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Analysis of stage-specific gene perturbations
and
characterisation of two novel F-box genes during
flower development in *Arabidopsis thaliana*

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, unless otherwise stated. I agree to deposit this thesis in the University's open access institutional repository or allow the Library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

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Summary

The model plant *Arabidopsis thaliana* has been used for past three decades to study the genetic and molecular processes underlying floral organogenesis. Flowers of this small plant consist of four concentric whorls, containing four sepals, four petals, six stamens, and a pair of fused carpels. Numerous studies in this field led to the proposal of the ABCE model of flower development, in which the combinatorial activities of four classes of master regulator transcription factors direct the formation of the floral organs. Extensive research has delineated the roles these transcription factors have with regard to specifying the floral organs, although gaps in our knowledge still remain concerning the downstream targets of these important regulators.

The first part of this thesis is focused on the development and optimisation of transgenic lines that can be used to reduce the activities of some of these homeotic genes at any given point during flower development, in order to study the gene networks that are regulated by these transcription factors at discrete developmental stages. This has been achieved through the use of artificial microRNAs (amiRNAs), designed to target and degrade mRNA transcripts of either the B-class regulator *APETALA3* (*AP3*) or the C-class regulator *AGAMOUS* (*AG*). These amiRNAs were controlled by a hormone-activated system, such that transcription of the amiRNA precursors only occurred upon application of the correct inducer. These were used in tandem with a second hormone-inducible construct, which allows for the user-defined initiation of hundreds of synchronously-developing floral buds, facilitating the collection of large amounts of tissue that can be used for downstream global analyses.

As both of these systems depend on the activation of transcription factors, that are translationally-fused to the ligand binding domains of related mammalian nuclear receptors, it was first demonstrated both systems could reliably activated independent of each other. It was also confirmed that reductions in the activities of *AP3* or *AG* occurred through decreasing mRNA levels, and a series of stage-specific knockdowns were carried out to monitor the phenotypic consequences of gene perturbations depending on the temporal context. These lines were then used to perform a second series of gene perturbations, at different stages during flower development, in order to analyse changes to global expression patterns using next generation sequencing technologies. From this it

was possible to examine the different genes and processes these transcription factors regulated depending on the flower's developmental stage, providing a greater insight into the dynamic roles of the floral homeotics.

The second part of the thesis focused on characterising two novel F-box genes that were thought to genetically interact with the F-box gene, and transcriptional co-regulator of B-class homeotic gene activity, *UNUSUAL FLORAL ORGANS (UFO)*. F-box genes encode for proteins that typically provide substrate specificity to multi-subunit E3 ligase complexes, which participate in protein modification and turnover through the conjugation of small ubiquitin moieties. Two closely-related genes, named *NANNY (NAN)* and *GRANNY (GRA)*, were identified through searches for F-box genes possessing similar expression profiles to that of *UFO*, in order to determine if they shared any functions related to flower development. Transcriptional reporter lines, and *in situ* hybridisations were performed, to establish the expression profiles of these genes, and their ability to interact with other subunits of E3 ligase complexes was verified. Several over-expression constructs were generated and studied, to attempt to place the genes in some developmental pathway, and finally the genome-editing technology CRISPR/Cas9 was employed to obtain full knockouts of both genes, separately and together, to learn more about any shared functions these genes might have.

For my late grandfather, Arthur Thomson

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List of Abbreviations

Term	Abbreviation
% Volume per volume (ml/100 ml)	(v/v)
% Weight per volume (g/100 ml)	(w/v)
17- β -Estradiol	EST
<i>ABERRANT TESTA SHAPE</i>	<i>ATS</i>
AGAMOUS	<i>AG</i>
Amino acid	aa
amiRNA	Artificial microRNA
<i>ANANTHA</i>	<i>AN</i>
Androgen Receptor	AR
<i>APETALA1</i>	<i>AP1</i>
<i>APETALA2</i>	<i>AP2</i>
<i>APETALA3</i>	<i>AP3</i>
<i>Arabidopsis</i> Genome Initiative	AGI
<i>Arabidopsis thaliana</i> (Columbia ecotype)	Col-0
<i>Arabidopsis thaliana</i> (Landsberg <i>erecta</i> ecotype)	L- <i>er</i>
<i>ARABIDOPSIS SKP1-LIKE1</i>	<i>ASK1</i>
<i>ARABIDOPSIS SKP1-LIKE13</i>	<i>ASK13</i>
<i>ARGONAUTE1</i>	<i>AGO1</i>
<i>ASYMMETRIC LEAVES2</i>	<i>AS2</i>
β -Glucuronidase	GUS
Base pair	bp
Beijing Genomics Institute	BGI
<i>CAULIFLOWER</i>	<i>CAL</i>
Cauliflower Mosaic Virus 35S promoter	35S::
Chromatin Immunoprecipitation	ChIP
<i>CLAVATA3</i>	<i>CLV3</i>
Clustered regularly-interspaced short palindromic repeats	CRISPR
Complementary DNA	cDNA
<i>CRABSCLAW</i>	<i>CRC</i>
CRISPR RNA	crRNA
<i>CUP-SHAPED COTYLEDON</i>	<i>CUC</i>
Deoxyribonucleic acid	DNA
Dexamethasone	DEX
<i>DICER-LIKE1</i>	<i>DCL1</i>
Differentially Expressed Gene	DEG
Dihydrotestosterone	DHT
Double strand break	DSB
<i>DOUBLE TOP</i>	<i>DOT</i>

<i>EGG-CELL 1.2</i>	<i>EC1.2</i>
Ethanol	EtOH
F-box (Kelch subfamily)	FBK
False Discovery Rate	FDR
<i>FAR-RED ELONGATED HYPOCOTYLS3</i>	<i>FHY3</i>
<i>FIMBRATA</i>	<i>FIM</i>
Floral Induction System	FIS
Floral Meristem	FM
Gene Ontology	GO
Gene Regulatory Network	GRN
<i>GIBBERELLIN 3-OXIDASE-1</i>	<i>GA3OX1</i>
Gibberellic Acid	GA
Glucocorticoid Receptor	GR
<i>GRANNY</i>	<i>GRA</i>
<i>HEN SUPPRESSOR1</i>	<i>HES01</i>
Herpes simplex virus protein vmw65	VP16
High-resolution melting	HRM
Homology Directed Repair	HDR
Homology to E6-AP C-Terminus	HECT
<i>HUA ENHANCER1</i>	<i>HUA1</i>
<i>INCURVATA2</i>	<i>ICU2</i>
Inflorescence meristem	IM
Insertion/deletion	Indel
<i>JAGGED</i>	<i>JAG</i>
<i>JAGGED AND WAVY</i>	<i>JAW</i>
<i>KANADI</i>	<i>KAN</i>
Kilobase	kb
<i>KIP RELATED PROTEIN2</i>	<i>KRP2</i>
<i>KIP RELATED PROTEIN4</i>	<i>KRP4</i>
<i>KNUCKLES</i>	<i>KNU</i>
<i>LEAFY</i>	<i>LFY</i>
Ligand Binding Domain	LBD
MCM1-AGAMOUS-SRF-DEFICIENS	MADS
Messenger RNA	mRNA
miRNA	MicroRNA
<i>NANNY</i>	<i>NAN</i>
Next Generation Sequencing	NGS
Non-Homologous End Joining	NEHJ
Nopaline Synthase	NOS
<i>NOZZLE/SPOROCTELESS</i>	<i>NZZ/SPL</i>
<i>NUBBIN</i>	<i>NUB</i>
Nucleotide	nt

<i>Octopine Synthase</i>	OCS
Open Reading Frame	ORF
Optical Density	OD
<i>PERIANTHIA</i>	<i>PAN</i>
<i>PISTILLATA</i>	<i>PI</i>
Polycomb Group	PcG
Polymerase Chain Reaction	PCR
Primary-miRNA	Pri-miRNA
Protospacer Adjacent Motif	PAM
<i>RABBIT EARS</i>	<i>RBE</i>
Really Interesting New Gene	RING
Reverse Transcription Quantitative Polymerase Chain Reaction	RT-qPCR
Revolutions Per Minute	RPM
Ribonucleic acid	RNA
RNA-guided endonucleases	RGEN
RNA-sequencing	RNA-seq
S-phase Kinase-associated Protein1	<i>SKP1</i>
<i>SEPALLATA1 - 4</i>	<i>SEPI - 4</i>
<i>SHATTERPROOF1</i>	<i>SHP1</i>
Shoot Apical Meristem	SAM
Single guide RNA	sgRNA
Skp1-Cullin-F-box	SCF
<i>SMALL RNA DEGRADING ENDONUCLEASE</i>	<i>SDN</i>
<i>SQUASOMA PROMOTER BINDING PROTEIN-LIKE 8</i>	<i>SPL8</i>
<i>SUPERMAN</i>	<i>SUP</i>
SUPERMAN Repressive Domain X	SRDX
T7 Endonuclease I	T7E1
<i>TERMINAL FLOWER1</i>	<i>TFL1</i>
Terminator	Ter
The <i>Arabidopsis</i> Information Resource 10	TAIR10
Tracking of Indels by Decomposition	TIDE
Transcription activator-like effector nuclease	TALEN
Transcriptional Start Site	TSS
Transcriptome-de Bruijn Graph	T-DBG
Transfer-DNA	T-DNA
<i>TRANSPARENT TESTA GLABRA2</i>	<i>TTG2</i>
<i>U6 SMALL NUCLEOLAR RNA26</i>	<i>U6-26</i>
<i>U6 SMALL NUCLEOLAR RNA29</i>	<i>U6-29</i>
<i>UNUSUAL FLORAL ORGANS</i>	<i>UFO</i>
<i>UTP:RNA URIDYLYLTRANSFERASE 1</i>	<i>URT1</i>
<i>WUSCHEL</i>	<i>WUS</i>

Zea mays codon-optimised Cas9	zCas9
Zinc finger nuclease	ZFN

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1. Introduction

This chapter is partially based on content from:

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Arabidopsis thaliana (*Arabidopsis*) is a small flowering plant belonging to the *Brassicaceae* family. During its life-cycle it undergoes two main stages of development: The vegetative phase and the reproductive phase. The vegetative phase encompasses the growth and development of rosette leaves and axillary meristems, which originate from a small collection of meristematic cells located within the centre of the plant, termed the Shoot Apical Meristem (SAM). All subsequent primordia originate in a spiral phyllotaxy along the periphery of the SAM such that individual organs are all at different stages of development.

The reproductive phase transition begins upon the culmination of several endogenous and environmental signals, which alter the developmental program of the SAM such that the main stem becomes elongated, and cauline leaves and axillary nodes are formed, and the SAM is now known as the Inflorescence Meristem (IM). This transition is controlled by the flowering time genes, which integrate to promote the expression of the floral meristem identity genes (Jack, 2004). The genes are controlled by four major pathways that respond

to these cues: Vernalisation, a long-day photoperiod, the biosynthesis of the phytohormone gibberellin (GA), and an autonomous pathway (Reviewed in Jack, 2004).

As reproductive development continues, the IM ultimately gives rise to Floral Meristems (FMs) along its flank, which then differentiate into the four organ types found within the flower: Sepals, petals, stamens, and the gynoecia. Floral organ identity is chiefly regulated by the homeotic genes, which when perturbed, result in deviations from the wild-type floral patterning and cause wide-ranging defects in organ identity, including the abolition of all floral organs. The homeotic genes encode transcription factors which establish the developmental program of the floral organs, in a spatially-specific manner, by directly or indirectly influencing the transcription of thousands of other genes.

1.1. Specification of the floral meristem

Incipient floral primordia arising from the inflorescence meristem undergo a series of transcriptional changes that allow them to adopt a determinate floral fate. In contrast, the cells of the inflorescence meristem are programmed to be indeterminate and resist the transition to floral organogenesis by the activity of shoot identity genes such as *TERMINAL FLOWER1 (TFL1)* (Bradley, 1997; Hanano and Goto, 2011; Baumann *et al.*, 2015). In the IM *TFL1* mRNA is located within a small group of cells below the most apical layer, and is notably not present within the developing floral primordia (Parcy, Bomblies and Weigel, 2002; Baumann *et al.*, 2015). *tfl1* mutant plants have a determinate meristem phenotype such that they produce a terminal flower in place of the inflorescence meristem shortly after bolting (Shannon and Meeks-Wagner, 1991; Schultz and Haughn, 1993).

The activation of the floral meristem identity genes is the main determining factor at the beginning of this process, as they promote the expression of key regulators of flower development while also repressing genes that function to suppress the developmental program. The meristem identity gene *LEAFY* (*LFY*) largely directs the specification of the floral meristem along with the partially redundant homeotic genes *APETALA1* (*AP1*), and *CAULIFLOWER* (*CAL*). *LFY* and *AP1* promote each other's expression, and it has been shown that both proteins bind directly to the *TFL1* promoter, with previous reports indicating that they directly result in its repression, allowing for the specification of the floral primordia (Kaufmann *et al.*, 2010; Moyroud *et al.*, 2011). However, recent studies have shown that *LFY* directly activates *TFL1* expression in the absence of *AP1/CAL* activity, and that *LFY* and *AP1* act in a partially antagonistic manner to specify the floral meristem by fine tuning the expression of floral activators and repressors through an incoherent feed-forward loop, where *AP1* and *LFY* activate repressors of *TFL1* while *LFY* simultaneously promotes *TFL1* expression (Goslin *et al.*, 2017). It is thought that this mechanism occurs to ensure that floral organogenesis only initiates when the levels of *AP1* and *CAL* are sufficient to efficiently promote the expression of their downstream targets, and override *LFY*'s antagonistic activity.

Loss-of-function phenotypes in *lfy* mutants result in a loss of competency of the incipient primordia to switch to a floral fate, producing co-florescences in place of floral organs (Schultz, 1991). While null *ap1* mutants have floral organ defects in the first and second whorls, and produce secondary floral meristems from the axils of the first whorl organs, *cal* mutants do not have a phenotype that is readily distinguishable from wild-type plants (For review see Yanofsky, 1995). However, *ap1-1 cal-1* double mutants fail to establish floral meristems, instead forming a large over-proliferation of inflorescence-like

meristems, giving the IM a cauliflower-like appearance (Ferrándiz *et al.*, 2000; Wellmer *et al.*, 2006). This indicates that *CAL* activity is capable of substituting for *AP1* in the specification of floral meristems, but not during floral organ formation.

The combined activities of *AP1* and *LFY* in the floral meristem result in the downregulation of shoot identity genes, with the concomitant activation of genes necessary to direct flower formation. These floral organ identity genes are grouped into 4 main classes depending on their spatial expression patterns throughout the presumptive whorls of the flower. Furthermore, the combinatorial activities of the different classes, as discussed below, results in the formation of each of the floral organs.

1.2. Floral organ specification

The ABC model of floral organ identity specification (Coen and Meyerowitz, 1991) has guided research on flower development over the past 25 years. The experimental basis, the history, as well as the modifications and extensions of the original model have been described in several recent reviews (Bowman, Smyth and Meyerowitz, 2012; O'Maoileidigh and Wellmer, 2014; O'Maoileidigh, Graciet and Wellmer, 2014; Prunet and Jack, 2014; Wellmer, Graciet and Riechmann, 2014; Sablowski, 2015). The model proposes that the activities of certain combinations of floral organ identity genes, which encode transcription factors of the MADS domain family, lead to the formation of sepals, petals, stamens, and carpels (Fig. 1.1, A and B). These transcription factors have been assigned to classes (A, B, C), and the activities of combinations of different classes directs floral organ specification. The model posits that A-class genes specify petals, A- and B-class genes together specify petals, B- and C-class genes specify stamens, and C-class genes specify the carpels, although specific aspects of the model (in particular, the

concept of “A function”) have been put into doubt in recent years (Litt 2007; Causier et al. 2010; Wollmann et al. 2010).

Mutations in the genes which code for these transcription factors have been shown to alter floral organ identity in adjacent whorls as their regions of action can overlap. Organ types may also be abolished or the ectopic expression of another organ type may occur. Additionally, mosaic organs often form, where tissues from more than one organ type are present. In *Arabidopsis*, ‘A’ class mutants lack the perianth organs of whorls 1 and 2 - sepals and petals respectively - which are replaced with carpels and stamens to give the phenotype: carpel, stamen, stamen, carpel. *API* and *APETALA2* (*AP2*) are A class genes, although *AP2* is a notable exception inasmuch that it does not code for a MADS domain transcription factor. ‘B’ class mutants such as *apetala3* (*ap3*), and *pistillata* (*pi*) affect whorls 2 and 3, substituting petals for sepals and stamens for carpels. Finally, the ‘C’ class mutant *agamous* (*ag*) affects whorls 3 and 4 to produce an indeterminate flower-within-flower phenotype, as the stamens in whorl 3 are replaced by petals and whorl 4 develops into a second mutant flower with the same characteristics. This phenotype suggests that *AG* functions as a gene that regulates the determinacy of the floral meristem as well as regulating organ development.

The molecular basis of the ABC model is summarised in the “quartet model” (Theissen 2001; Fig. 1.1C), which posits the existence of four regulatory complexes, each composed of four floral organ identity factors, that bind to two *cis*-regulatory elements [containing so-called CArG boxes; consensus: 5'-CC(A/T)₆GG-3'] in the promoter of a gene to induce DNA looping between the two binding sites. Each of these complexes was predicted to contain “E function” cofactors, termed *SEPALLATA1*, 2, 3, and 4 (*SEPI-4*), which also

belong to the family of MADS domain proteins. Both the *in vivo* existence of the floral organ identity factor complexes (Smaczniak *et al.*, 2012) as well as the occurrence of DNA looping (Melzer, Verelst and Theissen, 2009; Mendes *et al.*, 2013; Jetha, Theissen and Melzer, 2014) have been experimentally confirmed in recent years.

Despite this considerable progress in our understanding of the floral organ identity factors, key questions about their functions remain unsolved. In particular, it is currently not known how the MADS protein complexes act at the mechanistic level. The different floral organ identity factors have similar albeit not identical DNA binding specificities (Muiño *et al.*, 2014), and the results of genome-wide localisation studies (Kaufmann *et al.*, 2009; Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013; Pajoro *et al.*, 2014) showed that their global binding patterns exhibit large overlaps even in cases where two factors are not part of the same complex. At the same time, the sets of bona fide target genes, i.e. genes that are bound and respond to a perturbation of the different floral organ identity factors, are largely distinct (Yan, Chen and Kaufmann, 2016). Furthermore, the expression of the majority of genes bound by the floral organ identity factors is not, or only slightly, altered upon binding by the MADS protein complexes (Kaufmann *et al.*, 2010; Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013). Thus, it appears possible that a large number of their binding sites in the genome are non-functional at least in the context of floral organ identity specification (i.e. they might be functional in other processes during plant development when they are bound by additional members of the large MADS domain protein family).

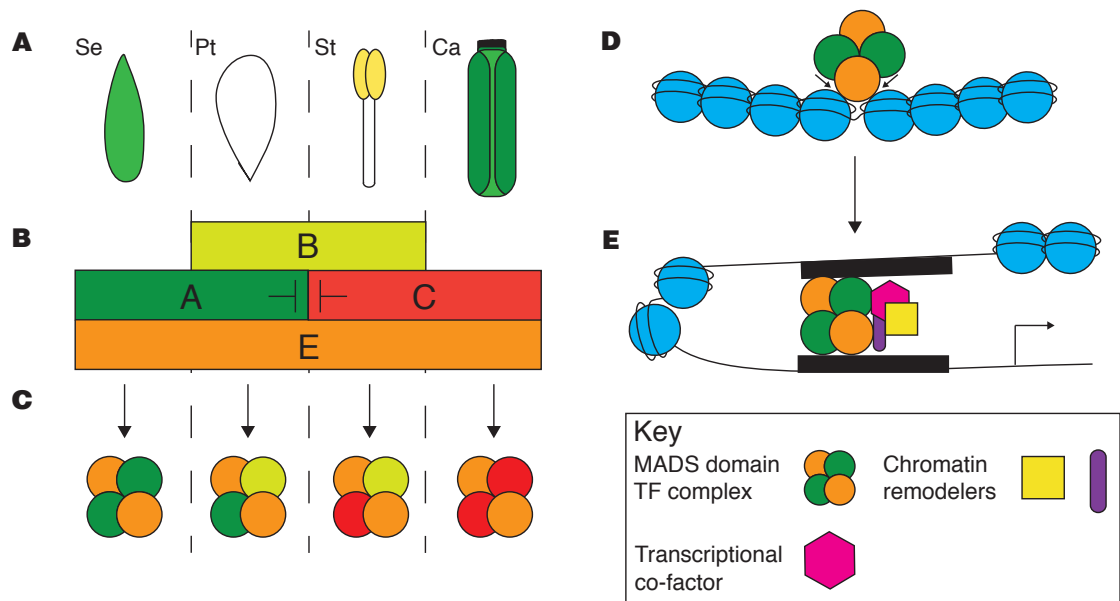


Figure 1.1 - Molecular aspects of the ABCE model. (A) Illustrations of the organs found in the outer to inner (left to right) whorls of the flower. Se, Sepal; Pt, petal; St, stamen; Ca, carpels. (B) The ABCE model of flower development (Theissen, 2001)). Specific classes of floral organ identity genes are active within each floral whorl. A class genes specify sepals in the first whorl; A and B class genes specify petals within the second whorl; B and C class genes specify stamens within the third whorl; C class gene function specifies carpel identity within the fourth whorl. The E class genes are active within all four whorls. (C) Combinatorial interactions of floral organ identity factors within each whorl form dimeric (not shown) and higher-order tetrameric complexes. (D) It has been proposed (Pajoro *et al.*, 2014) that the floral organ identity factors can act as pioneer factors, influencing chromatin accessibility throughout flower development. E, Higher-order MADS domain complexes likely interact with transcriptional cofactors and chromatin remodelers to regulate target gene expression. The complexes are predicted to induce short-range DNA looping by binding adjacent CArG box sequences (black rectangles) present within *cis*-regulatory elements (see text for details).

