row of white ray florets with many disc florets in the centre of the pseudanthium (Fig. 3A). When the developing pseudanthium is locally treated with 1-N-Naphthylphthalamic acid (NPA), an inhibitor of polar auxin transport, the ray florets fail to form or are severally stunted (Fig. 3C) (Geldner and Palme, 2001, Zoulias et al., 2014). As NPA strongly inhibits the ability of PIN1 to transport auxin in a polar manner, it was hypothesised that a reduction in PIN1 levels through VIGS would lead to similar phenotypes. When the developing inflorescent was infiltrated with *pTRV2-PIN1* the phenotypes observed in the flowers post-infection mirrored those treated with NPA (Fig. 3E, F), whereas when the developing flower head was infiltrated with empty pTRV2 there was no visible phenotype (Fig. 3B). The VIGS treated pseudanthium of *M. inodora* showed both phenotypes associated with NPA treatment of missing or shortened ray florets (Fig. 3E, F). The colour of VIGS treated pseudanthium was unaffected, in contrast to the overall shape of the remaining ray florets which were more tubular than wild-type pseudanthium (Fig. 3E, arrows). This indicates that although only part of the pseudanthium is showing PIN1 knockdown characteristics, the rest of the developing pseudanthium has also been affected. pTRV2-*PIN1* infiltrated flower heads that display tubular ray florets phenocopy flower heads that have been treated with indole-3-acetic acid (IAA) (Fig. 3D, arrows) (Zoulias et al., 2014).

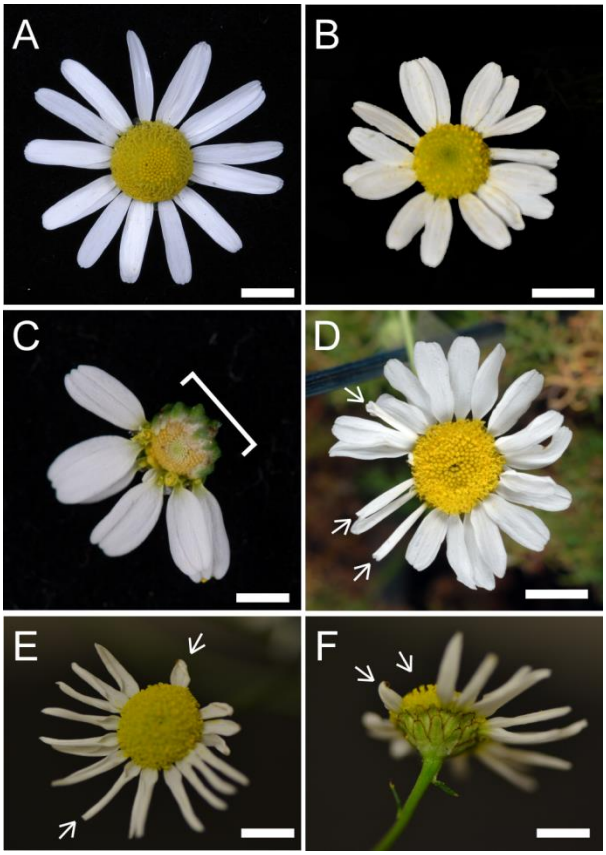


Figure 3. Effect of *PIN1* VIGS on the developing pseudanthium of *M. inodora*. (A) Wild-type pseudanthium of *M. inodora*.(B) *M. inodora* flower syringe infiltrated with the empty pTRV2 vector showing no phenotype. (C) NPA treated (Bracket-local induction) pseudanthium of *M. inodora*. (D) Auxin (IAA) treated flower head showing tubular ray floret phenotype. (E, F) *M. inodora* syringe infiltrated with pTRV2-*PIN1*, displaying typical absence of ray floret phenotypes associated with PIN1 polarisation inhibitors. Scale bars are all 5 mm.