Taken together, these observations led to the question of how the specificity of the different floral organ identity factor complexes is controlled. Current models of transcription factor function in eukaryotes (Slattery *et al.*, 2014) suggest that this control

of specificity might be based on a number of contributing factors. These could include the spatial arrangement of binding sites within larger cis-regulatory modules and differences in the affinities of the transcription factors for specific sites (“strong” binding sites are thought to more likely trigger differential expression than “weaker” sites (Slattery *et al.*, 2014). Also, the presence or absence of additional transcriptional regulators, which cooperatively or as part of a transcription factor collective (Junion *et al.*, 2012) act with the MADS domain proteins on a target gene promoter, may determine whether a gene is activated or repressed. The results of a recent proteomics study strongly suggest that the floral organ identity factor complexes indeed associate with other transcription factors as well as with proteins involved in the epigenetic control of gene expression (Smaczniak *et al.*, 2012; Fig. 1.1E). This idea is further supported by studies that specifically analysed the interaction of the MADS domain proteins with other transcription factors, such as members of the Auxin Response Factor family (Ripoll *et al.*, 2015) to name only one recent example. Because it was found that DNA binding by the floral organ identity factors often precedes an increase in DNA accessibility at target gene promoters, it has been suggested that they may act as pioneer factors (Pajoro *et al.*, 2014; Fig. 1.1D). Such pioneer factors bind to condensed chromatin and then alter chromatin conformation through the recruitment of epigenetic modifiers to facilitate or prevent binding by other transcriptional regulators (Zaret and Mango, 2016).

Whatever the precise mechanism of floral organ identity factor function, it is clear that these conserved master regulators have played a central role in the evolution of floral structures (e.g. Soltis and Solis, 2014; Gramzow and Theissen, 2015; Becker, 2016; Silva *et al.*, 2016). In fact, it appears likely that differences in the sets of target genes for a given factor can explain many of the alterations in floral morphologies found among the

angiosperms. At the same time, it would be important to identify, for each of the different classes of floral organ identity factors, core sets of targets that are shared between related species or even between angiosperms belonging to distinct families. These genes could provide the information necessary for establishing the basic blueprint for the different types of floral organs. While such studies have not yet been conducted systematically, the first steps in this direction have already been taken (Muino *et al.*, 2016) and will likely lead to much activity in this area in years to come.

1.3. Genes involved in floral organogenesis

Since the 1980s, forward and reverse genetic screens have led to the identification of dozens of key regulators of flower development (Causier and Davies, 2014; O'Maoileidigh, Graciet and Wellmer, 2014a; O'Maoileidigh, Graciet and Wellmer, 2014b; Prunet and Jack, 2014). The functions of many of the genes have subsequently been studied in great detail using a wide range of experimental approaches. With the advent of the genomics era, this work has been complemented by gene expression profiling experiments, in which the transcriptomes of developing (wild-type and mutant) flowers, individual types of floral organs, and different cell types were analysed. These studies yielded a comprehensive view of gene expression during flower formation. However, because the available data did not always provide detailed information on when and where genes are expressed, additional studies conducted in recent years have aimed at increasing the spatio-temporal resolution of the gene expression map for flower development (Jiao and Meyerowitz, 2010; Wuest *et al.*, 2010; Mantegazza *et al.*, 2014; Chen *et al.*, 2015; Ryan *et al.*, 2015; Villarino *et al.*, 2016). To this end, a wide range of methods, such as laser capture microdissection (Wuest *et al.*, 2010; Mantegazza *et al.*, 2014) or fluorescence-activated cell sorting, in combination with a flower

synchronisation system (O'Maoileidigh *et al.*, 2015; Villarino *et al.*, 2016), was used. While most of this work has focused on a few model species, in particular, *Arabidopsis* and rice (*Oryza sativa*), the development of next-generation DNA sequencing techniques has allowed the characterisation of the floral transcriptomes of several other species from different families, such as woodland strawberry (*Fragaria vesca*; Hollender *et al.*, 2014) and eucalyptus (*Eucalyptus grandis*; Vining, Romanel and Jones, 2014) to name but a few. This work has yielded valuable datasets, not only needed to establish additional model plants for flowering research, but also to better understand the genetic basis underlying the considerable differences in floral architecture found among the angiosperms.

The results of the studies described above showed that flower development involves the differential expression of thousands of genes, probably corresponding to as much as one-quarter of all the genes in a plant's genome (Ryan *et al.*, 2015). Because many of these genes are known or predicted to have regulatory functions, it appears that the gene networks that control floral organogenesis are exceptionally complex. However, attempts to systematically characterise the functions of these differentially expressed genes have not met with much success in spite of the powerful reverse genetics approaches available, especially for *Arabidopsis*. One reason for this may be that many of these genes play minor roles in flower formation so that their inactivation does not lead to readily discernible mutant phenotypes. Furthermore, the high degree of functional redundancy found among (often closely related) genes in plants likely hampers their analysis. With the invention of sophisticated genome editing approaches such as CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated; Wright, Nuñez and Doudna, 2016), the latter genes can now be studied more efficiently by generating full

loss-of-function alleles and by subsequently creating lines with multiple gene knockouts. Thus, many additional regulators of flower development will likely be discovered in the near future, and this will lead to an increasingly complete view of the gene sets that control floral organogenesis.

1.4. Genes acting downstream of the floral organ identity factors

How do the floral organ identity factor complexes control organ morphogenesis? García-Bellido, working with the fruit fly *Drosophila melanogaster*, proposed in the mid-1970s that master regulators (“homeotic selector genes”) such as the floral organ identity factors directly control the expression of (“realizator”) genes that are required for the terminal differentiation of organs (García-Bellido, 1975). That this idea is at least in part correct for the floral organ identity factors is suggested by the expression of the corresponding genes in floral organs throughout most of their morphogenesis (O’Maoileidigh, Graciet and Wellmer, 2014a, 2014b). Also, the results of perturbation experiments, in which the floral organ identity genes were specifically disrupted or activated at different floral stages, showed that these master regulators have crucial functions in processes that take place during intermediate and late stages of flower development (Ito *et al.*, 2007; Wuest *et al.*, 2012; O’Maoileidigh *et al.*, 2013). However, the now prevailing view is that they largely act by controlling the expression of other genes with regulatory functions. This conjecture is most strongly supported by the results of studies (which were already briefly mentioned above) in which the target genes and gene expression programs acting downstream of the floral organ identity factors were identified on a genome-wide scale (Kaufmann *et al.*, 2009, 2010; Wuest *et al.*, 2012; O’Maoileidigh *et al.*, 2013; Pajoro *et al.*, 2014). These studies not only showed that each factor has at least a few hundred direct

target genes and that these sets of targets exhibit considerable changes during the course of flower development, but also that they are highly enriched for genes with regulatory functions. The expression of these targets can be both activated and repressed, indicating that the floral organ identity factor complexes are bifunctional in their activity.

The systematic identification of the genes acting downstream of the floral organ identity factors triggered the question how these genes control the plethora of processes that take place during floral organ morphogenesis. Work conducted in recent years has led to several examples where we now understand in some detail the contributions to floral organogenesis of individual gene regulatory events that occur in response to floral organ identity factor activity. We will discuss a few of these cases in the following section.

1.5. Control of floral organ growth and development

An early example for a gene that is directly controlled by floral organ identity factors and that regulates an essential process during floral organ development is *NOZZLE/SPOROCTELESS* (*NZZ/SPL*), a master regulator for the formation of sporogenic tissues in *Arabidopsis* (Schiefthaler *et al.*, 1999; Yang *et al.*, 1999). It was shown that once activated by the C function regulator *AGAMOUS* (*AG*), which is involved in the specification of the reproductive floral organs, the *NZZ/SPL* transcription factor can trigger microsporogenesis in the absence of *AG* activity (Ito *et al.*, 2004).

Another example for a transcription factor-coding gene that acts downstream of floral organ identity factors in *Arabidopsis* is *JAGGED* (*JAG*). *JAG*, together with its paralog *NUBBIN* (*NUB*), controls the growth of leaf margins and floral organs, especially in their distal regions (Dinnyeny *et al.*, 2004; Ohno *et al.*, 2004; Dinnyeny, Weigel and Yanofsky, 2006). Both genes have been shown to be regulated by B, C, and E class floral

organ identity factors (Gómez-Mena *et al.*, 2005; Kaufmann *et al.*, 2009; Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013). Using computer modelling, and petal growth as an example, JAG was proposed as a possible distal-specific growth factor (Sauret-Güeto *et al.*, 2013). Furthermore, through three-dimensional analysis of cell geometry and DNA synthesis, it was shown that JAG promotes changes in growth patterns when organ primordia are initiated from a (floral) meristem and that it coordinates cell growth with the cell cycle (Schiessl *et al.*, 2012). Thus, it appears that JAG/NUB play roles in both organ patterning and growth control, possibly mediating between these two processes. The idea of a more complex role for JAG in lateral organ development is supported by the results of experiments that aimed at the genome-wide characterisation of JAG target genes (Schiessl, Muiño and Sablowski, 2014). Among the targets identified were regulators of diverse processes, including meristem development and organ growth, tissue polarity, cell wall modification, and cell cycle progression. Additional experiments showed that JAG directly represses the *KIP RELATED PROTEIN4* (*KRP4*) and *KRP2* genes, which control the transition to the Synthesis (S)-phase of the cell cycle, and that *krp2* and *krp4* mutant alleles largely suppressed the *jag* mutant phenotype (Schiessl, Muiño and Sablowski, 2014). Thus, JAG appears to regulate organ growth, at least in part, through direct cell cycle control.

A third example that illustrates the complexity of the gene regulatory networks downstream of the floral organ identity factors involves the *Arabidopsis* gene *RABBIT EARS* (*RBE*), which encodes a zinc-finger domain-containing transcription factor that mediates the control of petal development (Takeda, Matsumoto and Okada, 2004). *RBE* has been shown to act directly downstream of the B function regulators *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), which are involved in the specification of petals

and stamens. During petal initiation, RBE controls the microRNA miR164-dependent pathway (Huang *et al.*, 2012), which mediates boundary formation between organ primordia via regulation of *CUP-SHAPED COTYLEDON* genes (Sieber *et al.*, 2007). During this initial phase of petal development, RBE was found to repress the expression of certain TCP transcription factor-coding genes (Huang and Irish, 2015), which are thought to control the transition from cell division to cell expansion and differentiation during organ growth (Huang and Irish, 2016). This repression is lost or reduced as petal development progresses (Huang and Irish, 2015), leading to the promotion of growth and differentiation, thus contributing to the formation of mature petals.

The floral organ identity factors also control the expression of many genes involved in the metabolism of, or the response to, different phytohormones, including auxin, cytokinins, gibberellins, and jasmonic acid. In fact, these hormones have been shown to mediate diverse processes during flower development, ranging from the control of meristematic activity, to pattern formation and organ maturation (Chandler, 2011). How exactly these hormones act during floral organogenesis is in many cases still unknown, however significant progress toward a better understanding of their functions has been made in recent years, for example, by studying and comparing the patterns of auxin and cytokinin signalling at different stages of gynoecium development (Marsch-Martínez *et al.*, 2012). Such work has begun to address the extensive cross talk (and in some cases, antagonism) known to exist between the different hormone signal transduction pathways, and future research will undoubtedly aim at elucidating this interplay at the molecular level.

Taken together, the examples discussed above illustrate the impressive progress that has been made in recent years in understanding the molecular functions of key regulators of floral organogenesis, which act directly or indirectly downstream of the floral organ identity factors. Given that the number of regulatory genes with known or presumed roles in flower development is very large (see above), it will in all probability take many more years before we will have obtained a comprehensive view of the regulatory events that take place during floral organogenesis. Thus, the development of new experimental methods and protocols that allow a more high-throughput characterisation of floral regulators is urgently needed to expedite the systematic analysis of the gene network underlying floral organogenesis.

1.6. Control of floral meristem determinacy

In contrast to shoot apical meristems, which are indeterminate, floral meristems cease their activity once the formation of all floral organs has been initiated (at around stage 6 in *Arabidopsis*; stages according to (Smyth, Bowman and Meyerowitz, 1990). Molecularly, this loss of meristematic activity is often monitored by the expression of *WUSCHEL* (*WUS*), which encodes a homeodomain-containing transcription factor that functions as an important regulator of stem cell fate and is required for the maintenance of shoot and floral meristem identity (Mayer *et al.*, 1998). How is the determinacy of floral meristems regulated? It was shown 16 years ago that the floral organ identity factor AG, which is expressed in the centre of the floral meristem from around stage 3, is essential for terminating the expression of *WUS* in *Arabidopsis* (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001). This result explained the phenotype of *ag* mutant flowers, which not only show organ identity defects (i.e. an absence of reproductive floral organs) but also a strong over-proliferation of the floral meristem (Yanofsky *et al.*, 1990). Also, it

was found that other floral regulators involved in the control of floral meristem determinacy, such as *PERIANTHIA*, act upstream of *AG*. Thus, the floral meristem defects of these mutants are in all probability a consequence of *AG* mis-regulation. So how does *AG* repress *WUS*? It has been proposed that this occurs through both direct and indirect mechanisms (Fig. 1.2). It was shown that *AG* binds to CArG box sequences in the *WUS* promoter and recruits a Polycomb group (PcG) complex involved in the stable silencing of gene expression through covalent histone modifications (Liu *et al.*, 2011). As a consequence of this direct regulation, *WUS* expression declines over time. Indirect mechanisms are then required to terminate *WUS* expression immediately after all floral organ primordia have been initiated. The KNUCKLES (*KNU*) transcription factor (Payne, Johnson and Koltunow, 2004) has been shown to play a key role in this process. *KNU* is directly activated by *AG* at stage 6, exactly at the time when floral stem cell activity ceases (Sun *et al.*, 2009). *KNU* then represses *WUS* likely through direct regulation. The timing of the induction of *KNU* by *AG* has been shown to be a consequence of PcG protein eviction from the *KNU* promoter by *AG*, which over time (and coupled to cell division cycles) leads to a removal of repressive chromatin marks and an activation of *KNU* expression (Sun *et al.*, 2014).

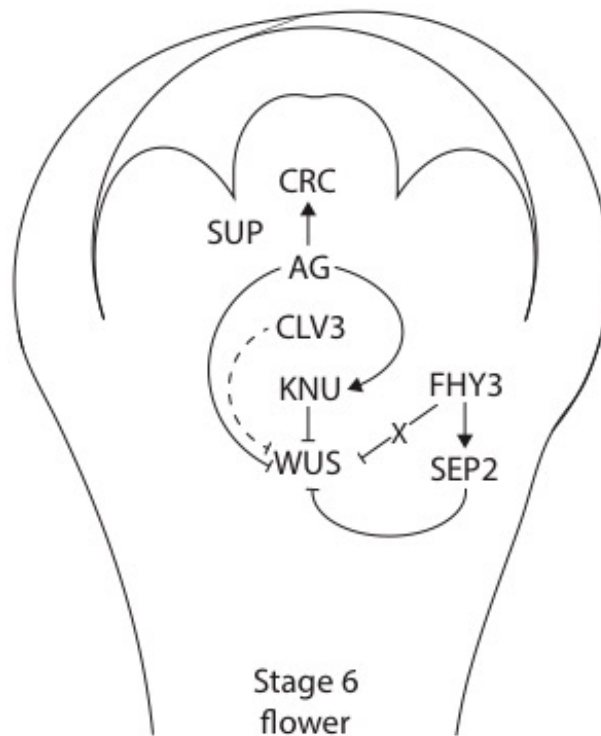


Figure 1.2 - Regulation of floral meristem determinacy. In *Arabidopsis*, stem cell activity within the developing flower ceases around stage 6. *WUS* is down-regulated by several pathways within the floral meristem, resulting in floral meristem termination (see the main text for details). Solid lines and dashed lines indicate direct and indirect regulation, respectively. “X” denotes a proposed unidentified factor whose activity is regulated by FHY3.

Despite the recent progress in our understanding of the molecular mechanisms underlying the control of floral meristem determinacy, several open questions remain. For example, why is the indeterminacy phenotype of *knu* mutant flowers much weaker than that observed for *ag*? One possibility is that other regulators act in parallel with KNU. A good candidate for this is the regulator of carpel and nectary development *CRABS CLAW* (*CRC*). *crc* mutant flowers show mild determinacy defects, which are much enhanced when *AG* activity is reduced (Bowman and Smyth, 1999). Also, like *KNU*, *CRC* is directly activated by *AG* (Gómez-Mena *et al.*, 2005; O’Maoileidigh *et al.*, 2013), and its

expression commences at stage 6 as well (Bowman and Smyth, 1999). Thus, it is tempting to speculate that the activation of *CRC* by AG may involve the same PcG protein eviction mechanism identified for *KNU*. However, one key difference between *KNU* and *CRC* is their expression patterns. While early *KNU* expression is found in the centre of the floral meristem, and especially in the organising centre where *WUS* is expressed (Sun *et al.*, 2009), *CRC* expression occurs on the abaxial side of emerging carpel primordia (Bowman and Smyth, 1999). Thus, the activation of these genes by AG in neighbouring but non-overlapping domains of the flower must involve additional spatial inputs.

In addition to the AG-dependent pathway, other regulatory genes have been identified that control floral organ determinacy. They include *SUPERMAN* (*SUP*; Sakai, Medrano and Meyerowitz, 1995), which encodes a transcription factor closely related to *KNU*, *JAG*, *NUB*, and *RBE* (Sakai, Medrano and Meyerowitz, 1995). *sup* mutants have supernumerary stamens and exhibit an increase or decrease of (abnormal) carpel tissue, depending on the allele. When combined with mutant alleles for *AG*, mutations in *sup* lead to a dramatic meristem over-proliferation phenotype (Bowman *et al.*, 1992), suggesting that *AG* and *SUP* function in parallel pathways. It has been suggested that *SUP* is involved in controlling the boundary between the third and fourth floral whorls, possibly by repressing the expression of the B function regulators *AP3* and *PI* (Prunet *et al.*, 2017). Furthermore, it has been shown that *SUP* controls cell proliferation and differentiation in a hormone-dependent manner (Nibau *et al.*, 2011). How *SUP* acts exactly to control floral meristem determinacy is currently unknown, though Prunet *et al.* (2017) have recently shown through live confocal imaging that the supernumerary stamens characteristic of *sup* mutants arise from fourth whorl cells that change their fate, in conjunction with the

floral stems cells proliferating for a longer period of time, adding to the evidence that SUP has some role in the temporal regulation of stem-cell determinacy.

Another, unexpected, regulator of floral determinacy was described in a recent study. The transcription factor FAR-RED ELONGATED HYPOCOTYL3 (FHY3), which is involved in photomorphogenesis, was shown to control the expression of the stem cell regulator *CLAVATA3* (*CLV3*) in the shoot apical meristem (Li *et al.*, 2016). In floral meristems, FHY3 controls determinacy by directly repressing *CLV3* and by activating the E class gene *SEP2*.

In summary, the results of the studies discussed above show that the genetic mechanisms that regulate floral meristem determinacy are surprisingly complex, and likely evolved to allow plants to precisely control the timing of stem cell termination and to tightly coordinate this process with the early development of floral organ primordia.

1.7. Strategies for gene perturbations

The complexity of the gene regulatory networks (GRNs) underlying floral organogenesis in *Arabidopsis* has been well documented (Gómez-Mena *et al.*, 2005; Schmid *et al.*, 2005; Wellmer *et al.*, 2006; Kaufmann *et al.*, 2010; Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013; Pajoro *et al.*, 2014; Ryan *et al.*, 2015; Goslin *et al.*, 2017). In recent years there have been significant advances in our understanding of the elaborate regulatory mechanisms that contribute to numerous developmental processes, however, large gaps still remain concerning their topologies. Studies focusing on gene perturbations have already contributed to the wealth of knowledge that is now available.

Perturbations to a gene's activity can be achieved by designing experiments that result in either a loss — or gain — of function. Such alterations are often static, such that the experiment employs mutants, transfer DNA (T-DNA) insertions, or constitutively expressed constructs which have the potential to affect the GRN under examination from the onset of development. The use of such techniques can therefore introduce problems when examining stage-specific aspects of development, as the gene of interest has been disturbed at all prior stages, possibly leading to systematic changes to other gene expression profiles that would not have occurred otherwise, and do not reflect the gene's endogenous temporal expression pattern.

In other cases, where it is appropriate to use static mutations for experiments and analyses, a challenge can be present if no suitable mutant line is pre-existing. While a large collection of curated T-DNA insertion lines (Alonso *et al.*, 2003), and chemically induced null mutants (e.g. the Nottingham *Arabidopsis* Stock Centre), is available to the *Arabidopsis* research community, oftentimes a complete disruption to the gene(s) of

interest is not available. Recent advances in the precision genome-editing technology, CRISPR/Cas9, has facilitated the production of user-defined null mutants with little apparent trade-off concerning unintended effects on the remainder of the genome. Some of the aspects and methods for both chemically-inducible gene perturbations, and perturbations via genome editing, are discussed below.

1.7.1. Chemically inducible systems

To circumvent a caveat of traditional analyses, in that using static perturbations can introduce systematic biases to the GRN under investigation, a small collection of inducible promoter systems is routinely used throughout the plant sciences community (Fig. 1.3) (Caddick *et al.*, 1998; Salter, Paine and Riddell, 1998; Zuo, Niu and Chua, 2000; Roslan *et al.*, 2001; Deveau *et al.*, 2003; Craft *et al.*, 2005). The gene's endogenous activity is altered by the exogenous application of specific chemical reagents. In this way, the plant effectively remains in a wild-type state until the transgene is 'switched on', allowing the user to precisely control the timing of the experiment and subsequently facilitate analyses in a stage-specific manner.

Three of the most common inducible promoter systems currently in use rely on two general components — 1) A constitutively expressed regulatory protein whose DNA-binding activity requires a specific chemical stimulus, and 2) a promoter which is bound by the protein in the presence of the stimulus, driving the expression of the transgene of interest. The specific regulatory sequences used in the promoters are bound to a minimal region of the constitutive 35S Cauliflower Mosaic Virus (CaMV) promoter to enhance their activity. Each system can be introduced into a single binary vector for efficient transformation into *Arabidopsis*.

The first inducible system utilises a translational C-terminal fusion of the rat glucocorticoid receptor (GR) ligand binding domain (LBD) to the chimeric transcription factor, LhG4, which is derived from a mutated version of the *Escherichia coli* LacI repressor that is bound to the GAL4 transcription-activation-domain-II of *Saccharomyces cerevisiae* (Fig. 3A) (Craft *et al.*, 2005). When expressed in plants, the GR domain of the fusion protein is bound by a heat shock protein in the cytoplasm, effectively sequestering its activity (Discussed further in Section 3.1). GR-LhG4 is released from this complex in the presence of dexamethasone, allowing its translocation into the nucleus. GR-LhG4 binds to the chimeric pOp promoter which is composed of *lac* operator multimers.

An ethanol-sensitive system is derived from the filamentous fungus *Aspergillus nidulans* (Fig. 3B) (Caddick *et al.*, 1998). In the presence of a metabolite of ethanol, acetaldehyde, the ALCR transcription factor is able to bind to regulatory elements of the *AlcA* promoter, driving transcription of the transgene. Ethanol vapour is used to activate ALCR as it is less toxic than acetaldehyde. It has been shown that direct application of dilute solutions is also sufficient to activate the transgene (Caddick *et al.*, 1998; Salter, Paine and Riddell, 1998; Roslan *et al.*, 2001).

The third system, termed XVE, is responsive to 17- β -Estradiol (EST) (Fig. 3A) (Zuo, Niu and Chua, 2000). The XVE protein is a fusion of the DNA binding domain of the LexA (X) bacterial repressor, the acidic transactivation domain of the Herpes Simplex Virus VP16 protein (V), and the human estrogen receptor regulatory domain (E). Upon application of EST to the transgenic plant, the XVE protein binds to a multimer of the *LexA* operator sequence, leading to the expression of the transgene of interest. Although widely used, we have found that a limitation of this system is that EST is highly insoluble

in water, leading to problems when inducing tissues that require the direct application of an aqueous solution.

As all three systems express their respective activator proteins constitutively, inducible expression of the transgene can occur at any region in the plant that the chemical is able to reach. However, all of the systems have the potential to be modified such that the activators are placed under the regulation of a tissue-specific promoter, enabling the user to control the spatial expression profile of the transgene in addition to the temporal expression.

In our laboratory we have taken advantage of the latter two systems to knock down floral homeotic genes using artificial microRNAs (amiRNAs) (Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013). We have also combined such constructs with floral induction systems, to produce large amounts of synchronously developing floral tissue which have the potential to be subjected to the effects of inducible transgenes in a stage-specific manner (This study; O'Maoileidigh *et al.*, 2015).

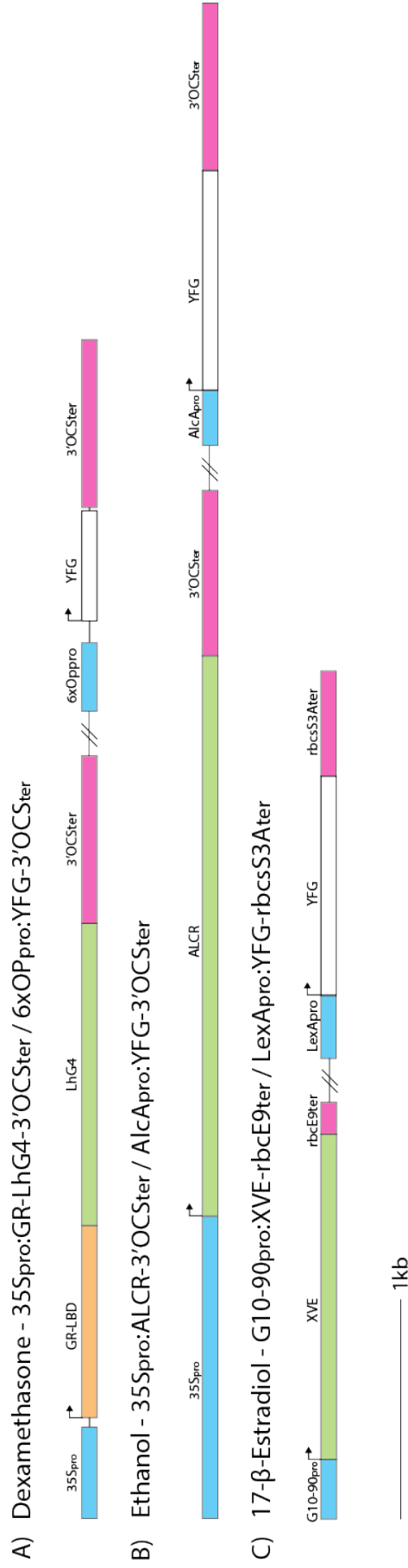


Figure 1.3 - Schematic representations of inducible promoter systems. (A) The gene of interest is placed under the control of the LexA promoter (LexA_{pro}) which can be activated by the chimeric XVE transcription factor, after treatment with 17-β-estradiol.

(B) The gene of interest is placed under the control of the 6xOp promoter (6xOp_{pro}), which can be activated by the GR-LhG4 chimeric transcription factor, after treatment with dexamethasone.

(C) The gene of interest is placed under the control of the AlcA promoter (AlcA_{pro}), which can be activated by the ALCR transcription factor, after treatment with ethanol. Arrows indicate transcription start sites. G10-90_{pro}: synthetic G10-90 promoter; rbcS E9_{ter}: rbcS E9 poly(A) addition sequence; rbcS3A_{ter}: rbcS3A poly(A) addition sequence; 35S_{pro}: Cauliflower Mosaic Virus 35S promoter; OCS_{ter}: terminator of octopine synthase gene from *Agrobacterium tumefaciens*; YFG: 'Your Favourite Gene'. Scale bar: 1 kb

1.7.1.1. Artificial microRNAs

MicroRNAs (miRNAs) are endogenous small non-coding RNA transcripts that have evolved to function in numerous developmental processes through post-transcriptional gene silencing, and translational repression, by the destruction of target mRNAs. Most miRNA targets are transcription factors, whose regulation is critical for the proper functioning of the gene regulatory networks in which they participate (Samad *et al.*, 2017). Multiple examples of miRNA mediated regulation of transcription factor activity have been documented during vegetative and reproductive development (Chen, 2004; Laufs, 2004; Nikovics *et al.*, 2006).

miRNAs are typically 20-22 nucleotides in length and their biogenesis in plants is well characterised. RNA polymerase II transcribes long single stranded primary miRNAs (pri-miRNAs), which adopt a hairpin-like secondary structure. Precursor miRNAs (pre-miRNAs) are then processed from these molecules by DICER LIKE 1 (DCL1), which cleaves them to produce shorter stem-loop structures (Chen, 2005). Mature miRNAs are produced by further processing of the precursor by DCL1, resulting in duplexed RNA molecules, one of which is the active miRNA and the other is its complementary strand. miRNA duplexes are subsequently methylated at their 3' termini by HUA ENHANCER 1 (HEN1), protecting them from degradation (Yu, 2005). Those that are not methylated are potentially subjected to degradation by HEN SUPPRESSOR 1 (HESO1) (Zhao *et al.*, 2012), a uridylyltransferase, promoting their degradation by members of the SMALL RNA DEGRADING NUCLEASE (SDN) family. Methylated miRNA duplexes are loaded into ARGONAUTE 1 (AGO1), which then interacts with and cleaves target mRNA. AGO1 directly interacts with HESO1 and cleaved mRNA fragments are promoted for degradation through 3' polyuridylation (Zhai *et al.*, 2013). Certain miRNAs

have also been shown to induce post-transcriptional gene silencing through monouridylation of their 3' terminus by UTP:RNA URIDYLYLTRANSFERASE 1 (URT) (Tu *et al.*, 2015).

miRNA function in plants has been extensively studied through computational analysis to determine the parameters that affect target specificity (Schwab *et al.*, 2005). In contrast to animal miRNA binding motifs, which are likely to contain multiple mismatches, miRNAs in analysed plant species have a much higher frequency of perfectly complementary binding sequences. This property has facilitated the accurate prediction of miRNA binding sites, though it has been shown that plant miRNAs are able to interact with several targets containing up to four or five mismatches (Palatnik *et al.*, 2003). Schwab *et al.* (2005) subsequently used genome-wide expression profiling to describe the factors determining plant miRNA activity and specificity. Using these parameters has led to the advent of artificial microRNAs (amiRNAs) (Schwab *et al.*, 2006), allowing the plant sciences community to target specific endogenous mRNAs and therefore use them as a tool for gene perturbation experiments (Alvarez *et al.*, 2009; Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013). Further, the online tool Web MicroRNA Designer 3 has been developed, facilitating simple target motif identification and amiRNA generation (Ossowski, Schwab and Weigel, 2008).

1.7.2. CRISPR-Cas9 genome editing

The development of effective RNA-guided endonucleases (RGENs) throughout the biological sciences is rapidly changing how genome editing studies are performed. Other enzyme-mediated strategies exist to facilitate genome editing, such as programmable zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), both of which function by engineering the proteins' DNA-binding domains to interact with the genomic sequences either side of the target region. The proteins are fused to the *FokI* nuclease domain, which functions as a dimer and facilitates double strand breaks (DSBs), causing the DNA repair machinery to reattach the severed strands (Wang, La Russa and Qi, 2016). However, as these strategies require the proteins to be cloned into site-specific DNA-binding domains for each locus being targeted, they are not suitable for high throughput applications.

The use of CRISPR (clustered regularly interspaced short palindromic repeats) / Cas (CRISPR associated) systems has therefore emerged as the prominent toolkit, in part due to the ability of the system to guide the endonuclease activity of the Cas protein using easily-produced short non-coding RNA sequences complementary to the genomic target site, thus facilitating high throughput studies as the user can simply insert the target sequence into their chosen vector using standard molecular biology techniques such as overlap-extension PCR or Golden Gateway reactions (Hyun *et al.*, 2014; Wang *et al.*, 2015).

The system, which originates from bacteria and archaea, was discovered in *E. coli* in the 1980s (Ishino *et al.*, 1987) and involves the coordinated action of a variety of Cas proteins under endogenous conditions depending on the species (Wright, Nuñez and Doudna,

2016). Native Cas proteins have multiple roles in adaptive immunity, being involved in CRISPR RNA (crRNA) biogenesis, immunity adaptation, and recognising PAM (protospacer adjacent motif) sequences present in foreign DNA. Short fragments of the invading DNA, called protospacers, are incorporated into CRISPR loci between crRNA repeats, which facilitate Cas recognition of the small RNA. From this point, the locus containing protospacers and crRNAs are expressed as a single precursor molecule, which is then processed to produce mature crRNAs that interact with Cas proteins. A noncoding trans-activating RNA (tracrRNA) is also required for crRNA processing and interaction with the nuclease. Cas interacts with the crRNA/tracrRNA and is recruited to the target site by a combination of Watson-Crick base-pairing of the guide to genomic DNA and a direct interaction between Cas and the PAM sequence. The nuclease activity of Cas causes double strand breaks (DSBs) to be induced, which are repaired mainly through Non-Homologous End Joining (NHEJ), and to a lesser extent Homology Directed Repair (HDR) (For a review, see Wright, Nuñez and Doudna, 2016).

Two classes of CRISPR systems exist depending on the nature of the effector proteins, with class I systems characterised by large multi sub-unit complexes while class II systems only require one effector protein to facilitate the recognition and cleavage of substrate DNA (For reviews see Makarova *et al.*, 2015; Wright, Nuñez and Doudna, 2016). Cas9, a class II effector protein derived from *Streptococcus pyogenes*, has been widely adopted since it was demonstrated that the protein could interact with a single guide RNA (sgRNA) chimera incorporating a crRNA and the tracrRNA (Jinek *et al.*, 2012), and induce genomic DNA cleavage at targeted loci in mammalian cells (Cong *et al.*, 2013). Since then the system has seen widespread use and modification to perform a variety of functions beyond genome editing, such as transcriptional regulation using a

deactivated form of Cas9 (dCas9) fused to different domains that can repress or activate transcription, or even induce epigenetic changes (Reviewed in Wang, La Russa and Qi, 2016).

1.7.2.1. CRISPR/Cas9 systems for mutation generation in plants

The generation of stable inheritable mutations in plants is of great importance, and up until recently this has mainly been achieved through chemical mutagenesis or insertional mutagenesis using T-DNA insertions. Indeed, in *Arabidopsis* a large repository of mutant lines has been developed and curated, facilitating genetic studies (Alonso *et al.*, 2003). These established methods of mutagenesis, however, have drawbacks insomuch as both methods rely on random mutagenic events that require labour-intensive screening to identify the affected loci. Further, especially in the case of insertional mutagenesis, the available lines possessing a T-DNA insertion in the vicinity of the gene of interest might not knock it out, instead resulting in the reduced transcription of the gene and the possibility that this mis-regulation does not accurately reflect a full loss-of-function of the gene. Moreover, the T-DNA could also affect the regulation of nearby genes that are not the focus of study, potentially contributing to erroneous conclusions.

The adoption of CRISPR/Cas9 systems in plants has therefore facilitated the generation of targeted mutations that are stably inherited. Furthermore, alterations of the genome have the potential to be isolated exclusive of the T-DNA carrying the sgRNA and Cas9 cassettes, producing genetically modified plants that do not harbour transgenes, possibly circumventing an important issue in the ongoing debate surrounding the release of genetically modified organisms into the environment (Jansson, 2015).

1.7.2.2.Strategies for detecting mutations

The DNA repair processes that result in the insertion or deletion of bases, disrupting a gene's function, often require detection using methods that are sensitive enough to distinguish between single nucleotide differences between alleles. Successful mutagenesis can be preliminarily detected through phenotype scoring if the gene of interest's mutant phenotype is already known, although in other cases this is not a viable method if the gene's function and phenotype are uncharacterised, or if other genes are functionally redundant.

Several methods exist to qualitatively detect small nucleotide insertions and deletions (indels) by taking advantage of the formation of heteroduplexes between wild-type and mutated DNA when the region is amplified using PCR. For instance, high-resolution melting analysis (HRM) detects this formation by comparing melting curves of amplified fragments in the presence of fluorescent dyes that are only active when they interact with double stranded DNA (e.g. Thomas *et al.*, 2014; Samarut *et al.*, 2016). Heteroduplex molecules will have a melt curve that is distinct from wild-type homoduplexes, allowing for their identification. Digestion of heteroduplex fragments is also possible using enzymes such as T7 endonuclease I (T7E1) (Déclais *et al.*, 2003) or Surveyor™ nuclease (Qiu *et al.*, 2004). It has been reported that T7E1 does not detect all single base substitutions, but has greater sensitivity towards indel mutations than Surveyor™ nuclease (Tsuji and Niida, 2008), thus these properties must be considered when performing mutant screens.

1.7.2.3. *Cas9-mediated off-target effects*

One of the main concerns surrounding the use of CRISPR systems is that Cas9 could erroneously bind to regions in the genome with high levels of homology to the target sequence. That is, the designed guide RNA could still bind to DNA, even in the presence of nucleotide mismatches in the genomic sequence, leading to unwanted genome editing events. Indeed, this has been demonstrated in human cell lines where off-target sites containing up to 5 nucleotide mismatches experienced similar levels of mutagenesis compared to the intended site (Fu *et al.*, 2013). However, it has also been shown that the position of mismatches within the target sequence influences the efficiency of Cas9 activity (Anderson *et al.*, 2015), and that off-target effects are particularly prevalent when a mismatch exists immediately distal to the PAM region (Tsai *et al.*, 2015), highlighting the importance of careful consideration of target sequence selection.

In *Arabidopsis* and other plant species it has been reported that Cas9 has very high specificity. Peterson *et al.* (2016) demonstrated that the specificity of Cas9 was such that unwanted mutations were not detectable, and others studies have also reported a lack of mutational events outside of their target sites, by way of whole-genome sequencing, Sanger sequencing, or amplification and restriction digestion of the loci predicted to contain potential off-target sites (Nekrasov *et al.*, 2013; Feng *et al.*, 2014; Xu *et al.*, 2015).

Despite the apparent lack of off-target activity of CRISPR systems in plants, it is still best practice to select target sequences that, when compared to the rest of the genome, do not have high homology with other loci. Online programs exist that perform this analysis, for instance *Cas-OFFinder* (Bae, Park and Kim, 2014) or *CRISPOR* (Haeussler *et al.*, 2016), allowing the user to select guides that have fewer potential off-targets. Despite this, off-

target prediction software is not completely accurate, as the molecular mechanisms governing CRISPR/Cas9 activity still have not been fully elucidated, so a bias is introduced wherein only selected off-target sites are examined for off-target effects. The use of high-throughput unbiased next generation sequencing methods is therefore necessary to ensure unintended mutations have not been created (Reviewed in Zischewski, Fischer and Bortesi, 2017).

1.8. Aims of this thesis

Broadly, this thesis has two focuses. Chapters 3 and 4 describe the generation and optimisation of floral induction systems, that possess inducible artificial miRNAs, for use in time course transcriptomics experiments. Several potential modifications to the originally described floral induction system (Wellmer *et al.*, 2006), a transgenic plant line that allows for user-controlled generation of large amounts of synchronously induced floral tissue, were examined and characterised. Two floral induction system lines were generated that possess two independent hormone-inducible constructs, to perturb homeotic gene activity at specified stages in flower development, and it was demonstrated that these lines can be reliably used with no ‘cross-talk’ between the two inducible systems. A series of stage-specific knockdowns was subsequently performed and subjected to transcriptomic analysis using next generation sequencing to explore the temporal changes to the gene regulatory network governing flower development as a result of these knock-downs.

Chapter 5 is an investigation and characterisation of two novel, and closely related, F-box genes thought to potentially be involved in some aspect of flower development, specifically the nature of their genetic interaction with the floral meristem and organ

identity gene *UNUSUAL FLORAL ORGANS (UFO)*. To explore this possibility, an analysis of several lines was performed between *ufo* mutants crossed with knock-down lines of the novel genes, with a focus on detecting alterations to the floral defects characteristic of *ufo* mutants. Several methods were used to attempt to characterise the genes, and assign them some function, including over-expression analyses and determination of their expression profiles. Last, using these genes as a proof of concept, the CRISPR/Cas9 genome editing technology was employed in the Wellmer laboratory for the first time to obtain full knockouts of both genes, separately and simultaneously, with the intention of dissecting each's function and to determine if they act redundantly.

2. Materials and Methods

2.1. Strains and growth conditions

2.1.1. Plant lines

The plant lines used in this study are:

Columbia (Col-0) wild-type

Landsberg *erecta* (L-*er*) wild-type

Col-0 *ufo-1*

Col-0 *ufo-13*

Col-0 *pi-1*

Col-0 SAIL 272_A02 (*nan-1 gra-1*)

Col-0 *nan-1 gra-1 ufo-1*

Col-0 *nan-1 gra-1 ap3-3*

Col-0 *nan-1 gra-1 pi-1*

Col-0 SALK_076200 (*gra-2*)

Col-0 *gra-2 ufo-1*

Col-0/L-*er gra-2 ufo-2*

L-*er ap3-3*

L-*er ufo-2*

L-*er ufo-2 x 35S::NAN&GRAamiRNA*

L-*er AP1::AP1-GR ap1-1 cal-1*

L-*er AP1::AP1-AR ap1-1 cal-1*

L-*er 35S::ALCR ALCA::AP1 ap1-1 cal-1*

L-*er G10-90::XVE LEXA::AP1 ap1-1 cal-1*

L-er 35S::LhG4-GR 6xOP::AP3amiRNA
L-er 35S::LhG4-GR 6xOP::AGamiRNA
L-er AP1::AP1-AR 35S::LhG4-GR 6xOP::AP3amiRNA
L-er AP1::AP1-AR 35S::LhG4-GR 6xOP::AGamiRNA
L-er GT_11847 (*nan-2*)
L-er nan-2 ufo-2
 Col-0 35S::NAN
 Col-0 35S::NAN-SRDX
 Col-0 35S::NAN-VP16
 Col-0 35S::GRA (Hanczaryk, 2014)
 Col-0 35S::GRA (Generated in this study)
 Col-0 35S::GRA-SRDX
 Col-0 35S::GRA-VP16
 Col-0 NAN::GUS
 Col-0 GRA::GUS
 Col-0 UFO::GUS
 Col-0 pGREEN U6-26::GRAsg1 ICU2::Cas9
 Col-0 pGREEN U6-26::GRAsg5 ICU2::Cas9
 Col-0 pHEE U6-26::NANsg10 EC1.2::zCas9
 Col-0 pHEE U6-26::NANsg7 U6-29::GRAsg9 EC1.2::zCas9

2.1.2. Bacterial strains

***Escherichia coli* strain Top10** (IBA Life Sciences): F⁻, mcrA, Δ(mrr-hsdRMS-mcrBC), φ80lacZΔM15, ΔlacX74, recA1, araΔ139, Δ(ara-leu)7697, galU, galK, rpsL (Str^R), endA1, nupG

***Escherichia coli* strain Stbl2** (Isolated by C. Brower): F⁻, mcrA, Δ(mcrBC-hsdRMS-mrr), recA1, endA1, gyrA96, thi-1, supE44, relA1, λ⁻, Δ(lac-proAB) - also carries unknown mutation that decreases recombination

***Agrobacterium tumefaciens* strain C58 pGV2260** (McBride and Summerfelt, 1990)

***Agrobacterium tumefaciens* strain C58 pGV3101** (Holsters et al 1980)

2.1.3. Yeast strain

***Saccharomyces cerevisiae* strain PJ69-4A** (MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) (James et al. 1996)

2.1.4. Bacterial growth media

LB (Luria-Bertani medium): 10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl. 1.5% (w/v) agar was added to make LB agar plates.

SOB (Super Optimal Broth): 20 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 0.5 g/L NaCl, 0.95 g/L anhydrous MgCl₂, 2.467g/l MgSO₄ (heptahydrate).

2.1.5. Plant growth media

2.1.5.1. Soil

Seeds were sown on sterilised soil composed of compost, vermiculite, and perlite at a ratio of 3:1:1 (volume). Seeds were stored at 4°C for three days before transfer into the growth facility. Plants were grown under constant illumination at ~20°C unless otherwise stated. Approximately 5 ml of Osmocote® Exact fertiliser was placed in the bottom of the growth trays, and 1.5 ml of Intercept 5GR insecticide was placed in the bottom of the growth trays to prevent aphid infection when necessary.

2.1.5.2. Murashige and Skoog medium

2.2g/L Murashige and Skoog (MS) powder, pH adjusted to 5.7 with KOH. 0.6% (w/v) agar was added to make MS agar plates.

2.2. Cloning and genotyping

2.2.1. Plasmid DNA extraction

2.2.1.1. Minipreps

Cultures of transformed *E. coli* colonies were grown in 5 ml of LB at 37°C overnight with the appropriate selective antibiotic. The cultures were centrifuged at 4000 RPM for 5 minutes and plasmids were extracted from the pellets using the EZNA Plasmid DNA Miniprep Kit supplied by Omega BioTek, according to the manufacturer's instructions.