5.5 Discussion

VIGS is an extremely promising tool that has the potential to speed up the long and slow process of tissue culture transformation in non-model species of plants (Shah et al., 2009). The *PDS* gene has been widely used as a control gene in order to evaluate the potential of VIGS in a new species (Burch-Smith et al., 2006, Wege et al., 2007, Romero et al., 2011). *PDS* is highly conserved, allowing it to be easily cloned using degenerate primers, which in turn is perfect for non-model species that may have unsequenced genomes (Kumagai et al., 1995). *PDS* orthologs were effectively cloned from both *S. vulgaris* and *M. inodora*, along with *LFY* and *PIN1* respectively (Fig. 1A). As previously reported, *PDS* performed very well as a positive control gene for VIGS (Wege et al., 2007). For both *S. vulgaris* (Fig. 2C, D, Table 2) and *M. inodora* (Table 2), visual phenotypes of *PDS* knockdown were visible. The gene silencing caused by VIGS in *S. vulgaris* and *M. inodora* was never systemic, that is to say that it was contained to the area of infiltration and never spread to new leaves (Fig. 2 C, D, Fig. 3E, F). Whilst, this is a promising first step for the optimisation of VIGS in *S. vulgaris* and *M. inodora*, the containment of the phenotype to the infiltrated leaves suggests that the host immune system may be able to stop the spread of the silencing. Gene silencing caused by VIGS has been well reported for being able to cause systemic infection, in some case the infection is able to persist through to subsequent generations (Burch-Smith et al., 2006, Wege et al., 2007, Gould and Kramer, 2007, Bruun-Rasmussen et al., 2007). In other Asteraceae species in which VIGS has been shown to be a successful technique, the phenotypic photobleaching due to the reduced levels of *PDS* has become systemic and spread to newly formed leaves (Deng et al., 2012). Due to the limited sample size, it is not currently clear whether the lack of systemic spread of VIGS in *S. vulgaris* is due to immunity or methodology, but the localised photobleaching points towards the latter.

Contrary to *S. vulgaris*, the use of VIGS in *M. inodora* appears to be more promising as the phenotypes seen in the *M. inodora* pTRV2-*PIN1* syringe infiltrated plants phenocopy those

produced by the NPA treatment of the developing pseudanthium (Table 2, Fig. 3.C, E, F). NPA inhibits the polarisation of PIN1, which in developing *M. inodora* pseudanthium disrupts the native auxin gradient and inhibits the formation of ray florets (Geldner and Palme, 2001). If the pTRV2-*PIN1* infilitration in *M. inodora* was able to successfully knockdown *PIN1* expression, the resulting phenotype should copy NPA treated pseudanthium (Fig. 3B-D). The phenotypes seen in the *M. inodora* pseudanthium indicate that knockdown of *PIN1* is not evenly distributed throughout development, as ray floret loss only appears in part of the pseudanthium. However, a more mild phenotype of tubular ray florets is seen through the rest of the pseudanthium, suggesting that there is some wide spread knockdown of *PIN1* when compared to native levels (Fig. 3E, F). Tubular ray florets were first seen in *M. inodora* when developing flower heads were treated with auxin (Fig. 3D) (Zoulias et al., 2014). The mimicking of the tubular ray floret phenotype by *PIN1* VIGS infiltrated plants further suggests that *PIN1* has been knocked down, as in order to achieve tubular ray florets auxin must be incorrectly transported. In *pin1 Arabidopsis* mutants, fused or cup shaped leaves are formed as a result of incorrect auxin transportation, which is analogous to the formation of tubular ray florets formed after *PIN1* VIGS on *M. inodora* flower heads (Reinhardt et al., 2003).