2.2.1.2. Midipreps

Cultures of transformed *E. coli* colonies were grown in 40 ml of LB at 37°C overnight with the appropriate selective antibiotic. The density of the cultures was measured and a final cell mass was of 80 was used. The volume for the final cell mass was calculated by dividing 80 by the OD₆₀₀ value of the culture. Midipreps were performed using the GenElute Plasmid Midiprep kit (Sigma-Aldrich) according to the manufacturer's instructions.

2.2.2. PCR product purification

PCR products were purified using the EZNA Cycle Pure kit (Omega BioTek) according to the manufacturer's instructions, with the following amendments: After the washing steps, columns were centrifuges at 15000 RPM for 5 minutes to remove residual ethanol. The columns were transferred to a clean 1.5 ml centrifuge tube and place in a heat block

at 42°C for 10 minutes to ensure the columns were dry. 30 µl of elution buffer was pipetted onto the columns and incubated at 42°C for 5 minutes.

2.2.3. Agarose gel extraction of DNA

PCR products and digested DNA samples were extracted from agarose gels after separation by electrophoresis using the EZNA Gel Extraction kit (Omega BioTek) according to the manufacturer's instructions, with the same amendments as described in the above section.

2.2.4. Quantification of nucleic acid concentrations

DNA and RNA concentrations were determined using the Nanodrop1000 spectrophotometer. Before use the Nanodrop1000 was blanked using the same solution in which the nucleic acid was dissolved.

2.2.5. Plant genomic DNA extraction

2.2.5.1. Cetyltrimethyl Ammonium Bromide (CTAB) genomic DNA extraction

This method was used to purify *Arabidopsis* genomic DNA to be used as template in PCRs that amplify regions of interest, for use in construct generation. Approximately 100 mg of plant tissue was ground in liquid nitrogen, and one volume of CTAB (Appendix 6.1) buffer (Appendix 6.1) (pre-heated to 65°C) is added. Tissue was incubated at 65°C for up to 1 hour, and two phenol-chloroform extractions are performed using phase-lock tubes (5-Prime) to separate the aqueous layer from the phenol layer. Genomic DNA was precipitated from the resulting aqueous layer by adding 0.7 volumes of isopropanol, gently mixed by inversion, and incubated at room temperature for 10 minutes. DNA was pelleted by centrifugation at 14000 RPM for 15 minutes at room temperature. The pellet was washed with 70% (v/v) ethanol and air dried. The pellet was suspended in 50 µl TE buffer or H₂O.

2.2.5.2. *Edwards DNA preparation*

This method was used to rapidly isolate plant genomic DNA for use in PCR genotyping assays. Briefly, a small section of leaf tissue was dissected from the plant and ground in 400 µl of Extraction buffer (Appendix 6.1) using a pestle. Samples were centrifuged at 14000 RPM for 1 minute to pellet cell debris, and 300 µl of the supernatant was transferred to a new tube containing 300 µl of isopropanol to precipitate the DNA. Tubes were briefly vortexed and centrifuged at 14000 RPM for 3 minutes to pellet the DNA. The supernatant was decanted and the pellet was washed with 70% ethanol. Tubes were centrifuged at 14000 RPM for 3 minutes and the supernatant was decanted. Tubes were dried inside a vacuum centrifuge and the genomic DNA was suspended in TE (Appendix 6.1) buffer. 2 µl was used in a typical genotyping PCR. All steps were performed at room temperature.

This method was also sufficient for use in PCRs with proofreading polymerases, for example Phusion polymerase (ThermoScientific), with the following amendments: The amount of plant tissue was kept constant by using a hole punch to dissect a ~5 mm diameter leaf disc. After decanting the isopropanol, and 70% ethanol, the tubes were placed upside down on absorbent tissue paper to remove a greater amount of the respective reagents. 200 µl ddH₂O was used to re-suspend the dried genomic DNA and 1 µl was typically used for PCRs, 20 µl final volume, intended to amplify genomic sequences.

2.2.6. *Preparation of chemically competent E. coli cells*

Competent cells were prepared according to the protocol established by Inoue *et al.* (1990).

2.2.7. Transformation of Escherichia coli

2.2.7.1. Stbl2

Plasmids were transformed using the chemically competent *E. coli* strain Stbl2. 100µL of competent cells per transformation were thawed on ice for approximately 20 minutes, added to the plasmid being transformed, mixed gently, and incubated on ice for a further 15 minutes. Cells were subjected to heat shock at 42°C for 1 minute and transferred immediately onto ice for at least 1.5 minutes. 1 ml of LB was added to the cells and they were incubated at 37°C for 60 minutes. The cells were pelleted at 7500 RPM for 1 minute and 1 ml of supernatant was removed. The cells were suspended in the remaining supernatant, plated on selective LB agar plates, and incubated at 37°C overnight. Note: Cells being transformed with plasmids conferring ampicillin/carbenicillin resistance did not require the period of incubation before plating.

2.2.7.2. TOP10

Plasmids were transformed using the chemically competent *E. coli* strain TOP10. 100 µl of competent cells per transformation were thawed on ice for approximately 20 minutes, added to the plasmid being transformed, mixed gently, and incubated on ice for a further 30 minutes. Cells were subjected to heat shock at 37°C for 5 minutes and transferred immediately onto ice for 5 minutes. 900 µl of LB was added to the cells and they were incubated at 37°C for 45 minutes. The cells were pelleted at 7500 RPM for 1 minute and 900 µl of supernatant was removed. The cells were suspended in the remaining supernatant, plated on selective LB agar plates, and incubated at 37°C overnight. Note: Cells being transformed with plasmids conferring ampicillin/carbenicillin resistance were pelleted immediately after addition of 1 ml of LB.

2.2.8. *Preparation of competent Agrobacterium tumefaciens*

In order to generate competent cells, a 5 ml culture of *A. tumefaciens* was grown overnight at 28°C. Then 250 mL of LB supplemented with 0.2 g/l MgSO₄ was inoculated with 0.5 ml of the 5 ml culture. This culture was grown at 28°C until an OD₆₀₀ between 1 and 2.5 was reached. The cells were then pelleted by centrifugation and washed with ice-cold 10 mM CaCl₂. Subsequently, the suspension was pelleted and suspended in 4 ml ice-cold 10mM CaCl₂. 100 µl aliquots were made and frozen in liquid nitrogen. In order to transform the competent cells, an aliquot of cells was thawed and incubated with the plasmid of interest for 5 min in liquid nitrogen. The cell suspension was then thawed at room temperature and 1 ml of LB was added. Afterwards, the cell suspensions were incubated at 28°C for 3-4 h. After incubation, the cells were pelleted through centrifugation and the pellet was suspended in 500 µl LB. An aliquot was then plated on an LB agar plate containing rifampicin (50 µg/ml), carbenicillin (100 µl/ml) and spectinomycin (100 µg/ml). Plates were incubated at 28°C for approximately 3 days.

2.2.9. *Transformation of Agrobacterium tumefaciens*

Plasmids were transformed using the competent *Agrobacterium* strains GV2260 and GV3101. 1 µg of purified plasmid was added to 100 µl of competent cells per transformation, which were thawed at room temperature for approximately 20 minutes beforehand. The cells were snap frozen in liquid nitrogen for 5 minutes, removed, and allowed to thaw at room temperature. 1 ml of LB was added before incubating at 28°C for 4 hours. Cells were pelleted at 5000 RPM for 5 minutes and 500 µl of supernatant was removed. Cells were suspended in the remaining supernatant, plated on selective LB agar plates, and incubated at 28°C for approximately 48 hours. Competent cells that were transformed with plasmids conferring kanamycin resistance were co-transformed with an equal quantity of the pSOUP helper plasmid.

2.2.10. Colony PCR of Escherichia coli

To determine the presence of relevant inserts within *E. coli* colonies after transformation, single colonies were picked and suspended in 20 µl LB. 1.5 µl of the suspension was used in subsequent genotyping PCRs, 15 µl total.

2.2.11. Colony PCR of Agrobacterium tumefaciens

Single colonies of transformed *Agrobacterium* were picked, suspended in 20 µl of lysis buffer (Appendix 6.1), and heated at 96°C for 10 minutes to lyse the cells. Cell debris was pelleted by centrifugation at 13000 RPM for 3 minutes. 2 µl of the supernatant was used for genotyping PCRs.

2.2.12. Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments, such the products of restriction digests and products derived from PCR amplification. All gels were composed of 1% (w/v) agarose and 1X TAE (Appendix 6.1) buffer, unless otherwise stated. 1/10th volume of 10X loading dye was added to samples before they were loaded into the wells.

2.2.13. Oligonucleotide design

Oligonucleotides were designed using the online tool OligoAnalyzer, provided by Integrated DNA Technologies (<http://www.idtdna.com>). Oligonucleotides designed using this tool were used in various PCRs. All oligonucleotides were designed such that a minimum amount of dimerization and hairpin structures occurred, to have a GC content between 40-60%, and a melting temperature between 50-60°C.

2.2.14. Amplification of coding sequences and genomic DNA

PCRs were carried out in order to amplify gene coding sequences from stock plasmids and *Arabidopsis* genomic DNA using the high-fidelity DNA polymerase Phusion (Thermo Scientific). This strategy was used to ensure conservation of the DNA sequence

being amplified, as the Phusion enzyme has an error rate 50x less than standard *Taq* polymerase (Frey and Suppmann, 1995).

2.2.15. Sequencing of DNA

Plasmids and PCR fragments were sequenced by GATC (www.gatc-biotech.com) using Sanger sequencing.

2.2.16. Dephosphorylation of DNA

DNA fragments and plasmids digested with restriction enzymes were 5' dephosphorylated by adding 1 µl Fast AP (Thermo Scientific) to digestion reactions. The reactions were run for 1 hour at 37°C. The alkaline phosphatase was deactivated by heating the reaction to 75°C for 10 minutes.

2.2.17. DNA ligations

Ligations of DNA fragments previously digested with compatible restriction enzymes were performed using T4 DNA Ligase (ThermoScientific). Unless otherwise specified, ligation reactions were carried out under the following conditions: 100ng digested and dephosphorylated vector, 3 molar equivalent digested insert DNA, 2 µl 10X T4 DNA Ligase buffer, 0.2 µl T4 DNA Ligase, ddH₂O to 20 µl. Reactions were performed at 22°C for 10 minutes.

2.2.18. Ligation of PCR products into pGEM-Easy T Vector System I

A-tailed PCR fragments from the previous section were ligated into the pGEM-Easy T vector (Promega) according to the manufacturer's instructions. The ligations were carried out at room temperature for 1 hour and 10 µl of the reaction was used to transform chemically competent Stbl2 *E. coli* cells. Transformed cells were plated on LB-agar supplemented with 100 µg/ml carbenicillin. Single colonies were genotyped for the

presence of inserts using an M13 forward primer and a reverse primer specific to the insert, unless otherwise stated.

2.2.19. RNA extraction

Total RNA was isolated from plant material using the Spectrum Total Plant RNA Kit (Sigma Aldrich) according to the manufacturer's instructions. On-column DNaseI digestion was performed on samples to remove as much residual genomic DNA as possible. RNA quality was measured using a Nanodrop1000 spectrophotometer to check OD_{260/230} and OD_{260/280} ratios. Purified RNA was stored at -80°C.

2.2.20. cDNA Synthesis

cDNA was synthesised using Revertaid H Minus Reverse Transcriptase (Thermo Scientific) and Oligo(dT)18 primer (Thermo Scientific). Unless otherwise stated, 500ng of total RNA and 0.5 µl Reverse Transcriptase was used to synthesise cDNA in each 20 µl reaction. The cDNA was diluted by the addition of 80 µl ultra-pure H₂O, and 2 µl was used for quantitative PCR and reverse transcription PCR. cDNA synthesis was carried out at 42°C in a thermal cycler for 60 minutes, and the reverse transcriptase was inactivated by heating the reaction to 75°C for 10 minutes. cDNA was stored at -20°C.

2.3. Plant transformation and selection of transformants

2.3.1. Floral dip transformation of Arabidopsis

Agrobacterium mediated transformation of *Arabidopsis* was performed according to the protocol described in Clough & Bent (1998).

2.3.2. Seed sterilisation

Seeds were sterilised within a sealed container by exposing them to chlorine gas released from the reaction between bleach (Domestos) and concentrated HCl. 3 ml of HCl was added to 100 ml of bleach in a glass beaker, and left within the chamber for two hours. After sterilisation seeds were immediately transferred to 0.5X MS agar with the appropriate herbicide and stored at 4°C for two days before being transferred to the growth facility.

2.3.3. Selection of Arabidopsis transformants

2.3.3.1. Selection on soil

Transgenic seeds that possessed a BAR resistance cassette were selected on soil using 0.15% (V/V) ammonium-glufosinate (BASTA™) supplied by Bayer. Seedlings were sprayed three times at two-day intervals. Those that formed true leaves were transplanted to fresh soil and genotyped for the presence of the transgene of interest.

2.3.3.2. Selection on MS agar plates – Hygromycin

Transgenic seeds that possessed a hygromycin B resistance cassette were grown on 0.5X MS agar supplemented with 25 µg/ml hygromycin (Melford). Seedlings were grown for 10 days at 21°C under long day conditions, at which point seedlings with extended

hypocotyls, roots, and expanded cotyledons were transferred to soil and genotyped for the transgene of interest after sufficient time to allow for the seedlings to grow.

2.3.3.3. Selection on MS agar plates – DL-Phosphinothricin

Transgenic seeds that possessed a BAR resistance cassette were grown on 0.5X MS agar supplemented with 50 μ M DL-Phosphinothricin (Duchefa Biochemie). Seedlings were grown for 10 days at 21°C under long day conditions, at which point seedlings with extended hypocotyls and expanded cotyledons were transferred to soil or collected for use in RNA extractions and RT-qPCR.

2.4. Crosses and isolation of mutants

Plant lines of interest were crossed by manual pollination of one line with the pollen of the other. Individual flowers to be pollinated had their stamens removed by dissection before anthesis. Pollinated flowers were isolated and left to mature until seeds had fully developed. To determine if the cross had occurred successfully, plants of the next generation (F1) were genotyped for the presence of transgenes, mutations, or T-DNA insertions of interest. Those that had been crossed successfully were brought to the F2 generation, at which point homozygous individuals were isolated.

2.5. Quantitative PCR

Primers were designed to have a melting temperature of 55-60°C. Relative enrichment of cDNA was quantified using the Light Cycler 480 (Roche) and the SYBR Green Master I reagent (Roche). Each reaction consisted of 2 μ l of a 1:5 dilution of cDNA, 1 μ l of 5 μ M primer mix, and 5 μ l of SYBR Green Master I, and 2 μ l ultra-pure H₂O. A master mix consisting of appropriate amounts of the three prior reagents was made up, and 8 μ l was used in each reaction. Plates were centrifuged at 1200 RPM with a swing-bucket rotor for

30 seconds before insertion into the Light Cycler 480. An equivalent time of 60s per 1 kb of DNA was given to generate the amplicons. Annealing temperatures between 58°C and 60°C were used depending on the primer pairs. The enrichment observed was calculated relative to the input sample. Therefore, for each locus analysed, the enrichment was calculated as follows: $2^{Cp(REF)-Cp(GENE)}$, where Cp is the crossing point calculated by the Lightcycler 480, Cp(REF) is the Cp value for the chosen reference gene and Cp(GENE) is the Cp value for the chosen target gene. The reference gene used for qRT-PCR was DM-242/DM-243 (AT1G13320), which was chosen from Czechowski *et al.* (2005).

2.6. β -Glucuronidase (GUS) staining of plant tissue

Transgenic plants harboring transcriptional GUS reporter fusions were stained and sectioned to examine the expression profiles of each construct. Briefly, tissue was collected and immediately placed ice-cold 90% acetone until sample collection had finished. A vacuum was applied for 10 minutes at room temperature to allow the acetone to infiltrate the tissue completely. Samples were transferred in to staining solution (Appendix 6.1) that did not contain the X-gluc (5-Bromo-4-chloro-3-indolyl- β -D-glucuronide) substrate and a vacuum was applied for 15 minutes on ice. Samples were transferred next in to staining solution containing X-gluc (Appendix 6.1) and three successive rounds of vacuum infiltration on ice were performed for 15 minutes each, or until the tissue was no longer buoyant. Samples were incubated within the staining solution at 37°C for the appropriate amount of time to allow sufficient staining to occur.

2.7. Fixation of plant tissue

GUS-stained plants were dehydrated through a progressive ethanol series (20%, 30%, 50%) in H₂O, incubated in FAA (Appendix 6.1), then underwent incubation in a further ethanol series (70%, 80%, 90%). All incubations were for 30 minutes at room temperature. Samples were incubated overnight in 95% ethanol with EosinY. Tissue was washed twice in ethanol, and cleared in 25% and 50% (V/V) tert-butanol (TBA) in ethanol. All incubations were for 30 minutes at room temperature. Tissue was incubated at 60°C overnight in 100% TBA.

2.8. Embedding of plant tissue

To embed fixed tissue in paraffin wax, tissue was incubated in 50% Paraplast (Sigma) in TBA at 60°C overnight. The solution was replaced with 100% Paraplast and incubated at 60°C for 6 hours. The Paraplast was changed once before placing the tissue and wax in a small plastic boat. Samples were stored at 4°C.

2.9. Sectioning of plant tissue

Embedded tissue samples were sectioned at 8 µm thickness using the Leica RM2255 Automated Microtome. Charged glass slides were pre-warmed to 42°C and 3-4 ml of H₂O was placed on top to aid in the placement of wax ribbons. Wax ribbons were placed on the slide and excess H₂O was removed after a few minutes. Slides were incubated at 42°C overnight. Paraffin was removed by washing the slides in HistoClear II (National Diagnostics) (2 x 10 minutes). Slides were mounted using Histomount (National Diagnostics) and left to cure for several hours. Slides were visualised with bright field microscopy.

2.10. *In situ* hybridisation

Non-radioactive *in situ* hybridisations were performed as previously described (Long and Barton, 1998). Probes generated against the 3'UTR sequences of AT4G19865 and AT4G19870 were hybridised to 8 µm sections of whole inflorescence tissue from Col-0 plants.

2.11. Microscopy techniques

2.11.1. Light microscopy

Floral organs were counted by dissection of individual flowers using a sharpened no. 5 tweezers under an Olympus SZX7 microscope. Photographs were taken using an Olympus SP-500UZ digital camera mounted to the microscope eyepiece.

2.11.2. Epifluorescence microscopy

Visualisation of GFP was performed using an Olympus BX61 fluorescence microscope with excitation wavelengths of 460/480 nm and emission wavelengths of 495- 540 nm.

2.12. Image manipulation and figure construction

Photographs and agarose gel images were altered using *Adobe Photoshop CS6*. Images were altered to reduce the overall size, or to improve the contrast of samples against the background to aid in visualisation. No alterations to the subject matter have been made. Figures were constructed using *Adobe Illustrator CS6*.

2.13. Prediction of potential off-target sites of Cas9

Guide sequences to be used in the construction of sgRNAs targeting the genes in this study were checked for potential off-target sites in the *Arabidopsis* genome using the *Cas-*

OFFinder program (<http://www.rgenome.net/cas-offfinder>) (Bae et al., 2014). Query sequences were screened for sites within the genome containing up to 4 mismatches. Unless otherwise specified, all selected guide sequences did not have any potential off-target sites with 1 or 2 mismatches. Genomic regions surrounding off-target sites with 3 or 4 mismatches to the guide sequence were amplified by PCR and Sanger sequencing was used to detect any deviation from the published wild-type sequence.

2.14. Identification and sequence analysis of CRISPR/Cas9

transformants

Genomic DNA that had been PCR amplified from CRISPR/Cas9 transformants was directly sequenced after purification, or used in heteroduplex formation detection analyses (outline below).

2.15. Detection of DNA heteroduplex formation

2.15.1. SurveyorTM nuclease

To detect the presence of mutations at sgRNA target sites, Phusion polymerase (Thermo Scientific) was used to amplify a ~1000bp region surrounding the predicted site in a 20 µl reaction. After verification of successful amplification via agarose gel electrophoresis, 1 µl of the PCR product was diluted in 15 µl of ultra-pure H₂O. 1 µl of the diluted PCR product was used in a second PCR, with nested primers, to generate sufficient amounts of PCR product for digestion. To promote heteroduplex formation of the PCR products 10 µl of the reaction was heated to 95°C in a thermal cycler for 10 minutes, cooled to 85°C (ramp rate -3°C/s) for 1 minute, then cooled to 25°C at intervals of 10°C (ramp rate -0.3°C/s) for 1 minute. 1 µl of SurveyorTM nuclease and 1 µl of SurveyorTM Enhancer

(Integrated DNA Technologies) was directly added to the PCR mixture and incubated at 42°C for 1 hour. A stop solution was added to 1/10th the volume of the reaction after the incubation, and the digested DNA was visualised on a 2% (w/v) agarose gel.

2.15.2. T7 Endonuclease I

T7E1 endonuclease (NEB) was used to detect the presence of targeted mutations mediated by CRISPR/Cas9 activity in transgenic plants. Genomic DNA of transformants was extracted from cauline leaves or inflorescences of T1 individuals using the Edward's preparation method (Section 2.2.5.2), with the exception that the DNA pellet was suspended in 300 µl dH₂O. 1 µl of the template was used in a 20 µl PCR, using Phusion polymerase, to amplify fragments ranging from approximately 600-1000bp. 5 µl of the unpurified PCR product was mixed with 2.3 µl 10X NEB 2 buffer and 15.7 µl dH₂O. To induce heteroduplex formation the mixture was heated to 95°C for 2 minutes, cooled to 85°C (-2°C/s), and cooled to 16°C (-0.1°C/s) in a thermal cycler. 2 µl of 1.25U/µl T7E1 endonuclease (diluted in 1x NEB buffer 2) was added to the mixture and incubated at 37°C for 60 minutes to digest the fragments at sites where DNA mismatches had occurred. The reactions were run on a 2% (w/v) agarose gel until adequate band separation occurred. Samples that contained digested DNA agreeing with the predicted band sizes were identified, and further rounds of PCR were carried out to re-amplify the fragments, which were purified (Section 2.2.2) and sequenced to confirm the mutations.

2.16. Transgenic line generation

2.16.1. Over-expression lines

2.16.1.1. *pML-BART 35S::GRANNY-3'OCS*

The coding sequence for *GRANNY* (AT4G19870) was PCR amplified from Col-0 genomic DNA using the primers BT157/200. The PCR fragment was digested with the *PstI* and *Acc65I* restriction enzymes. The digested fragment was ligated into the pBJ36 35S-3'OCS plasmid, digested with the same enzymes, and transformed into *E. coli*. Once a plasmid stock had been established, the sequence integrity was verified against the TAIR10 listing. pBJ36 35S::GRANNY-3'OCS was digested with *NotI* and ligated into the binary plasmid pML-BART that had been digested with the same enzyme and 5' dephosphorylated using FastAP (Thermo Scientific). *Agrobacteria* transformed with a pML-BART 35S::GRANNY-3'OCS plasmid stock were used to transform *Arabidopsis*. Transformed seedlings were selected using BASTA™.

2.16.1.2. *pML-BART 35S::NANNY-3'OCS*

The coding sequence for *NANNY* (AT4G19865) was PCR amplified from Col-0 genomic DNA using the primers KH285/286. The PCR fragment was digested with the *ClaI* and *XbaI* restriction enzymes. The digested fragment was ligated into the pBJ36 35S-3'OCS plasmid, digested with the same enzymes, and transformed into *E. coli*. Once a plasmid stock had been established, the sequence integrity was verified against the TAIR10 listing. pBJ36 35S::NANNY-3'OCS was digested with *NotI* and ligated into the binary plasmid pML-BART that had been digested with the same enzyme and 5' dephosphorylated using FastAP (Thermo Scientific). *Agrobacteria* transformed with a

pML-BART 35S::NANNY-3'OCS plasmid stock were used to transform *Arabidopsis*. Transformed seedlings were selected using BASTA™.

2.16.1.3. *pML-BART 35S::GRANNY-SRDX-3'OCS*

The coding sequence for *GRANNY* (AT4G19870), excluding the stop codon, was PCR amplified from Col-0 genomic DNA using the primers AR11/AR15. The PCR fragment was digested with the *EcoRI* and *XbaI* restriction enzymes. The digested fragment was ligated into the pBJ36 35S-SRDX-3'OCS plasmid, digested with the same enzymes, and transformed into *E. coli*. Once a plasmid stock had been established, the sequence integrity was verified against the TAIR10 listing. pBJ36 35S::GRANNY-SRDX-3'OCS was digested with *NotI* and ligated into the binary plasmid pML-BART that had been digested with the same enzyme and 5' dephosphorylated using FastAP (Thermo Scientific). *Agrobacteria* transformed with a pML-BART 35S::GRANNY-SRDX-3'OCS plasmid stock were used to transform *Arabidopsis*. Transformed seedlings were selected using BASTA™.

2.16.1.4. *pML-BART 35S::NANNY-SRDX-3'OCS*

The coding sequence for *NANNY* (AT4G19865), excluding the stop codon was PCR amplified from Col-0 genomic DNA using the primers AR11/16. The PCR fragment was digested with the *EcoRI* and *XbaI* restriction enzymes. The digested fragment was ligated into the pBJ36 35S-SRDX-3'OCS plasmid, digested with the same enzymes, and transformed into *E. coli*. Once a plasmid stock had been established, the sequence integrity was verified against the TAIR10 listing. pBJ36 35S::NANNY-SRDX-3'OCS was digested with *NotI* and ligated into the binary plasmid pML-BART that had been

digested with the same enzyme and 5' dephosphorylated using FastAP (Thermo Scientific). *Agrobacteria* transformed with a pML-BART 35S::NANNY-SRDX-3'OCS plasmid stock were used to transform *Arabidopsis*. Transformed seedlings were selected using BASTA™.

2.16.1.5. *pML-BART 35S::GRANNY-VP16-3'OCS*

The coding sequence for *GRANNY* (AT4G19870), excluding the stop codon, was PCR amplified from Col-0 genomic DNA using the primers AR11/AR17. The PCR fragment was digested with the *EcoRI* and *HindIII* restriction enzymes. The digested fragment was ligated into the pBJ36 35S-VP16-3'OCS plasmid, digested with the same enzymes, and transformed into *E. coli*. Once a plasmid stock had been established, the sequence integrity was verified against the TAIR10 listing. pBJ36 35S::GRANNY-VP16-3'OCS was digested with *NotI* and ligated into the binary plasmid pML-BART that had been digested with the same enzyme and 5' dephosphorylated using FastAP (Thermo Scientific). *Agrobacteria* transformed with a pML-BART 35S::GRANNY-VP16-3'OCS plasmid stock were used to transform *Arabidopsis*. Transformed seedlings were selected using BASTA™.

2.16.1.6. *pML-BART 35S::NANNY-VP16-3'OCS*

The coding sequence for *NANNY* (AT4G19865), excluding the stop codon, was PCR amplified from Col-0 genomic DNA using the primers AR12/AR17. The PCR fragment was digested with the *EcoRI* and *HindIII* restriction enzymes. The digested fragment was ligated into the pBJ36 35S-VP16-3'OCS plasmid, digested with the same enzymes, and transformed into *E. coli*. Once a plasmid stock had been established, the sequence

integrity was verified against the TAIR10 listing. pBJ36 35S::NANNY-VP16-3`OCS was digested with *NotI* and ligated into the binary plasmid pML-BART that had been digested with the same enzyme and 5` dephosphorylated using FastAP (Thermo Scientific). *Agrobacteria* transformed with a pML-BART 35S::NANNY-VP16-3`OCS plasmid stock were used to transform *Arabidopsis*. Transformed seedlings were selected using BASTA™.

2.16.2. Expression analysis lines

2.16.2.1. *pML-BART GRA::GUS-3`OCS*

A 2kb region directly upstream of the *GRANNY* (AT4G19865) start codon was PCR amplified from Col-0 genomic DNA using the primers BT146/148. The PCR fragment was digested with the *PstI* and *Acc65I* restriction enzymes. The digested fragment was ligated into the pBJ36 GUS-3`OCS plasmid, digested with the same enzymes, and transformed into *E. coli*. Once a plasmid stock had been established, the sequence integrity was verified against the TAIR10 listing. pBJ36 GRA::GUS-3`OCS was digested with *NotI* and ligated into the binary plasmid pML-BART that had been digested with the same enzyme and 5` dephosphorylated using FastAP (Thermo Scientific). *Agrobacteria* transformed with a pML-BART GRA::GUS-3`OCS plasmid stock were used to transform *Arabidopsis*. Transformed seedlings were selected using BASTA™.

2.16.2.2. *pML-BART NAN::GUS-3`OCS*

A 680bp region directly upstream of the *NAN* (AT4G19870) start codon was PCR amplified from Col-0 genomic DNA using the primers BT163/164. The PCR fragment was digested with the *PstI* and *Acc65I* restriction enzymes. The digested fragment was ligated into the pBJ36 GUS-3`OCS plasmid, digested with the same enzymes, and

transformed into *E. coli*. Once a plasmid stock had been established, the sequence integrity was verified against the TAIR10 listing. pBJ36 NAN::GUS-3'OCS was digested with *NotI* and ligated into the binary plasmid pML-BART that had been digested with the same enzyme and 5' dephosphorylated using FastAP (Thermo Scientific). *Agrobacteria* transformed with a pML-BART NAN::GUS-3'OCS plasmid stock were used to transform *Arabidopsis*. Transformed seedlings were selected using BASTA™.

2.16.3. Generation of CRISPR/Cas9 lines targeting NANNY and GRANNY

2.16.3.1. *pGREEN 0029 U6-26::sgRNA ICU2::CAS9*

Cas9 guide sequences was generated using the program *CasDesigner*, available at <http://www.rgenome.net/cas-designer/> (Bae, Park and Kim, 2014; Park, Bae and Kim, 2015). Selected sequences did not have any homologous regions in the *Arabidopsis* genome, including up to 2 nucleotide mismatches. Two rounds of PCR were performed, using synthesised Oligonucleotides (IDT) that contained the 20 base target region, to amplify a) U6-26::sgRNA and b) sgRNA-U6-26_{ter}. A third round of overlap extension PCR was performed, using the first two PCR products, with primers annealing to the U6-26 promoter and terminator, incorporating *SpeI* restriction sites on both ends. The pGREEN 0029 *ICU2::CAS9* binary vector (Supplied by George Coupland, Hyun *et al.*, 2014) and the purified PCR product, were digested with *SpeI* and ligated using T4 DNA ligase (Thermo Scientific). The vector was also dephosphorylated prior to ligation using FAST AP (Thermo Scientific). 100 µl of chemically competent Stbl2 *E. coli* cells were transformed with 10 µl of the mixture and plated on LB agar supplemented with 50 µg/ml kanamycin. Transformed *E. coli* and *Agrobacterium* colonies were genotyped using primers that amplified a portion of the vector and the insert (BT208/BT235). Seeds generated from plants that had been transformed were sprayed with a BASTA™ solution

to select for transformants. Transformants were genotyped for the presence of the transgene and screened for the occurrence of mutations caused by the Cas9 nuclease using T7E1 nuclease (NEB).

2.16.4. *pHEE401 U6-26::sgRNA EC1.2::zCAS9*

The pHEE401 (Wang *et al.*, 2015, supplied by Dr. Peter Ryder, The National University of Ireland Galway) vector contains two *BsaI* sites for use in cloning guide RNAs to be expressed in *Arabidopsis*. 20 nt guides were designed, as described above, possessing 4nt overhangs complementary to the overhangs produced during *BsaI* digestion of pHEE401. The guide sequences were generated by annealing complementary oligonucleotides to each other. Equal amounts of 100 µM complementary oligonucleotides were mixed to a concentration of 50 µM, heated at 95°C for 5 mins, and cooled to 20°C at a rate of 0.1°C/s. A one-step reaction was performed to insert the guide sequences into the binary vector. 200 ng of pHEE401, 5 nmol of the guide sequence, 1.5 µl 10x T4 DNA Ligase buffer (NEB), 1.5 µl 1 mg/ml BSA, 1 µl T4 DNA Ligase HC (NEB), 1 µl *BsaI* (NEB), and ddH₂O to 15 µl. The reaction was incubated at 37°C for 4 hours, 16°C for 30 minutes, 50°C for 5 minutes, and 80°C for 10 minutes. 100 µl of chemically competent Stbl2 *E. coli* cells were transformed with 5 µl of the mixture and plated on LB agar supplemented with 50 µg/ml kanamycin. Colonies were genotyped using primers flanking the insertion site of the vector (BT436/437). An amplified DNA fragment of 423bp indicated successful insertion of the guide sequence. Seeds generated from plants that had been transformed were sterilised and sowed on 0.5X MS agar plates supplemented with 25 µg/ml Hygromycin. After 10 days those seedlings which had extended hypocotyls, roots, and expanded cotyledons were transferred to soil.

2.16.5. Construction of *EC1.2pro* CRISPR vectors containing 2 sgRNA cassettes

pHEE vectors containing two sgRNA cassettes were generated using a modified protocol available on Addgene (https://media.addgene.org/data/plasmids/71/71286/71286-attachment_1NlSpD2Dqztp.pdf) (Xing *et al.*, 2014). Overlapping PCR was used to generate a single Golden Gateway compatible fragment containing both cassettes. Briefly, primers containing the guide sequences of interest were used to amplify the sgRNA-U6-26_{ter}-U6-29_{pro}::sgRNA fragment from the template plasmid pCBC-DT1T2. A second set of primers present in the PCR, containing a *BsaI* restriction sites and the corresponding guide sequences, was included to generate the final sgRNA-U6-26_{ter}-U6-29::sgRNA2 fragment. The purified fragment was ligated into the pHEE401 vector and transformed into chemically competent Stbl2 *E. coli* cells in the same manner as above. Transformed plants were identified as above.

2.17. RNA-seq library construction

Library construction from purified total RNA was performed by Beijing Genomics Institute (BGI, Hong Kong). Briefly, Oligo (dT) magnetic beads were used to select mRNA with polyA tails, after which the target RNA was purified. The target RNA was fragmented and reverse transcriptase was used to synthesise single-strand cDNA. Double-strand cDNA (dscDNA) was subsequently synthesised with random hexamer primers. End repair and A-tailing of the 5' phosphates of the dscDNA was performed, and adaptors were ligated to both the 5' and 3' ends. Two specific primers were used to amplify the ligation product and the PCR product was denatured by heating. The single strand DNA was cyclised by using splint oligonucleotides and DNA ligase. The circular single-

stranded DNA templates were then used to create DNA nanoballs, which were attached to a flow cell. Single-end sequencing was then performed on the prepared library using a BGISEQ-500 high-throughput sequencer.

2.18. Bioinformatics methods

2.18.1. Sequencing quality control

Quality control of the raw sequencing reads was performed using the *FastQC* (Version 0.11.1) software (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).

2.18.2. Indexing of an annotated transcriptome

To perform pseudo-alignments of raw reads to the *Arabidopsis* transcriptome, the transcriptome file (downloaded from http://www.Arabidopsis.org/download_files/Genes/TAIR10_genome_release/TAIR10_NCBI_mapping_files/TAIR10_NCBI_REFSEQ_mapping_RNA) was indexed using the *Kallisto* software (Version 0.43.1).

2.18.3. Quantification and pseudo-alignment of reads

Transcript abundances were quantified against the indexed *Arabidopsis* transcriptome using the pseudo-alignment method employed in *Kallisto* software (Version 0.43.1), with the following arguments: --single: Specifies single-end sequencing, -b: Bootstrapping (100), -l: Average fragment length, -s: Standard deviation of fragment lengths.

2.18.4. Importation of pseudo-aligned reads

Quantified transcript abundance estimates were imported and summarised, for use in downstream Bioconductor packages, with the *tximport* (Version 3.5) package in *R Studio* (available from: www.bioconductor.org/packages/release/bioc/html/tximport.html).

2.18.5. Analysis of quantified transcript abundance estimates

Imported and summarised transcript abundance estimates were analysed using the *DESeq2* (Version 3.5) package (available from: www.bioconductor.org/packages/release/bioc/html/DESeq2.html) in *R Studio*. Transcripts were identified as differentially expressed if the false discovery rate (Benjamini and Hochberg, 1995) adjusted *p*-value was less than 0.05 for genes that were upregulated, or downregulated, according to each transcript's mean Log₂-fold change.

2.19. Tissue collection

2.19.1. Synchronously developing floral tissues

Collection of developing floral tissues on the inflorescences of Floral Induction System plants was performed under an Olympus light microscope using sterile 22 surgical blades (Swann-Morton). Tissue was then immediately placed into 1.5 ml centrifuge tubes immersed in liquid Nitrogen. Samples were stored at -80°C until they were used.

2.19.2. Inflorescences

Young stage floral buds and inflorescence meristems were dissected from plants using a sharpened No. 4 jeweler's forceps and placed immediately in 1.5 ml centrifuge tubes immersed in liquid Nitrogen and stored at -80°C until they were used. The inflorescences were quickly dissected beforehand to removed older buds and obstructing tissues.

2.20. Treatments of floral induction system inflorescences

2.20.1. Steroid hormone treatments

Dexamethasone: Inflorescences of *L-er ap1-1 cal-1* plants harboring the AP1::AP1-GR or 35S::LhG4-GR 6xOP::amiRNA transgenes were treated with an aqueous solution

containing 10 μ M dexamethasone, 0.1% ethanol, and 0.015% Silwet L-77. For mock-treated samples, the dexamethasone was omitted.

Dihydrotestosterone: Inflorescences of *L-er ap1-1 cal-1* plants harboring the AP1::AP1-AR transgene were treated with aqueous solutions containing from 10-1000 μ M dihydrotestosterone, 0.2-1% ethanol, 0-2.5 mg/ml (2-Hydroxypropyl)- β -cyclodextrin, and 0.015% Silwet L-77. For mock-treated samples, the dihydrotestosterone was omitted.

17- β -estradiol: Inflorescences of *L-er ap1-1 cal-1* plants harboring the G₁₀₋₉₀::XVE LEXA::AP1 transgene were treated with an aqueous solution containing 500 μ M 17- β -estradiol, 1% ethanol, 2.5 mg/ml (2-Hydroxypropyl)- β -cyclodextrin, and 0.015% Silwet L-77. For mock-treated samples, the 17- β -estradiol was omitted.

2.20.2. Chemical treatments

Ethanol: *L-er ap1-1 cal-1* plants harboring the 35S::ALCR ALCA::AP1 transgene were treated sealing the plants in a growth chamber with a transparent lid in the presence of two 10 ml tubes of ethanol. Treatments lasted for 24 hours. For mock-treated samples, ethanol was replaced with H₂O.

3. Chapter 3 – Characterisations of floral induction systems for stage-specific gene perturbation analyses

This chapter is partially based on content from: O'Maoileidigh, D.S., Thomson, B., Raganelli, A., Wuest, S.E., Ryan, P.T., Kwasniewska, K., Carles, C.C., Graciet, E., and Wellmer, F. (2015) Gene network analysis in *Arabidopsis thaliana* flower development through dynamic gene perturbations. *Plant Journal*, 83, pp. 344-358.

3.1. Introduction

The emergence of flowers in *Arabidopsis* follows a spiral phyllotaxy, such that no two flowers are at the exact same stage of development on any one inflorescence. Consequently, it is impossible to collect stage-specific tissue without the use of time consuming techniques such as laser capture microdissection (for an overview see Kerk *et al.*, 2003). A floral induction system (FIS), which allows the synchronisation of flowers from an inflorescence-like meristem, has been developed to circumvent this difficulty (Wellmer *et al.*, 2006).

The floral induction system exploits the phenotype of the *ap1-1 cal-1* floral meristem identity double mutant. *API* and *CAL* are related and have partially redundant activities, such that mutations to both genes prevent the specification of the floral meristem, and consequently prevent flower development (Bowman *et al.*, 1993; Ferrándiz *et al.*, 2000). However, while *ap1* mutants have floral organ identity defects in the first and second whorls, and cause partial transformations of flowers into inflorescences, *cal* mutants are phenotypically indistinguishable from wild-type plants (Bowman *et al.*, 1993). Upon entering into the reproductive phase, *ap1-1 cal-1* plants do not form flowers, but rather produce an abundance of inflorescence-like meristematic tissue, resulting in a structure resembling a cauliflower head. Thus, *API* is sufficient for floral meristem initiation in *cal*

mutants, but *CAL* cannot fully compensate for a lack of AP1 activity (with respect to the organ defects in *ap1* mutants), and their simultaneous knockout temporarily halts floral meristem initiation. After a prolonged period of time flowers begin to form sporadically from *ap1-1 cal-1* inflorescences, although they lack sepals and petals. Mutations to the *FRUITFUL (FUL)* gene, which encodes a MADS domain protein closely related to *AP1* and *CAL*, abolishes floral meristem identity completely (Ferrándiz *et al.*, 2000).

The original floral induction system was based on hormone-dependent activation of AP1 that is translationally fused to the rat glucocorticoid receptor (AP1-GR), and ubiquitously and constitutively expressed using the Cauliflower Mosaic Virus 35S promoter (Wellmer *et al.*, 2006) (**Fig. 3.1 A**). Upon application of a solution containing the synthetic corticosteroid hormone dexamethasone (DEX), floral meristem initiation occurs at hundreds of sites around the inflorescence, resulting in the synchronous development of a large number of flowers. Despite AP1-GR being expressed at all stages in the plant, the protein is effectively inactivated by its sequestration to the cytosol, mediated by interactions with ATP-dependent heat shock proteins (HSPs) (**Fig. 3.1 C**), molecular chaperones that play roles in protein synthesis, folding, turnover, and regulation. It has been demonstrated in animal systems that the rat GR protein binds to HSP90 in the absence of a ligand (Howard *et al.*, 1990), and that HSP90 is also required for the activation of GR through ligand binding (Picard *et al.*, 1990). Once activated, AP1-GR translocates to the nucleus and is available to direct flower development (Wellmer *et al.*, 2006; Kaufmann *et al.*, 2010) (**Fig. 3.1 D & G**).

Since its inception, numerous studies have used the floral induction system, and more recently developed versions discussed below, to study the gene regulatory network

governing flower development (Ito *et al.*, 2007; Das *et al.*, 2009; Sun *et al.*, 2009; Jiao and Meyerowitz, 2010; Kaufmann *et al.*, 2010; Smaczniak *et al.*, 2012; Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013; Pajoro *et al.*, 2014; Ryan *et al.*, 2015; Villarino *et al.*, 2016; Goslin *et al.*, 2017). One issue arising from the use of the 35S::AP1-GR transgene is that the expression pattern of AP1-GR does not reflect that of the endogenous gene, and so the system has the potential to produce results that do not accurately reflect the wild-type condition. To enhance the usefulness of this system, we have focused on mimicking the endogenous *API* expression pattern and expanding the repertoire of chemicals that can be used to activate AP1. This has been partially addressed previously in the laboratory by generating a construct containing the genomic *API* locus, fused to the coding sequence for the GR ligand binding domain, to produce the *ap1-1 cal-1* AP1::AP1-GR (AP1::AP1-GR) line (O'Maoileidigh *et al.*, 2013). Three more novel floral induction systems have also been developed and analysed in order to achieve this, with assistance from Dr. Diarmuid O'Maoileidigh and Dr. Andrea Raganelli.

The first system functions in a similar manner to the AP1::AP1-GR system. However, the GR fusion has been replaced with the human androgen receptor (AR), which is activated upon exposure to dihydrotestosterone (DHT) (**Fig. 3.1 C**). The purpose of this is to allow us to use the GR fusion protein as a functional component of other inducible systems that are expressed in parallel, such as the modified LhG4-GR/xOP system (Moore *et al.*, 1998). Second, an estrogen-based system is assessed, in which 17- β -estradiol is used to activate the chimeric XVE transcription factor, which in turn directs the expression of *API* fused downstream of a transgenic promoter termed LEXA. Third, an ethanol-activated floral induction system has been developed which utilises a modified *ALC* gene

expression system, endogenous to the fungus *Asperilligus nidulans* (**Fig. 3.1 E & F**) (Salter, Paine and Riddell, 1998).