Traditional tissue culture methods will always have one distinct advantage over VIGS, which is that the phenotypes seen are stable and not transient or localised like those caused by VIGS. This was extremely apparent when looking at knocking down *LFY* expression in *S. vulgaris.* Using VIGS methodologies, no phenotype was record for pTRV2-*LFY* although there were phenotypes for *PDS*. In comparison, when using traditional tissue culture methods to make antisense *LFY*, phenotypes were found for several lines (Data not shown). Another issue of the transient nature of VIGS for most species is that the phenotypes are not inherited, so replicate experiments have to come from a new set of biological samples. This is especially problematic for developmental studies as it makes it harder to separate out particular phenotypes from biological noise. VIGS is as known to not be very efficient at knocking down expression of the targeted gene (Liu et al.,

2002). Although this can be a disadvantage, in developmental studies having a range of expression levels is advantageous. VIGS was recently used in a developmental study to assess the function of two genes in the formation of cotton fibres (Qu et al., 2012). One of the genes used in the study was *KATANIN*, a gene well known for microtubule dynamics and organisation (Burk et al., 2001, Wightman et al.). VIGS was successful at both reducing expression levels of *KATANIN* and the distribution of microtubule arrangements, which resulted in shorter and fewer cotton fibres (Qu et al., 2012). In the non-model species *Eschscholzia californica* (California poppy) VIGS has been used with great success to study flower development genes (Orashakova et al., 2009, Lange et al., 2013). The aforementioned studies offer an insight to how a VIGS could impact our understanding of flower head development.

VIGS does possess further substantial advantages over traditional tissue culture methods, which are its speed and ease to perform when compared to tissue culture. After cloning, which needs to be completed for either method, VIGS can be completed within a month. In contrast to VIGS, tissue culture methods may take 6-8 months before it is possible to observe phenotypes and includes many stages in which the transformation may be lost along the way. Also, the skill and specialist equipment needed to complete VIGS is minimal when compared to tissue culture. Finally, there is no published method for the tissue culture transformation of *M. inodora*, whereas we were quickly able to establish a basic protocol for VIGS. New VIGS based methodologies are being developed on a regular basis, which improve the potential of VIGS. One such methodology is the expression or over expression of genes using viral promoters, this will greatly compliment VIGS studies through a more complete functional analysis of genes (Rybicki and Martin, 2014).

VIGS appears to be a promising technique for use in the newly developing model species of *M. inodora* and *S. vulgaris*. Although further experimentation is necessary to confirm the reproducibility of the technique as well as to confirm on a molecular level that the genes of interest are being knocked down/out. Overall VIGS in both *S. vulgaris* and *M. inodora* has been

successful and provides the possibility of a quick and easy method of reverse genetics in these two future model species.

Chapter 6

General Discussion

6.1 Study Overview

The flower head of the Asteraceae family is one of the most successful flower forms in the Angiosperms (Bremer, 1994). Originating in South America, it has spread throughout the world with the exception of Antarctica and the extreme Artic (Barreda et al., 2010). It is also an important economical crop family globally, with the flower head being the main organ of consumption in Sunflowers (*Helianthus annuus*) and artichokes (*Cynara cardunculus var. scolymus*). In the United Kingdom alone, 60 grams of lettuce (*Lactuca sativa*) are consumed per person per week (Hospido et al., 2009). In contrast to the economic importance, the Asteraceae family also contains some of the top noxious and injurious plants described in the Weeds act of 1959, such as *Senecio jacobaea*, *Cirsium arvense* and *Cirsium vulgare* (Defra, 1959). Yet, despite the importance of Asteraceae crops to global economics and agriculture, there is little known about the reproductive development of the Asteraceae family.

This thesis aimed to understand the complex flower head development of the Asteraceae family. The focus of this thesis was the role of the plant hormone auxin in the flower head development in two potential model species *S. vulgaris* and *M. inodora*. Initially, developing flower heads of *M. inodora* were sprayed with auxin (IAA) and an auxin transport inhibitor (NPA) in order to disrupt native auxin concentration and movement respectively (Geldner and Palme, 2001). The results of which indicated that auxin plays a major role in organ identity and pattern formation in the developing flower head of *M. inodora*. Spraying developing flower heads with different concentrations of IAA, suggested that both organ identity and patterning are in fact auxin concentration dependant.

In order to understand the native auxin patterning within the flower head, a construct containing the auxin responsive promoter, DR5, located upstream of the visual reporter gene GUS was inserted into *S. vulgaris* (Ulmasov et al., 1997b). GUS patterning in the developing flower heads confirmed that the distribution of auxin appeared to be at the most concentrated during the

formation of phyllaries and least concentrated during the formation and development of disc florets. Sectioning of GUS stained *S. vulgaris* flower heads revealed the existence of an auxin gradient. After different auxin treatments, the specific responses of known and candidate lateral organ identity genes (Kim et al., 2008), were tested using qPCR and *in situ* hybridisations. The expressional regulation of *MiLFY* and *MiRAY2* to different concentrations of auxin further confirmed the role of auxin in lateral organ identity and pattern formation. SEM analysis of *M. inodora* flower heads allowed for in depth developmental studies of individual lateral organs and provided a greater understanding of overall flower head development.

Virus induced gene silencing (VIGS) was assessed as an experimental method to perform reverse genetics on both *S. vulgaris* and *M. inodora*. VIGS has been used successfully in many species including those that do not have a traditional tissue culture transformation method (Wege et al., 2007). Using a fragment of *PDS* as a positive control (Liu et al., 2002), VIGS on both *S. vulgaris* and *M. inodora* resulted in the appearance of photobleached leaves that are typical of a *PDS* knock down/out phenotype. Significantly, VIGS of *M. inodora PIN1* on developing *M. inodora* flower heads resulted in flower heads which phenocopied those that had been treated with the auxin inhibitor NPA (Zoulias et al., 2013). This suggested that VIGS is functional in *M. inodora* and a good candidate as a reverse genetics tool.

6.2 Auxin and the flower head

Auxin has been extensively researched as a hormone of great importance in plant growth and development for the past three centuries. Whilst there has been an extensive amount of research into the function of auxin in model species such as *A. thaliana*, there has been little or no research into the role of auxin in non-model species. In order to understand the role of auxin in flower head development, developing flower heads of *M. inodora* were treated with exogenous auxin (IAA) and a polar auxin transport inhibitor (NPA) (Geldner and Palme, 2001). When developing

flower heads are treated with NPA there can be a resulting complete or partial loss of ray florets (Zoulias et al., 2014). In this investigation, local application of NPA to developing flower heads caused only the site of local application to show the loss of ray floret phenotype. The loss of a lateral organ following treatment with NPA is consistent with the literature. For example, in both *A. thaliana* and *Solanum lycopersicum* (Tomato), meristems treated with NPA lead to a loss of flower or leaf primordial respectively (Reinhardt et al., 2000, Koenig et al., 2009). In contrast to *A. thaliana* treated with NPA which produces no lateral organs (Reinhardt et al., 2000), developing flower heads of *M. inodora* still produced a lateral organ (disc florets) but lose the formation of another lateral organs (ray florets) (Chapter 3, Fig. 1). This suggests that either polar auxin transport does not play a role in the formation of disc florets in the flower head or the effects of NPA have diminished by the time of their formation.

Contrary to NPA application, when flower heads are treated with IAA they show both changes in lateral organ identity and pattern formation. The new patterns always reflect the original patterning of phyllaries > ray florets > disc florets, with the conversions always appearing to be disc floret to phyllary or ray floret. The ability to maintain the original flower head pattern formation after wounding has been observed in developing sunflower heads (Hernandez and Palmer, 1988), which is consistent with the results of auxin treatment of developing flower heads. Hernandez and Palmer showed that the carving of a circular wound around the developing sunflower meristem produced converted lateral organs in the original pattern on the flower (Hernandez and Palmer, 1988).

The conversion of one lateral organ type to another lateral organ type through change of auxin concentration has never been documented in plants before. Usually auxin treatment of meristematic tissue results in a change of phyllotaxy or the formation of ectopic organs (Marchant et al., 2002, Reinhardt et al., 2003, Koenig et al., 2009). In order to investigate whether the conversion to phyllary or ray floret from disc floret was auxin dependant, two different auxin

concentrations were sprayed on developing flower heads of *M. indora.* The results of the spraying experiment indicated that the type of lateral organ conversion was auxin concentration dependant. For example, at a higher auxin concentration the tendency was to convert from disc florets to phyllaries, whereas at a lower auxin concentration the tendency was to convert from disc florets to ray florets. Taken together, the preservation of the lateral organ patterning in the flower head along with the concentration dependant organ patterning suggests that an auxin gradient is controlling flower head formation in *M. inodora*.