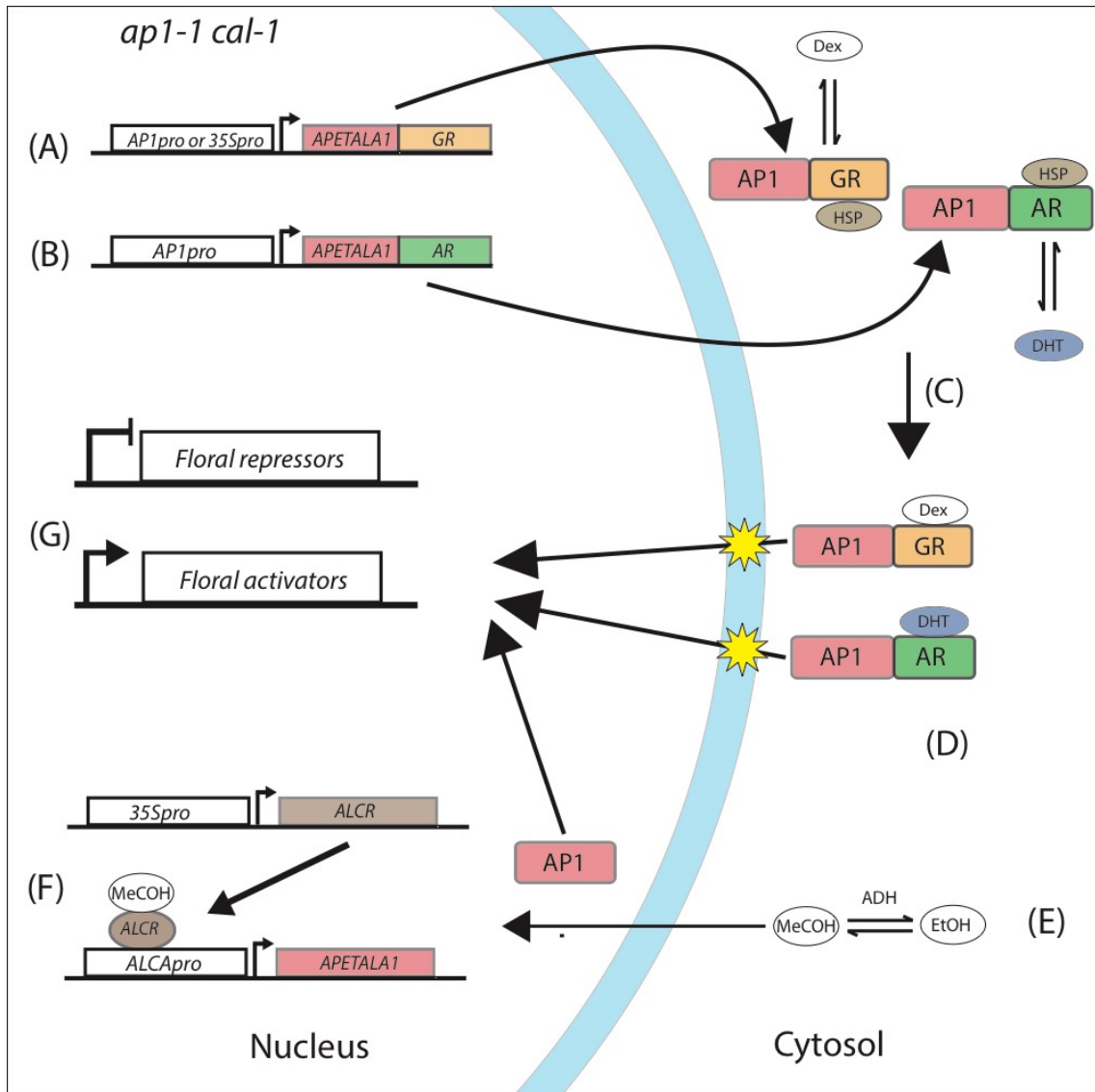


Figure 3.1 - Simplified schematic of separate functional Floral Induction Systems.

(A & B) APETALA1 (AP1) fused to the ligand binding domain of either the rat glucocorticoid (GR) or androgen receptor (AR). The coding sequences are expressed using either the endogenous *API* promoter or the constitutive 35S promoter. The fusion proteins are sequestered to the cytosol in their inactive form, bound by heat shock proteins (HSP). (C) Dexamethasone (DEX) or dihydrotestosterone (DHT) activate AP1-GR and AP1-AR, respectively, resulting in their dissociation from HSP and

translocation into the nucleus via active transport **(D)**. **(E)** Ethanol (EtOH) vapor is reduced to acetaldehyde (MeCOH) by alcohol dehydrogenase (ADH). **(F)** AP1 is expressed from the 35S::ALCR/ALCA::AP1 system. Acetaldehyde is required to activate the ALCR transcription factor, which upregulates transcriptional activity of the ALCA promoter, driving *AP1* expression. **(G)** AP1 is a master regulator of floral meristem identity, contributing to the dynamic expression of the gene regulatory network controlling flower development (Kaufmann et al., 2010). All systems are present in an *ap1-1 cal-1* double mutant background.

3.1.1. Chapter aims

This chapter focuses on the characterisation, assessment, and optimisation of some of the floral induction systems outlined above. In particular, the DHT-responsive line was optimised and the system's usefulness was highlighted through the simultaneous employment of two hormone inducible transgenic constructs: 1) The DHT-responsive floral induction system (AP1::AP1-AR), and 2) DEX-inducible artificial microRNAs (amiRNAs, discussed in Section 1.7.1.1) targeting the floral homeotic genes *APETALA3* (*AP3*) and *AGAMOUS* (*AG*) in a stage-specific manner. The specificity of each system's response to its respective activating hormone has been demonstrated, the induction kinetics of the activated amiRNAs were assessed, and the suitability for these systems with regard to stage-specific gene perturbations was investigated.

3.2. Results

3.2.1. Optimisation of treatment conditions for the DHT-responsive floral induction system

To be able to use two independently inducible systems to study stage-specific perturbations to flower development, the DHT-responsive *apl-1 cal-1* AP1::AP1-AR (FIS-AR) line was initially developed by Dr. Andrea Raganelli with the intention that a stably responsive line would be crossed with DEX-inducible amiRNAs (Raganelli, 2013).

Previous work in the Wellmer laboratory has shown that 10 μ M aqueous solutions of dexamethasone are sufficient to induce synchronous flower formation in the *apl-1 cal-1* AP1::AP1-GR (AP1::AP1-GR) and *apl-1 cal-1* 35S::AP1-GR (35S::AP1-GR) transgenic lines after a single treatment (Wellmer *et al.*, 2006; O'Maoileidigh *et al.*, 2013, 2015). To determine the range of concentrations that will induce synchronous flower formation in the FIS-AR transgenic line, a series of aqueous solutions were prepared with dihydrotestosterone, a potent endogenous ligand for the AR ligand binding domain (Gao, Bohl and Dalton, 2005).

FIS-AR plants were grown at 16-18°C under constant illumination until they had bolted and the stem had extended more than 2 cm. The plants were transferred to a warmer temperature (20-22°C) and left for 24 hours before treatment. Solutions containing 10, 50, 100, 200, 500 μ M, or 1 mM DHT and 0.015% Silwet L-77 were tested by saturating the inflorescence with the solutions until they turned a dark green colour, and after 10 days the plants were examined for the presence of synchronously developing flowers that agreed with the developmental stages outline by Smyth *et al.* (1990). Plants treated with 10 and 50 μ M DHT (**Fig 3.2 A and B**) solutions did not appear to be fully induced, as a

very low level of flower formation was observable. Most flowers resembled those of non-treated inflorescences that had transitioned to the flowering state autonomously. Furthermore, floral structures that did form lacked perianth organs, suggesting that AP1 had not been sufficiently activated to direct flower development. Plants treated with more concentrated solutions possessed greater numbers of synchronously developing buds with sepals and petals, though in the cases of 100 μ M and 200 μ M DHT (**Fig 3.2 C and D**) petal formation was rare, again indicating that AP1 activity had not been restored to a level that was permissive for complete 2nd whorl development in most instances. Plants treated with 500 μ M and 1 mM solutions (**Fig 3.2 E and F**) both had greater frequencies of developing buds, though the frequency between the two was similar, with petal formation markedly more apparent.

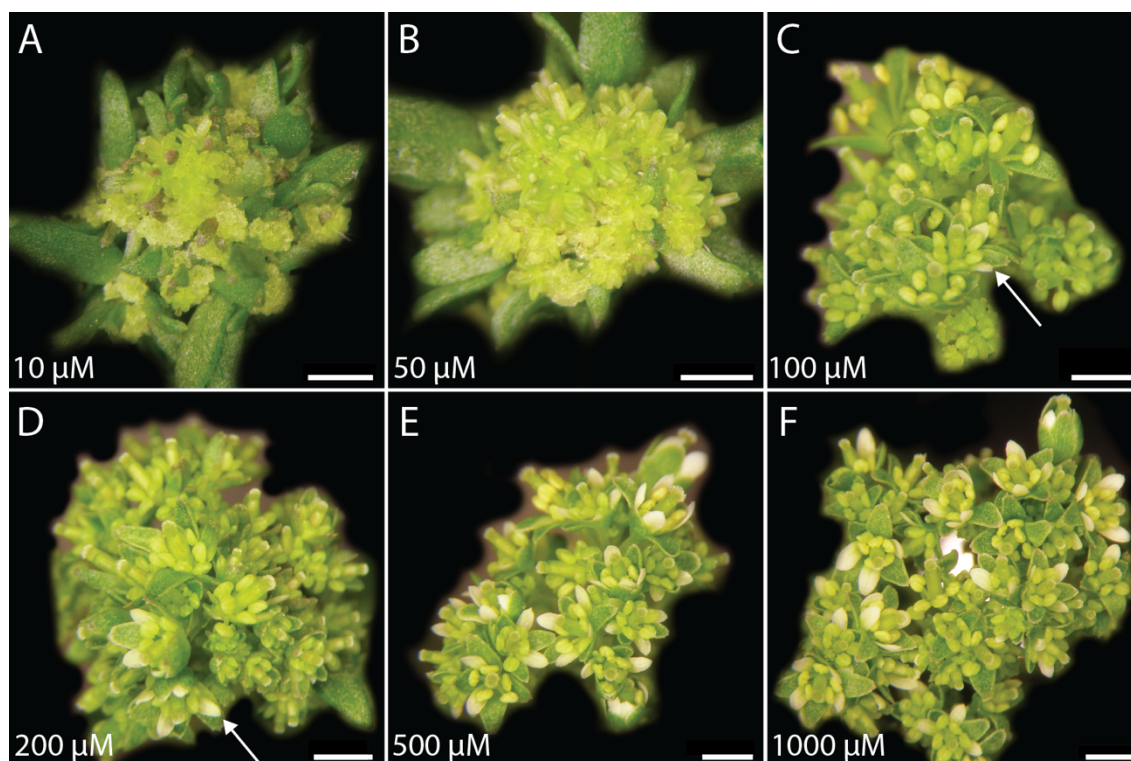


Figure 3.2 – Response of FIS-AR inflorescences to varying DHT concentrations.

Plants were treated with aqueous solutions containing 0.015% Silwet L-77, 1% ethanol, and 10; 50; 100; 200; 500; or 1000 μM DHT (A-F, respectively). Plants treated with 10 μM (A) and 50 μM DHT (B) did not undergo any visually detectable floral induction, and any floral structures apparent lack sepals and petals. Plants treated with 100 μM (C) and 200 μM DHT (D) possess synchronously developing flowers with sepals and, though infrequent, petals (white arrows). Those treated with 500 μM (E) and 1 mM DHT (F) solutions experienced the highest levels of floral induction, with a large proportion of flowers developing synchronously. The majority of flowers have produced the full complement of sepals and petals, as well as stamens and carpels. All images were taken 10 days after application of the DHT solutions. Scale bars: 1 mm.

3.2.2. *Improving the solubility of DHT in an aqueous solution*

DHT is a hydrophobic molecule and therefore sparingly soluble in H_2O , with concentrations beyond 50 μM resulting in precipitation of the compound as the ethanol stock solution is added to the water. The preparation of DHT solutions beyond this concentration led to obvious precipitation of the compound, and as such likely reduces its

bioavailability due to aggregation. To determine if improving its aqueous solubility led to a greater activation of the AP1-AR protein in FIS-AR plants, (2-Hydroxypropyl)- β -cyclodextrin was added to the solution.

Cyclodextrins, a family of cyclic oligosaccharides, are compounds that consists of varying glucopyranose units that form a rigid toroidal shape. Cyclodextrins that are composed of 6, 7, or 8 glucopyranose units are referred to as α -, β -, and γ -cyclodextrins, respectively. The arrangement of the hydroxyl groups at the C2, C3, and C6 positions form a hydrophobic cavity on the inside of the molecule, while the outside remains hydrophilic, allowing for lipophilic molecules to complex with the ring and therefore increase their solubility in an aqueous solution. Cyclodextrins have been used to assist in lipophilic drug delivery for pharmaceutical applications, and might also improve the stability of the complexed molecule, protecting it from degradation by hydrolysis or photodecomposition (Rasheed, Kumar and Sravanthi, 2008). (2-Hydroxypropyl)- β -cyclodextrin was chosen for experimentation because it has been previously shown to enhance testosterone solubility (Stuenkel, Dudley and Yen, 1991).

The concentration of (2-Hydroxypropyl)- β -cyclodextrin (referred to as cyclodextrin from herein) required to fully complex with DHT was titrated by monitoring the OD₆₀₀ absorbance value of a 500 μ M DHT + 0.015% Silwet L-77 + 1% ethanol solution, against increasing concentrations of cyclodextrin, until the absorbance approached 0. Cyclodextrin at a concentration of 2.5 mg/ml was sufficient to achieve this (**Fig. 3.3**).

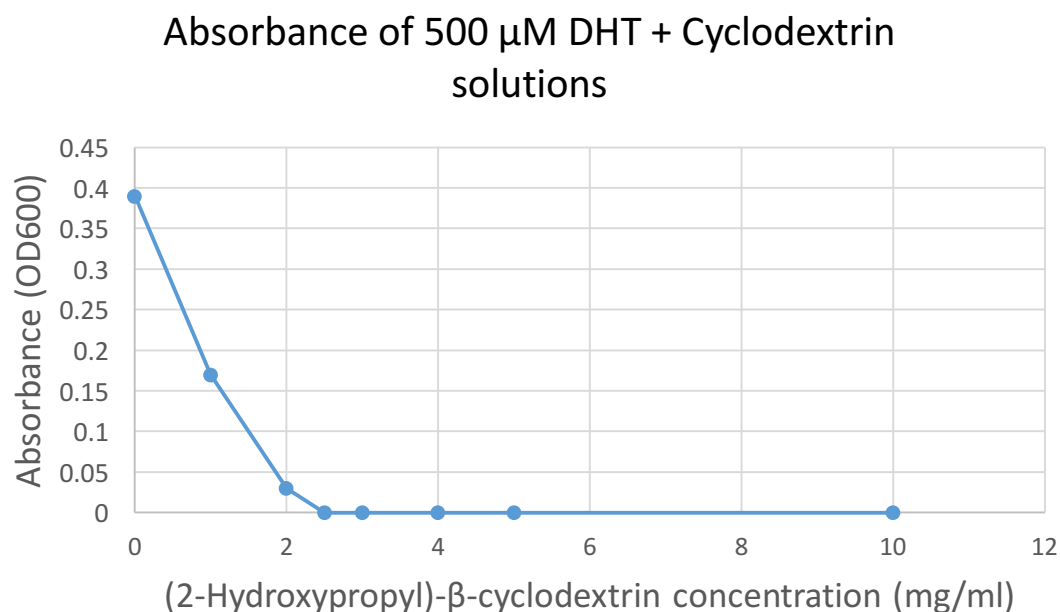


Figure 3.3 – Titration of cyclodextrin concentrations to solubilise DHT in an aqueous solution. The absorbance (OD₆₀₀) of 500 μ M DHT + 0.015% Silwet L-77 + 1% ethanol solutions (y-axis) was measured against increasing concentrations of cyclodextrin (x-axis). At 2.5 mg/ml cyclodextrin, the absorbance reading was 0 and the solution had become clear.

The efficacy of different DHT/cyclodextrin solutions was tested on the FIS-AR line to determine if this addition improved the plants' response to the hormone. The same DHT concentrations as above were used, with linearly-scaled cyclodextrin concentrations (Table 3.1).

Table 3.1 – Concentrations of DHT and cyclodextrin used in FIS-AR response optimisation

DHT concentration (μ M)	Cyclodextrin concentration (mg/ml)
10	0.05
50	0.25
100	0.5
200	1.25
500	2.5
1000	5

DHT/cyclodextrin-treated plants responded more thoroughly to lower concentrations of DHT than plants treated solely with DHT. While application of the 10 μ M solution only resulted in a few buds forming (**Fig. 3.4 A**), inflorescences of plants treated with 50-1000 μ M DHT solutions had a progressively larger number of synchronously developing floral buds (**Fig. 3.4 B-F**), indicating that the addition of cyclodextrin is sufficient to increase the amount of AP1-AR fusion protein being activated upon its application. In agreement with the result that increased DHT + cyclodextrin concentrations result in a greater level of synchronous floral induction, stage 14-16 flowers were also examined, from the induced plants, to determine at what DHT concentration the full complement of floral organs becomes apparent for the majority of flowers. Flowers from 10 μ M DHT-treated plants developed sepals but rarely petals, indicating that even with the addition of cyclodextrin the concentration of DHT is too dilute to activate AP1-AR to a sufficient degree (**Fig. 3.4 G**). Those plants that had been treated with 50 μ M DHT produced flowers with variable numbers of petals, up to the full complement, though in many cases one or two were missing (**Fig. 3.4 H**). The majority of flowers treated with 100-1000 μ M DHT (**Fig. 3.4 I-L**) all possessed the full complement of floral organs. Plants treated with the higher concentrations did not appear to be visually affected by the presence of greater quantities of the hormone and cyclodextrin.

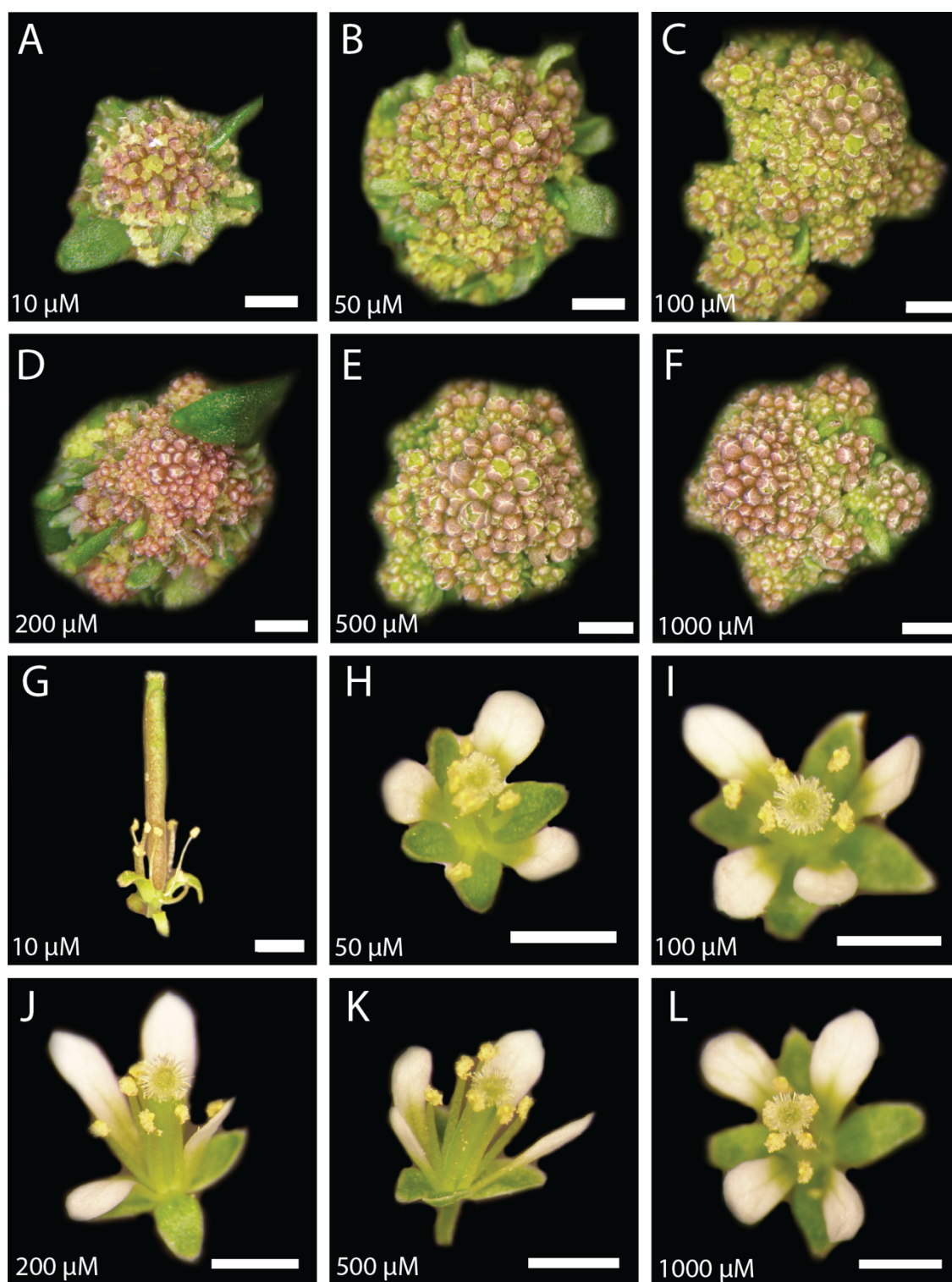


Figure 3.4 – Response of FIS-AR plants to varying DHT and cyclodextrin concentrations. Plants were treated with aqueous solutions containing 0.05 - 5 mg/ml cyclodextrin (See **Table 3.1**), 0.015% Silwet L-77, 1% ethanol, and 10; 50; 100; 200; 500; and 1000 μM DHT (**A-F**, respectively). Plants treated with 10 μM DHT (**A**) developed few synchronous buds, while plants treated with 50 μM DHT (**B**)

possessed a greater amount. Plants treated with 100-1000 μ M DHT (C-F) possessed the highest number of developing buds. Images were taken 7 days after treatment. (G-L) Representative mature flowers from the plants imaged in (A-F). (G) A 10 μ M DHT-treated flower lacking 2nd whorl organs. (H) A 50 μ M DHT-treated flower possessing 3 petals, and the full complement of all other floral organs. (I-L) Flowers from 100-1000 μ M DHT-treated plants possessing all floral organs in the correct quantities. Images were taken 14 days after treatment. Scale bars for all panels: 1 mm.