6.3 Auxin as a morphogen

In treating developing *M. indora* flower heads with different concentrations of IAA it was revealed that the formation of phyllaries and ray florets may be auxin concentration dependant. To further investigate the role of auxin in flower head development and pattering, *DR5::GUS* was transformed into *S. vulgaris* to enable the visualisation of auxin *in situ* (Ulmasov et al., 1997b, Ulmasov et al., 1997a). Auxin visualisation confirmed the presence of an auxin gradient in developing flower heads, with the highest concentration being evident when the flower heads are forming phyllaries and the lowest concentrations occurring during the formation of disc florets. The activity of GUS was quantified using the flurometric MUG assay, which showed that young *S. vulgaris* flower heads had significantly more activity than the more developed flower heads. Whilst there is current evidence of auxin working in a gradient in the literature (Sabatini et al., 1999, Friml et al., 2002, Friml et al., 2003, Grieneisen et al., 2007, Pagnussat et al., 2009), there has never be such a clear example of the gradient *in planta* (Chapter 3 Fig. 2). This highly suggests that auxin is working in a similar manner to the classic animal morphogen, *BICOID*, which works in a concentration dependant manner to determine cell fates (Driever and Nüsslein-Volhard, 1988b, Driever and Nüsslein-Volhard, 1988a).

Morphogens are able to change gene regulation based on their concentration in the developing cells (Tabata and Takei, 2004). In order to assess whether auxin acts in a concentration dependant manner, it was therefore necessary to test whether auxin is able to change the expression of lateral organ identity genes in a concentration dependant manner. *SvRAY2* is known to be required for the formation of ray florets in *S. vulgaris* (Kim et al., 2008), whilst homologs of *SvRAY2* are known to be important for ray floret formation in *Helianthus annuus* and *Gerbera hybrid,* indicating that its function is highly conserved (Broholm et al., 2008, Chapman et al., 2012). qPCR and *in situ* hybridisations on *MiRAY2* showed an increase in expression after low concentration auxin treatment, suggesting that the increase in production of ray florets seen in low auxin treated flower heads is caused by an increase of *MiRAY2*. An increase in *MiRAY2* expression consequently leading to an increase in ray floret production is consistent with the known role of *SvRAY2* and its homologs in other Asteraceae species (Kim et al., 2008, Broholm et al., 2008, Chapman et al., 2012).

There are no current studies evaluating the direct interactions between *CYCLOIDEA* (*RAY2* ortholog) and auxin, but the interactions between other *TEOSINTE BRANCHED, CYCLOIDEA, PROLIFERATING CELL FACTORS* (TCP) genes and auxin have been investigated (Aguilar-Martinez et al., 2007, Finlayson, 2007). Apical dominance and shoot branching both rely on auxin and TCP genes for correct function (Aguilar-Martinez et al., 2007, Finlayson, 2007). In *Arabidopsis BRANCHED1* (*TEOSINTE BRANCHED* homolog) is indirectly negatively regulated by auxin to suppress the development of lateral shoots. Hence when auxin is exported from developing buds the repression on *BRANCHED1* is relieved and the lateral shoot matures (Aguilar-Martinez et al., 2007, Finlayson, 2007, Bennett et al., 2006, Shinohara et al., 2013). Shoot branching highlights how auxin acts indirectly to regulate the function of TCP genes, which could be consistent with our data that shows a direct or indirect positive regulation of *MiRAY2* with auxin. Preliminary analysis of a *CYC* homologue *pTCP1*::*GUS* which was transformed in *A. thaliana* shows an increase

of *GUS* expression post auxin treatment, suggesting that the up-regulation of *CYC-like* genes by auxin is conserved (personal communication, Minsung Kim).