From these data, it can be surmised that the addition of cyclodextrin to aqueous solutions of DHT is sufficient to improve the rates of synchronous flower development in the FIS-AR line at all tested concentrations. The mechanism by which this occurs is not clear, but one possibility is that precipitated DHT in aqueous solutions forms aggregates, reducing the number of molecules that are available to cross the plant cell wall and cell membrane. As the addition of cyclodextrin effectively eliminates the issue of aqueous solubility, by providing a hydrophobic space for the DHT to occupy in an otherwise hydrophilic environment, it could be that the quantity of DHT available to diffuse into the inflorescence meristematic tissue becomes sufficient to activate AP1-AR and induce the synchronous development of floral buds.

3.2.3. *Attempts to optimise an estradiol-responsive floral induction system*

The *ap1-1 cal-1* G10-90::XVE LEXA::AP1 (LEXA::AP1) transgenic line, developed by Dr. Andrea Raganelli, requires the steroid hormone 17- β -estradiol (EST) as an XVE-activating ligand, which binds to the LEXA promoter to drive transcription of *API*. Studies working with the XVE-LEXA system have noted that EST-mediated activation requires the plant tissue in question to be in constant contact with a medium containing the hormone (Zuo, Niu and Chua, 2000). Furthermore, it has been demonstrated in the

laboratory, through RT-PCR, that transcription of *API* in seedlings occurs when they are grown on 0.5X MS agar supplemented with 5 μ M EST for 24 hours (O'Maoileidigh *et al.*, 2015). EST is structurally similar to DHT, as is its solubility in aqueous solution.

Synchronous floral induction using the above transgenic line has not been previously achieved using 10 μ M aqueous solutions (Raganelli, 2013). However, considering the higher DHT concentration required to induces flower development in the FIS-AR line, it remained a possibility that the EST concentration was simply not optimised and that improving its solubility could ameliorate the lack of *API* activation. To test if the addition of cyclodextrin would have a similar positive effect on the induction of flower development, a solution containing 500 μ M EST + 2.5 mg/ml cyclodextrin + 0.015% Silwet L-77 + 1% ethanol was applied to the inflorescences of LEXA::AP1 plants. Despite the increased solubility of EST due to cyclodextrin (not shown), no induction of flower development was noted. This line was not further optimised, due in part to the hazardous nature of EST, and because the previous establishment of the two other hormone-inducible lines is sufficient to carry out gene perturbation studies.

3.2.4. Attempts to optimise an ethanol-inducible floral induction system

The ethanol-responsive *ap1-1 cal-1* AP1::ALCR ALCA::AP1 (35S>>AP1^{EtOH}) line, developed in collaboration with Dr. Diarmuid O'Maoileidigh (Max Planck Institute for Plant Breeding Research, Cologne, Germany), was tested to determine whether ethanol-mediated induction of *API* transcription was sufficient to direct synchronous flower formation. Plants were treated by placing the trays inside a transparent sealed container, with either 2 x 10 ml vials of ethanol or water (mock-treated plants), with a duration of 3 hours per day for 10 consecutive days. The inflorescences of plants treated with water

were phenotypically indistinguishable from *ap1-1 cal-1* plants, and flower development was not induced (**Fig. 3.5 A & C**). Approximately 15% of plants treated with ethanol vapour did respond to the treatment, although those flowers that did develop were delayed in their development by several days when compared to the other systems described (**Fig. 3.5 B**). Furthermore, the restoration of sepal and petal formation was not fully achieved, with most flowers possessing only partly restored sepals, and one or two petals (**Fig. 3.5 D**). RT-qPCR analysis of *AP1* mRNA levels in inflorescence tissue obtained from 35S>>AP1^{EtOH} plants, which had been treated with ethanol vapour for 5 consecutive days (3 hours per day), showed a substantial accumulation of *AP1* transcripts (**Fig. 3.5 E**), indicating that the lack of flower formation is not necessarily due to a low level of *AP1* induction. Finally, GO term analysis from microarray data of an amiRNA line targeting *AGAMOUS*, expressed under the control of the ALCA/ALCR system, showed an enrichment of stress-related terms, indicating that the activation of the ALCR transcription factor results in unwanted negative effects (O'Maoileidigh *et al.*, 2015). This is in contrast to a similar line, where the same amiRNA was driven by the DEX-inducible LhG4-GR/6xOP system, where terms related to stress responses were much less abundant.

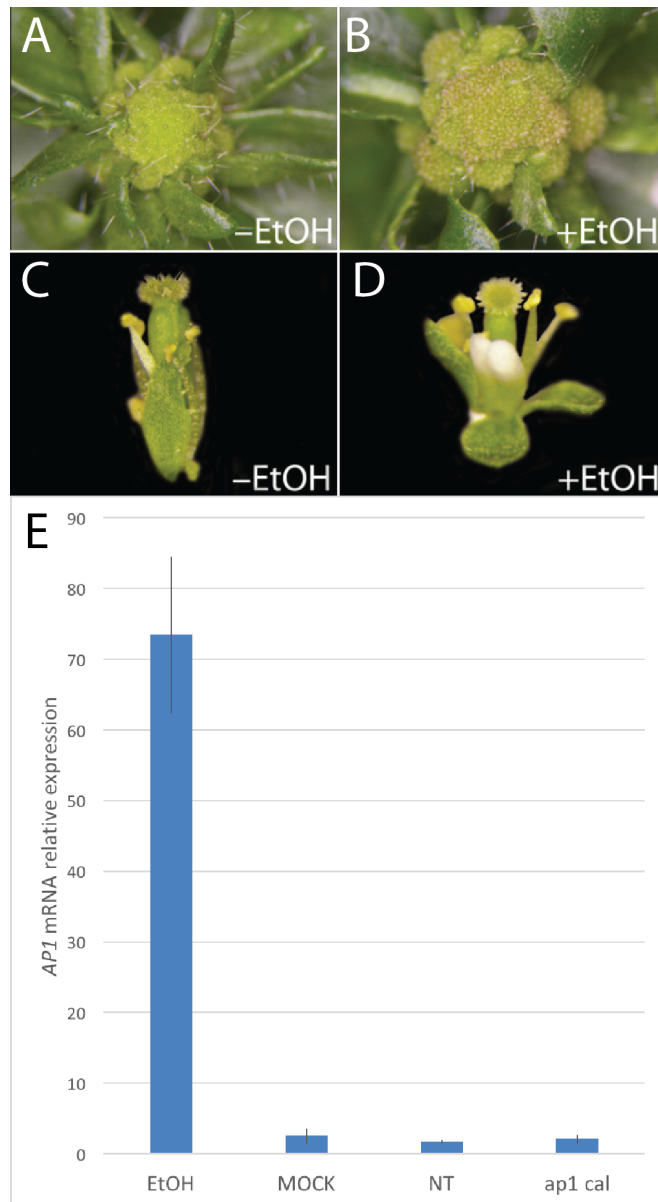


Figure 3.5 – Characterisation of the ethanol-inducible floral induction system. (A-D) Response of the 35S>>AP1^{EtOH} line to treatment with ethanol (EtOH) vapour. (A-B) Response of the inflorescence to mock treatment (A) or ethanol treatment (B). Treatments consisted of daily 3 hour exposure to ethanol vapour for 10 days. (C-D) Mature flowers of mock-treated (C) or ethanol-treated (D) plants. (E) Quantitative RT-PCR results of *API* mRNA levels in ethanol-treated 35S>>AP1^{EtOH} and *ap1-1 cal-1* plants. Plants were treated for 3 hours per day, for 5 days. Values are averages from three independent biological replicates, and two technical replicates, for each category. Error bars represent standard deviations from the mean. NT: No treatment.

3.2.5. *Assessment of autonomous flower formation in floral induction systems*

The over-proliferation of inflorescence-like meristematic tissue characteristic of *apl-1 cal-1* plants results in a ‘cauliflower head’ phenotype, due to the plants’ reduced ability to specify the floral meristem (highlighted in Section 3.1). Eventually, *apl-1 cal-1* plants begin to autonomously transition into the reproductive phase, and sporadic flower development commences, producing flowers that lack petals and sepals due to a lack of *API* activity. The time increment between bolting and transitioning to flower development is governed in part by the temperature of the growth facility, likely in combination with other factors that typically promote or repress the vegetative-reproductive phase transition. After bolting, *apl-1 cal-1* plants that were grown in cooler temperatures took longer to form flowers, and the time increment became shorter with an increase in temperature.

The molecular mechanisms underpinning the hormone-inducible floral induction systems rely on the transcription of the *API* coding sequence, fused to a mammalian nuclear receptor ligand binding domain, that is effectively sequestered to the cytoplasm until application of its ligand. After binding of the ligand, the protein is translocated into the nucleus and AP1 is then able to take part in the GRN governing flower development. In the case of the ethanol-inducible ACLA/ALCR system, the ALCR transcription factor promotes the expression of the *API* coding sequence fused downstream of the *ALCA* promoter, which is activated upon ALCR’s interaction with acetaldehyde, a metabolite of ethanol. In all cases the potential for the systems to be ‘leaky’ needs to be assessed. A consequence of AP1 activation (or *API* transcription) before the desired time could lead to precocious flower formation.

To this end, the tendencies of the floral induction systems (developed in the Wellmer laboratory) to transition into a flowering state, without any application of their respective inductive stimuli, was assessed (**Fig. 3.6**). *ap1-1 cal-1* plants harbouring the following transgenes were examined: 35S::AP1-GR; AP1::AP1-GR; and FIS-AR; (35S>>AP1^{EtOH}). These lines were grown under constant illumination at temperatures between 18-24°C and observed until they had begun to autonomously develop floral organs. The point of transition was defined as the length of time between the emergence of the flowering stem and the point when autonomous floral organ formation was visible.

Plants grown at 18°C took approximately 20-40 days before autonomous flower formation occurred. 35S::AP1-GR plants took the least amount of time, at approximately 20 days, while AP1::AP1-GR, FIS-AR, and 35S>>AP1^{EtOH} plants began to form flowers after 30-40 days. The behaviour of *ap1-1 cal-1* plants was similar to that of the latter three lines, developing flowers lacking perianth organs after approximately 37 days. The time required for all lines to transition was markedly reduced, to 3-5 days, in plants that were grown at 24°C. The similar responses of all transgenic lines, apart from 35S::AP1-GR, to that of *ap1-1 cal-1* plants suggests that the presence of the transgenic AP1-expressing constructs does not cause precocious flower formation in the absence of the correct stimulus.

The tendency for 35S::AP1-GR plants to form flowers earlier than the rest of the tested lines, especially at 18°C, is unclear. It could be due to the spatial expression pattern of AP1-GR, its higher level of expression compared to the other lines, or both, although these properties of the line do not necessarily translate into a greater quantity of AP1-GR entering the nucleus without DEX application. Additionally, the successful development

of the AP1::AP1-GR line, which more closely mimics *AP1*'s endogenous expression pattern and produces flowers with more comparable organ sizes to wild-type *L-er* plants (O'Maoileidigh *et al.*, 2015), places the constitutive line into obsolescence, as studies dissecting the GRN of flower development in a stage-specific manner require plant lines that resemble the wild-type developmental state as much as possible.

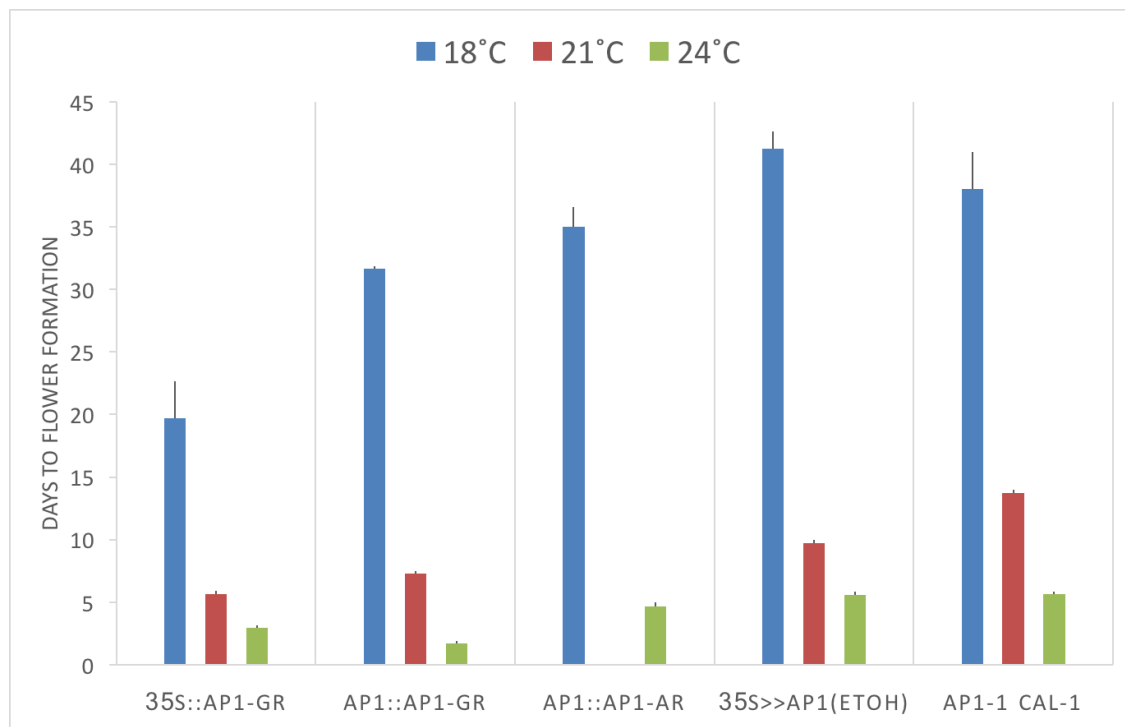


Figure 3.6 – Time to autonomous flower formation in floral induction systems.

Flower formation was timed without the application of each line's respective chemical inducer, at 18, 21, and 24°C. Error bars represent the standard error. $n = 27$ for the counts performed at 18°C, and $n = 40$ for the counts performed at 21°C and 24°C. Counts for AP::AP1-AR (FIS-AR) at 21°C are presently unavailable.

3.2.6. Isolation of stable FIS-AR lines doubly transgenic for dexamethasone-inducible artificial microRNAs that target floral homeotic genes

The FIS-AR line optimised in this study was crossed with transgenic *L-er* lines, harbouring inducible amiRNA cassettes that target the floral homeotic genes, *AP3* or *AG*. The expression of these amiRNAs is controlled using a DEX-inducible system, 35S::LhG4-GR / 6xOP, and the resulting lines are referred to as FIS-AR AP3amiR^{DEX} or FIS-AR AGamiR^{DEX}. Briefly, as with the other hormone-responsive lines used in this study, the LhG4-GR chimeric transcription factor is located in the cytoplasm until a DEX-containing solution is applied to the inflorescences of the plants, at which point it translocates to the nucleus and binds the 6xOP promoter, activating the transcription of the amiRNA precursor, which is then processed into the functional amiRNA and participates in the degradation of its target endogenous mRNA. The amiRNAs used in this study were previously developed and validated by Dr. Diarmuid O'Maoileidigh and Dr. Samuel Wuest in the Wellmer laboratory (Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013).

Stamens from FIS-AR plants were used to pollinate the carpels of transgenic *L-er* plants possessing 35S::LhG4-GR 6xOP::AGamiR^{DEX} / 35S::LhG4-GR 6xOP::AP3amiR^{DEX} cassettes. Seeds arising from the crosses were grown and allowed to self-fertilise. F2 progeny were first phenotypically screened for the presence of the *ap1-1 cal-1* mutations. Those that produced inflorescence-like meristems typical of *ap1-1 cal-1* double mutants were treated with 500 µM DHT to determine the presence of the FIS-AR transgene. Further to this, those plants which responded by forming flowers were PCR genotyped for the presence of the amiRNA-carrying transgenes, and the individuals for which the genotyping assay returned a positive result were brought through to the next generation.

To isolate *ap1-1 cal-1* lines homozygous for both transgenes, segregation analysis was performed on several F3 populations of plants by PCR genotyping for the two cassettes. Approximately 24 plants were used for each line being assessed. A line for each amiRNA cassette was selected that returned 100% positive results for both genotyping assays.

3.2.7. Treatment of FIS-AR plants with DEX does not induce synchronous flower formation

The purpose of generating the FIS-AR amiR^{DEX} lines was to be able to activate flower development and amiRNA transcription independently, thus allowing us to study the effects of stage-specific gene perturbations, and ultimately gain new insights into the dynamic roles of the homeotics throughout the reproductive cycle. As the receptors of inducing molecules, dihydrotestosterone (DHT) and dexamethasone (DEX), belong to the same nuclear receptor subfamily, it was necessary to determine if FIS-AR plants treated with DEX, and the DEX-responsive AP1::AP1-GR (referred to as FIS-GR) / LhG4-GR systems treated with DHT, unintentionally responded to the wrong stimuli.

To determine the effects of dexamethasone treatment on FIS-AR plants harbouring a DEX-inducible amiRNA transgene, plants were grown until inflorescence-like meristematic tissue had developed. Before the plants began to transition to the flowering state, the inflorescence tissue was treated once with a 10 μ M DEX solution in order to investigate whether DEX treatment alone was sufficient to induce synchronous flower formation, possibly due to the aberrant interaction with AP1-AR protein present within the tissue.

In contrast to treatment with DHT (**Fig. 3.7 A**), FIS-AR AP3amiR^{DEX} plants that were treated with DEX did not form flowers synchronously. Instead, asynchronous flower development was observed that partially matched the development of plants treated with a mock solution, as the inflorescence-like meristems began to transition into flower development autonomously (**Fig. 3.7 B and C**). Developing flowers in both treatment categories lacked sepals and petals, likely due to a lack of AP1 activity. DEX-treated flowers, however, additionally lacked stamens and gynoecium development was disrupted. This modification of the asynchronous flowering phenotype, characteristic of FIS plants that have not been treated with an AP1-activating hormone, is likely due to the activation of transcription of the amiRNA targeting *AP3* mRNA. RT-qPCR analysis of cDNA generated from these tissues showed that the DEX-mediated induction of amiRNA transcription is abundant (**Fig. 3.10**).

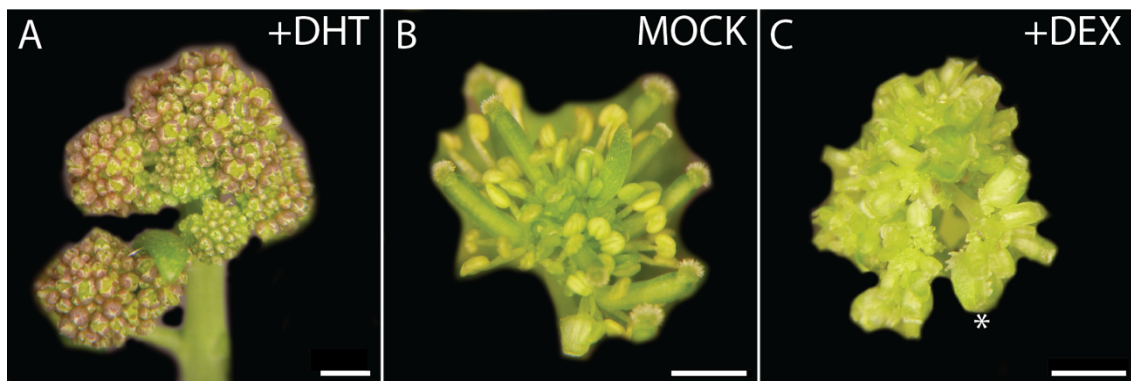


Figure 3.7 – Sole application of dexamethasone to FIS-AR AP3amiR^{DEX} plants does not induce flower development. (A) Inflorescence treated once with 500 μ M DHT and 2.5 mg/ml cyclodextrin. (B) Inflorescence treated with a mock solution. Numerous flowers lacking sepals and petals have developed in a non-synchronous manner, due to autonomous transition of the plant to the flowering state. (C) Inflorescence treated with 10 μ M DEX. A similar phenotype has been achieved, as in (B), with the difference that the 3rd and 4th whorl organs show developmental defects consistent with a reduction in *AP3* function. Stamens are absent and morphological

defects in the gynoecia are abundant (*). All images were taken 8 days after treatment. Scale bars: 1 mm.

3.2.8. Treatment of FIS-GR plants with DHT does not induce synchronous flower formation

FIS-GR plants were grown at 18°C under constant illumination until the main stems had bolted and reached a height of above 5 cm. To investigate if the application of DHT to the plants caused erroneous activation of the AP1-GR fusion protein, the inflorescences were treated once with solutions containing 0-1000 µM DHT and examined after 6 days, at which point the onset of synchronous flower development becomes obvious in the FIS-GR line when treated with DEX. No response to the stimulus was visually detected for any of the DHT concentrations used, and the treated individuals were indistinguishable from mock-treated or ‘No treatment’ plants, whereas those treated once with a 10 µM DEX solution had synchronously responded as intended (**Fig. 3.8**). This indicates that the DHT molecule is unable to interact with the GR ligand binding domain, and thus the ectopic activation of AP1-GR is likely not a concern when DHT solutions are applied if AR-dependent systems are used in tandem, and vice versa.