LEAFY (*LFY*) was seen as an ideal candidate gene to investigate phyllary identity, because *LFY* mutation in *A. thaliana* and *Antirrhinum majus* results in an altered phenotype whereby the plant produces cauline leaves instead of flowers, which may be seen as analogous to when flower heads make phyllaries rather than florets (Weigel et al., 1992, Coen et al., 1990). *MiLFY* expression analysis (qPCR and *in situ* hybridisations) showed that *MiLFY* was negatively regulated at higher auxin treatments and showed no change at lower auxin treatments (Chapter 3 Fig. 3). The relationship between low *LFY* expression and the conversion of disc florets to phyllaries, is similar to that of *A. thaliana lfy* mutants making cauline leaves instead of flowers (Weigel et al., 1992). The negative regulation of *LFY* by auxin is in contrast to what has been observed in previous studies (Li et al., 2013, Yamaguchi et al., 2013), in which two recent reports have both found that in the floral meristem of *A. thaliana* auxin positively regulates *LFY* expression. The difference in the positive or negative regulation of *LFY* by auxin therefore appears to be species dependant. It may be concluded that, even though *LFY* is known to be a highly conserved gene, it has evolved different functions throughout its evolutionary history (Wang et al., 2008). This functional diversity may explain the contradictory negative regulation of *LFY* expression by auxin observed in this study.

When the collective evidence is evaluated, it allows for a model of Asteraceae flower head development to be constructed. When flower heads are young, they have the highest levels of auxin that in turn represses *LFY* expression and allows for the formation of the phyllaries. As the flower head expands the auxin concentration decreases (Chapter 3, Fig. 2), this may occur through an increase in flower head volume, a decrease in auxin production or a collaborative effect of both. This decrease in auxin concentration stops the repression of *LFY* and increases the expression of *RAY2*. In turn, this has a knock on effect of allowing the reproductive lateral organs

to be produced in the form of a ray floret. The flower head continues to increase in size and therefore the auxin concentration continues to fall, which activates the genes responsible for disc floret formation. The evidence presented in this thesis has clearly shown that auxin meets one of the criteria of a morphogen. The homeotic conversion of disc florets to phyllaries or ray florets clearly shows that altering the auxin concentration changes cell fate. Further work will be needed to show that auxin acts directly on its target genes.

The concentration dependant gene regulation by auxin also suggests indicates that auxin is behaving as a morphogen in the developing flower head. This model is robust enough to explain the unique cultivars seen throughout horticultural species of the Asteraceae family. Species with multiple layers of ray florets or comprised of all ray florets have an increased growth stage with a medium concentration of auxin which promotes *RAY2* expression and therefore ray floret formation. Similarly, cultivars that form ray florets at the centre of the flower head after producing disc florets have acquired mutation that allows for high auxin expression at the end of flower head development and therefore the production of ray florets.

6.4 Flower head growth and morphology

In furtherance of the understanding of flower head development, a detailed developmental study was necessary. To achieve this, *M. inodora* was harvested at several developmental stages for investigation by SEM. Analysis of the SEM images of the developing flower heads revealed a number of interesting morphological features that presented during development. When looking at the developing flower head as a whole, it is initially noticeable that the ray florets have their development arrested shortly after formation (Chapter 4 Fig. 2). This arrested development will serve two purposes; firstly it will protect the ray florets from damage whilst the rest of the flower head develops. Secondly, it will synchronise development, as there is no point in attracting pollinators with ray florets when the disc florets are not ready to be pollenated. The arrest of petal outgrowth has been seen in several *A. thaliana* mutants, which suggests a possible

molecular mechanism by which ray floret petal outgrowth may be inhibited. *UNUSUAL FLORAL ORGANS* (*UFO*) has been found to play a role in early petal outgrowth, with loss of *UFO* resulting in dramatic floral phenotypes including homeotic organ transformations (Ingram et al., 1995). It is thought that *UFO* works in early petal outgrowth by antagonising the inhibitory effects of the class C gene *AGAMOUS* (Durfee et al., 2003, Laufs et al., 2003). Therefore in the early petal development of ray florets there could be an inhibition of *UFO,* which allows for *AGAMOUS* to inhibit growth. As the flower head develops the expression of *UFO* increases relieving the inhibition of *AGAMOUS*, which could allow petal growth.

Several other interesting observations were made through the monitoring of the developing ray florets. Although the petals in ray florets finish development with a large amount of asymmetry, during the early stages of development the petals are symmetrical. The symmetry in the petals of developing ray florets indicates that the two dorsal petals are inhibited rather than the ventral petals being elongated, which is consistent with the evolutionary theories of floret formation that suggests that they originate from a single ancestor (Cronquist, 1981, Barreda et al., 2010). This pattern of development is consistent with that observed in other five petal zygomorphic flowers. For example, in *Primulina heterotricha* early development, the five petal primordial are symmetrical and maintain that symmetry for several stages of development before asymmetry begins to appear (Yang et al., 2012). When the petals of the ray florets are being elongated auxin is also being accumulated in the floret (Chapter 4, Fig. 3), this suggests that auxin is causing rapid cell expansion or cell division allowing the petals to expand quickly (Tao et al.). Further investigation will be necessary to determine whether floret elongation is due to cell division or cell elongation, as auxin is known to play a role in both of these processes (Tao et al., 2008). In shade avoidance, auxin is rapidly synthesised and transported to the site of elongation to allow the plant to move to an optimal photosynthetic position (Tao et al., 2008). In contrast to auxin's

role in cell expansion, auxin is also known to trigger cell division through G-protein-mediated signal transduction (Campanoni and Nick, 2005). Whilst it appears that auxin is involved in both cell expansion and cell division, further analysis has suggested that different auxins participate in different signalling pathways of cell division and expansion. The study of developing disc florets revealed that, like ray florets, they have an asymmetrical stage of development. In contrast to the ray florets which maintain the asymmetry, disc florets lose the asymmetry and revert back to being symmetrical. This further supports the hypothesis that both ray and disc florets are evolved from a single origin. The asymmetrical stage of the disc florets also draws parallels with the development of *Mohavea confertiflora*, a five petal flower with actinomorphoric flowers that shows asymmetry during development (Hileman et al., 2003). *Mohavea confertiflora* is a close relative of the model species *Antirrhinum majus* but has acquired symmetry through the change of *CYC* and *DICHOTOMA* expression domains, further suggesting that the change in symmetry from ray to disc floret is caused by TCP genes (Hileman et al., 2003). When combined with the molecular data from auxin manipulation experiments, it appears that the simple genetic traits that are responsible for flower asymmetry have been reinvented in order to create the complex patterning of the Asteraceae flower head.

6.5 Virus induced gene silencing, tool of the future?

As plant genetics has advanced and developmental biologists have moved away from the simple model species to evaluate more complex systems the need for robust genetic tools has become greater than ever. Virus induced gene silencing (VIGS) is an effective tool that has been shown to work throughout the plant kingdom, with the tobacco rattle virus (TRV) based vectors proven to work in several different dicotyledon families (Lu et al., 2003, Burch-Smith et al., 2006, Wege et al., 2007, Deng et al., 2012). In this study, VIGS was evaluated as a potential tool for reverse genetics in both *M. inodora* and *S. vulgaris*. *Phytoene desaturase* (*PDS*) was used as a positive control gene for both species throughout the initial testing phases (Wege et al., 2007). The typical

PDS phenotype of photobleached leaves was observed for both species after the induction of VIGS. The photobleaching was generally detained to the site of inoculation, suggesting that VIGS is unable to become systemic in the two species tested. In contrast to our data, the ability of VIGS to develop a systemic infection is widely reported in the literature and has been reported in another Asteraceae species (Lu et al., 2003, Burch-Smith et al., 2006, Wege et al., 2007, Deng et al., 2012). There are two likely possibilities to why VIGS was not able to go systemic; the first possibility is that the host immune system is able to limit the viral infection. The second likely possibility is that the methodology is not completely optimised and with more refinement VIGS may show increased dissemination into the plant tissue (Broderick and Jones, 2014). A gene fragment of *M. inodora PIN-FORMED1* (*MiPIN1*) was introduced into the pTRV vector and was inoculated into the developing inflorescent of *M. inodora*. In the limited sample size available, the *M. inodora* flower heads that developed after inoculation displayed shortened or missing ray florets. The phenotype of shortened or missing ray florets is consistent with previous work which showed that application of an auxin transport inhibitor (1-N-Naphthylphthalamic acid) induced the formation of shortened and missing ray florets when applied to developing flower heads of *M. inodora* (Zoulias et al., 2013). This suggests that VIGS using the *MiPIN1* fragment is able to knock down/out native *MiPIN1* expression leading to disruption of flower head development. The lack of lateral organ formation is consistent with *A. thaliana pin1* plants and NPA treated inflorescence which cannot form lateral organs (Geldner and Palme, 2001).

6.6 Future work

Whilst this study furthered our understanding of flower head development in the Asteraceae family, it also created new opportunities for future research. Future evaluation should focus around the role of the auxin gradient in the developing flower head. Importantly this would include the generation of DR5::reporter *M. inodora*, as the small size of the *S. vulgaris* flower head produced limitations in terms of the physical manipulation of the flower head. The larger flower

head of *M. inodora* would allow us to wound its flower head meristem, laser ablate and compress whilst using the DR5 promoter to monitor auxin flow providing a much more thorough understanding of the influence of auxin on the flower head developmental stages in this species

Using the targeted approach of treating flower heads with auxin prior to assessing the expression profiles of candidate lateral organ genes allowed us to gain significant insight into the developing flower head, however, using RNAseq would allow for a much broader evaluation. By comparing the developing meristems of non-treated and auxin treated flower heads, we would be able to observe the differences in mRNA expression induced by the various treatments. RNAseq would also further confirm that there is a concentration dependant response to auxin at a genetic level. *LEAFY* knock down plants were generated towards the end of the study but time restraints inhibited assessment. Further research should allocated towards the study of *LEAFY* and its known antagonist *Terminal Flowering1* to assess their role in flower head development, as *in situ hybridisation* data showed that *LFY* is expressed throughout the developing flower head. Understanding how these two key floral meristem genes behave in the Asteraceae flower head would greatly increase our understanding of flower head development.

Although several PIN1 antibodies were assessed for use in immunolocalisation procedures throughout this study, none showed a clear expression pattern. The generation of an Asteraceae flower head with a fluorescently tagged PIN1 would allow for detailed analysis of auxin flow throughout the developing flower head, helping to indicate the mechanism by which the auxin gradient is established and maintained.

Further investigation into the potential role of VIGS as a genetic tool should be made a priority. The traditional tissue culture methods used to generate transgenic *S. vulgaris* plants currently takes 6-8 months to complete whereas the limited testing of VIGS revealed that knock down/out can be achieved within 1 month. As VIGS appears to be able to greatly speed up reverse genetics a thorough protocol should be completed.

6.7 Closing remarks

The Asteraceae family flower head has inspired and puzzled brilliant minds throughout the ages, yet little is known about its growth, development and evolution. Understanding the basic development of such an iconic flower form would greatly enhance agriculture and horticulture. Our results have shown that plants have evolved to use morphogen-like signals in a similar manner to animals. Furthermore, our data corroborates with the literature to suggest auxin's role as a morphogen is wide spread throughout angiosperms. Auxin's role as a morphogen provides a foundation behind which complex pattern formation in plants can occur, preliminary testing (personal commutation Minsung Kim) has shown that this is holding true for a pseudanthium from the monocotyldens, *Spathiphyllum cochlearispathum* (Peace Lilly). Although the basic mechanisms underpinning the development and pattern formation of the flower head have now been perceived, the rest of the story has yet to be written.

Chapter 7

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