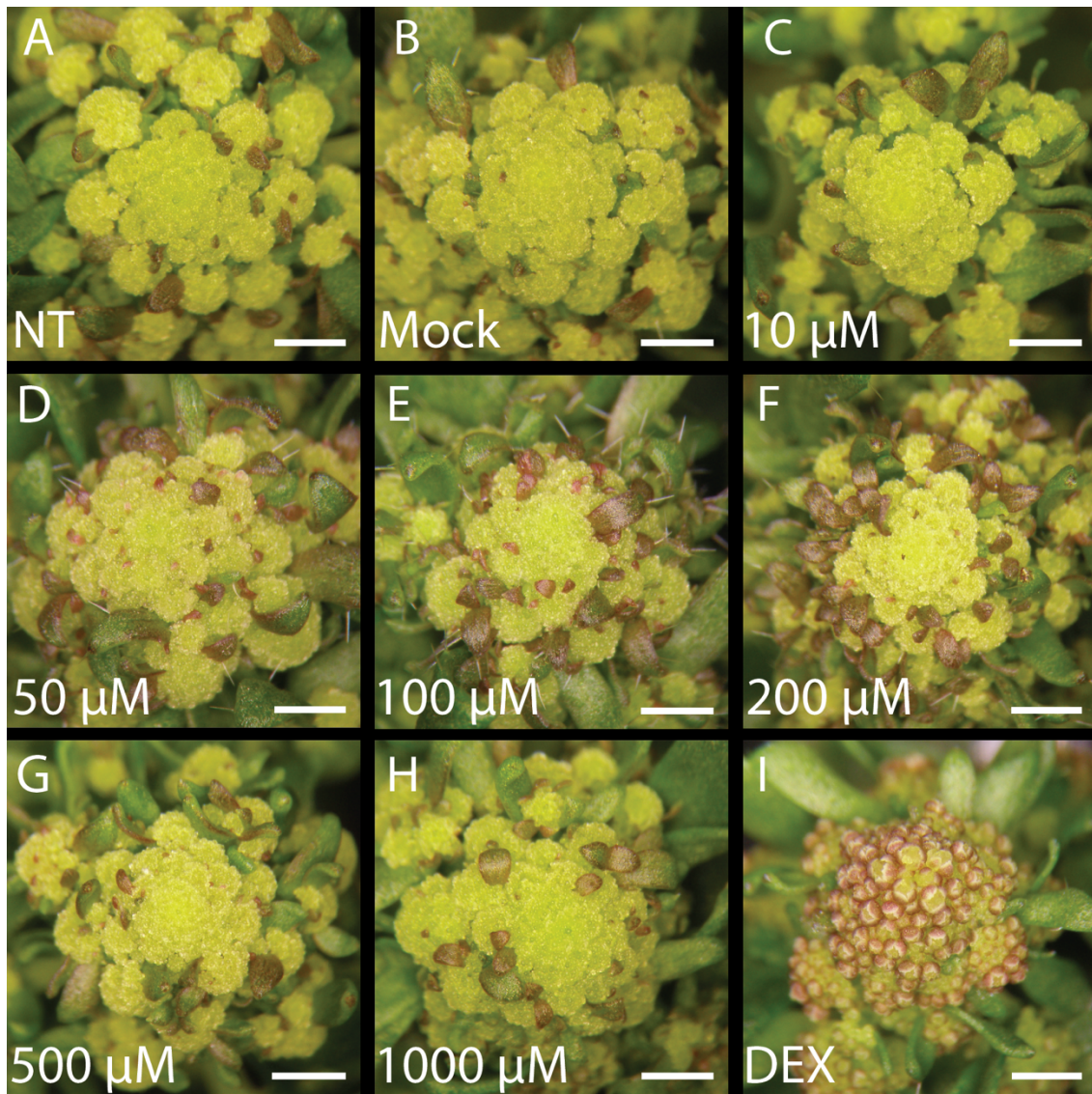


Figure 3.8 – Response of AP1::AP1-GR *ap1-1 cal-1* (FIS-GR) plants to dihydrotestosterone. Inflorescences of plants that were treated with aqueous solutions containing 10-1000 μM DHT. (A) No treatment (NT), (B) Mock solution, (C) 10 μM DHT, (D) 50 μM DHT, (E) 100 μM DHT, (F) 200 μM DHT, (G) 500 μM DHT, (H) 1 mM DHT, (I) 10 μM DEX. DHT solutions contained varying cyclodextrin concentrations, as outlined in Table 3.1. The mock solution did not contain cyclodextrin. All images were taken 6 days after treatment. Scale bars: 1 mm.

3.2.9. *DHT treatment does not induce amiR^{DEX} transcription in FIS-AR*

AP3amiR^{DEX} plants

In a similar vein to above, FIS-AR AP3amiR^{DEX} plants were treated with a range of concentrations of DHT to investigate whether the treatment would lead to the ectopic activation of amiRNA transcription through an interaction with the LhG4-GR fusion protein. The inflorescences of the plants were treated with 0 (mock), 10, 50, 100, 200, 500, and 1000 μ M solutions of DHT and the top layer of tissue was collected 24 hours post treatment. RT-qPCR analysis of cDNA generated from these tissues examined the relative levels of amiRNA precursor molecules 24 hours after application of the solutions. The result indicates that all concentrations of DHT tested did not contribute to any unwanted activation of the LhG4-GR transcription factor, or subsequently the transcription of the amiRNA (**Fig. 3.9**). In agreement with this, flowers derived from DHT-treated plants in FIS-AR AGamiR^{DEX} or FIS-AR AP3amiR^{DEX} lines do not exhibit morphological defects characteristic of reduced amiRNA target function. This indicates that the simultaneous use of both DHT and DEX, to induce synchronous flower formation and amiRNA transcription respectively, does not result in unintended activation of the other system.

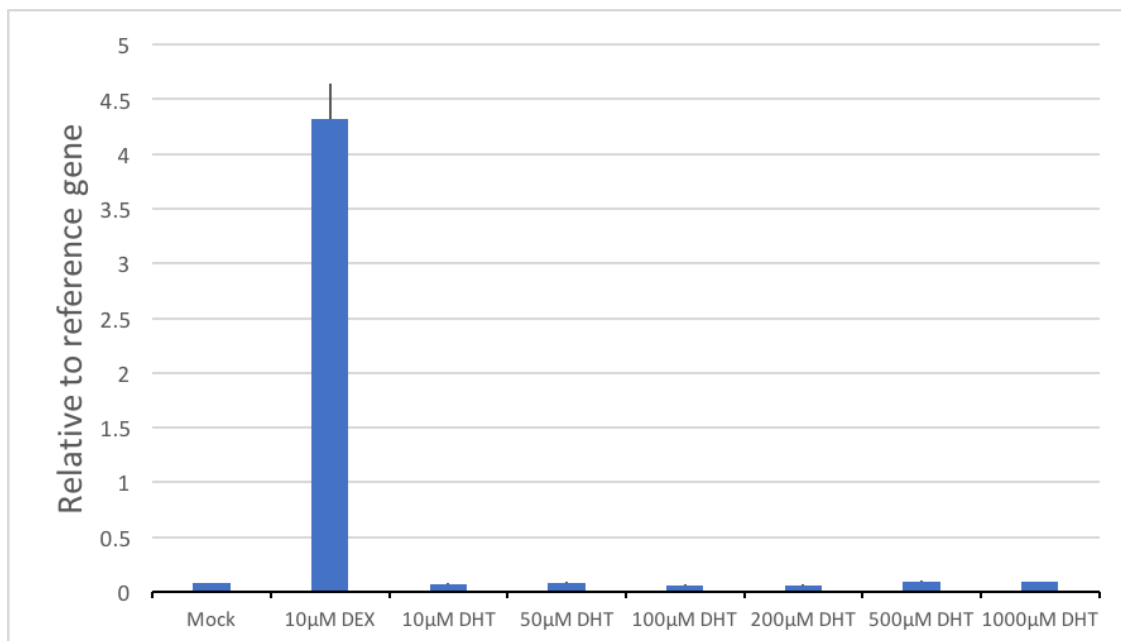


Figure 3.9 – Induction of amiRNA transcription by DHT. RT-qPCR results of AP3amiR precursor mRNA levels after application of DHT solutions, relative to the reference gene used (REF1). Non-transitioned FIS-AR AP3amiR^{DEX} inflorescences were treated with either a mock solution (0.015% Silwet L-77, 1% EtOH), 10 µM DEX solution, or DHT solutions with concentrations ranging from 10-1000 µM. Tissue was collected 24 h after treatment. Three independent biological replicates, and two technical replicates, were used for each treatment condition. Error bars represent the SEM. All DHT solutions contained cyclodextrin at the concentrations listed in Table 3.1.

3.2.10. Activation kinetics of dexamethasone-inducible artificial microRNA in the DHT-responsive floral induction system

To examine the transcriptional response of the AP3amiR^{DEX} transgene, the inflorescence tissue of FIS-AR AP3amiR^{DEX} was treated with 500 μ M DHT, and after 3 days 10 μ M DEX was subsequently applied. The top layer of floral tissue was collected at 0h, 12h, 24h, 48h, 72h, and 96h post treatment to monitor the activation and decline of amiRNA transcription. RT-qPCR was performed on cDNA, synthesised from total RNA extracted from the tissue, using primers that amplify the amiRNA precursor (Fig. 3.10).

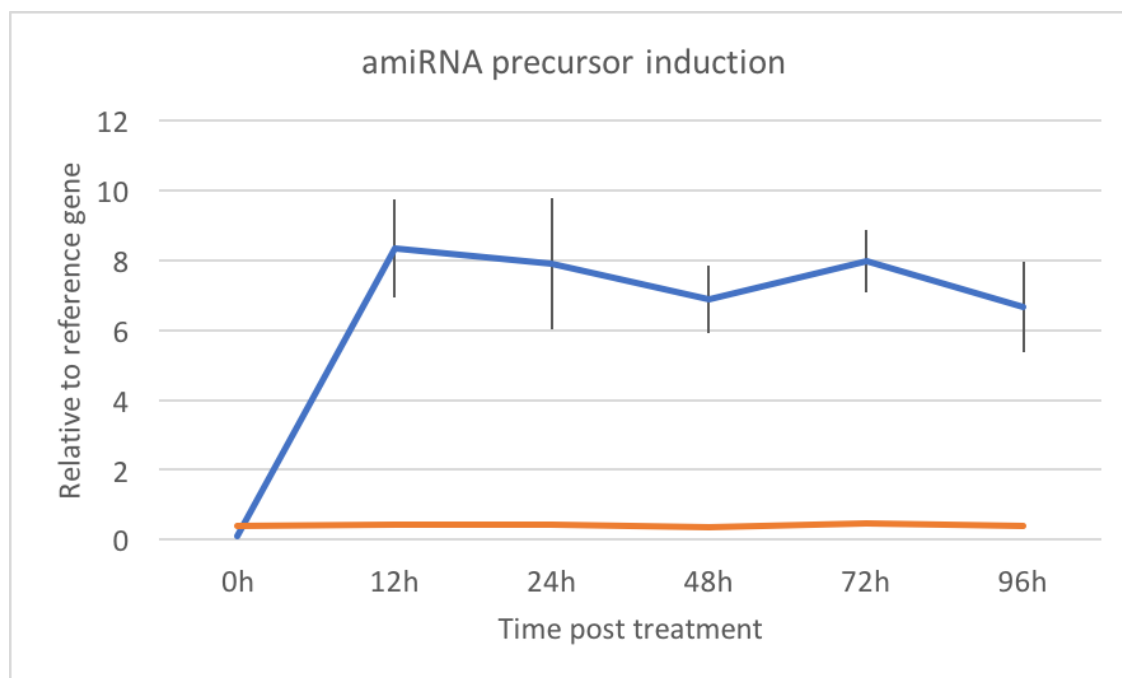


Figure 3.10 – Activation of transcription of the AP3amiR^{DEX} transgene in response to a single application of DEX. RT-qPCR results of amiRNA precursor levels relative to the reference gene used (REF1) (blue line). Floral tissue was collected from FIS-AR AP3amiR^{DEX} plants after treatment with DEX. Error bars represent the SEM. Three independent biological replicates, and two technical replicates, were used for each time-point. DEX was applied to the developing floral tissue 3 days (0h) after treatment with 500 μ M DHT. The x-axis time-points correspond to the amount of time passed after DEX treatment. The orange line represents RT-qPCR performed using

samples in which the reverse transcriptase was omitted from the cDNA synthesis reaction.

Analysis of the qPCR data shows that there is a rapid onset of transcription upon application of the DEX solution, as the relative amount of the amiRNA precursor is at its peak level from 12-24 hours post induction, with a 92.6-fold change over the 0h time-point. All of the later time-points also show a persistently high level of transcription for the amiRNA, with a small decline towards the end of the time course at 96h post induction. This result suggests that the sustained activation of the LhG4-GR transcription factor occurs for several days after a single application of DEX, and that the molecule is stable under the physiological conditions present in the developing floral tissue. Furthermore, as DHT had been applied to the tissue prior to DEX, the low level of transcription seen at the 0h time-point indicates that DHT does not appreciably activate the LhG4-GR transcription factor.

3.2.11. Knockdowns of AP3 and AG in the DHT-responsive floral induction system using DEX-inducible artificial microRNAs

Recent work in the Wellmer laboratory has used the floral induction system as a tool for performing transcriptomic analyses in order to study the gene regulatory network governing flower development over time, providing important insights into how differential gene expression correlates with specific events in the flower's development (Ryan *et al.*, 2015). Furthermore, microarray analyses have been performed using strong mutant alleles for floral homeotic genes, such as *ap3-3* and *ag-1*, examining the overarching changes to the transcriptome when compared to wild-type plants (Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013). However, to date we have not studied the transcriptomic changes caused by knocking down these floral regulators in a stage-

specific manner. A central question therefore remains: what regulatory roles do these floral regulators have at any specific time during flower development? To begin to answer this, DEX-inducible artificial microRNAs have been combined with the DHT-responsive floral induction system (as described above) to be able to knock down the activities of some of the homeotic genes in a temporally-controlled manner. In this way, the effects of stage-specific gene perturbations have been monitored through phenotypic analysis of the resulting flowers, as well as through next generation sequencing approaches, that are discussed in Chapter 4.

To validate that the induction of amiRNAs targeting *AP3* or *AG* resulted in a reduction in the mRNA levels of both genes in the FIS-AR AP3amiR^{DEX} and FIS-AR AGamiR^{DEX} systems, inflorescences of both lines were first treated with 500 μ M DHT to induce flower formation. After 3 days, the floral buds were treated once with a 10 μ M DEX solution, or a mock solution excluding DEX, and the top layer of tissue was collected 24 hours later. RT-qPCR was performed on cDNA synthesised from total RNA that had been extracted from this tissue, revealing that the relative amount of *AP3* and *AG* transcripts present, compared to the mock solutions, was around 12% in both cases (**Fig. 3.11**). These results provide further confidence that both lines are suitable for use in gene perturbation experiments.

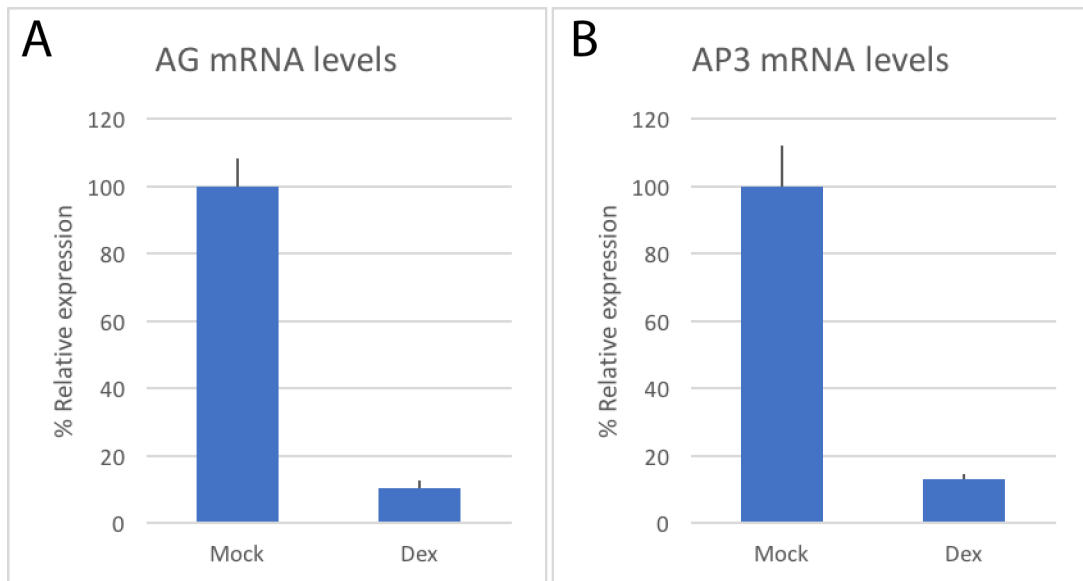


Figure 3.11 – Reduction of mRNA levels through dexamethasone-dependent induction of amiRNAs. Results of RT-qPCR assays monitoring *AG* (A) and *AP* (B) mRNA levels in FIS-AR AGamiR^{DEX} and FIS-AR AP3amiR^{DEX} plants. Inflorescences were treated once with a 10 μ M DEX solution. Results shown are the mean of three independent biological replicates, and two technical replicates, for each condition tested. Error bars represent the standard deviations. Plants were treated with 500 μ M DHT 3 days prior to treatment with DEX or the mock solution.

3.2.12. Phenotypic analysis of stage-specific *AG* knockdowns during flower development

To investigate the phenotypic consequences of reducing *AG* activity at specified times in the development of the flower, FIS-AR AGamiR^{DEX} plants were treated at several time-points with DEX, after DHT treatment, according to the schematic shown in **Fig. 3.12 A**. Plants that were treated with the DEX solution were allowed to develop, for approximately one to two weeks, until the flowers had matured and were easily observed under a microscope. In general, those plants that were treated with DEX at earlier stages experienced a greater conversion of stamens to petals (**Fig. 3.12, B-C**), as well as determinacy defects within the 4th whorl. Plants treated at 4 days after DHT still possessed 3rd whorl organs resembling petals, although it was clear that stamen identity had not been

fully abolished, due to the presence of anther tissues at the apical region of the petals (**Fig. 3.12 D**). Stamen identity was maintained in plants treated at 7 days, presumably because many of the organ tissues had already formed as the developing flowers would have normally progressed beyond stage 7 (according to Smyth *et al.* (1990)), and the stamen primordia arise around stage 5. However, it appears as if the developmental program was halted or decreased, as the filaments did not become extended in the mature flowers, nor did they produce pollen (**Fig. 3.12 E**), demonstrating the prolonged requirement for AG activity during flower development.

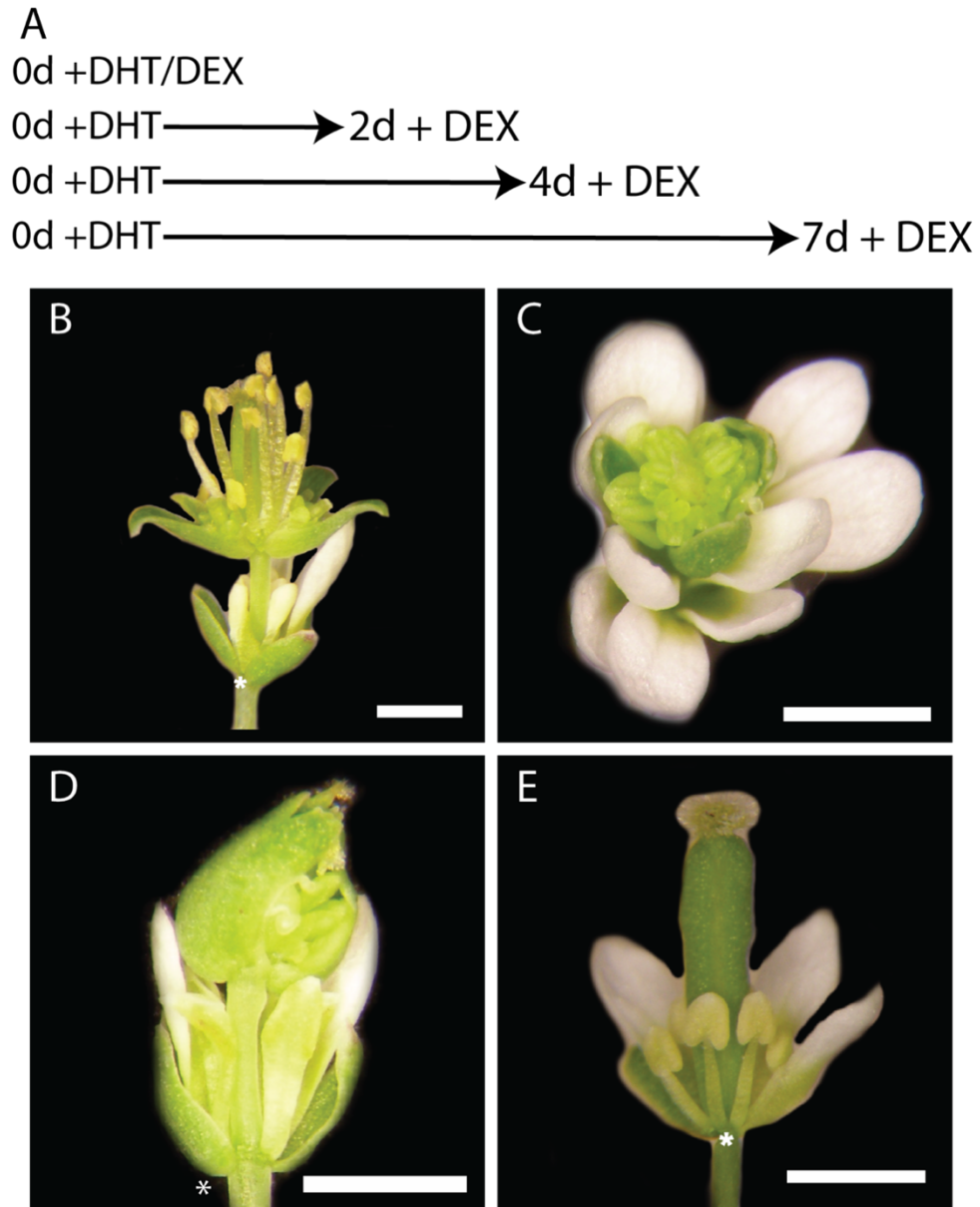


Figure 3.12 – Stage-specific knock down of *AG* activity in FIS-AR plants. (A) Schematic of knockdowns performed in FIS-AR AGamiR^{DEX} plants. DHT treatments were performed using a 500 μ M DHT, 2.5 mg/ml cyclodextrin, 0.015% Silwet L-77 solution. Plants were treated with a 10 μ M DEX solution at the specified time-points after DHT treatment. (B-E) Mature flowers of plants treated with DEX at 0d (B), 2d (C), 4d (D), and 7d (E) post DHT treatment. Photos were taken between 8-15 days after DEX treatment. Sepals and petals have been removed where necessary to aid visualisation (*). Scale bars: 1 mm.

Changes to the development of the 4th whorl in plants treated at 0d, 2d, and 4d, were all substantial. In agreement with the indeterminacy phenotype of strong *ag* mutants, the unspecified floral meristem persisted within the presumptive 4th whorl of plants treated with DHT/DEX simultaneously, and at 2 days post treatment with DHT. At 7 days post induction, these plants possessed floral buds that appeared superficially similar to mock-treated plants. Dissection of the buds at this stage revealed the presence of meristematic tissue within the centre of the developing flower, instead of 3rd and 4th whorl organs, whereas stamens and carpels were already recognisable in mock-treated plants. Petals were still present in the DEX-treated plants (**Fig. 3.13**).

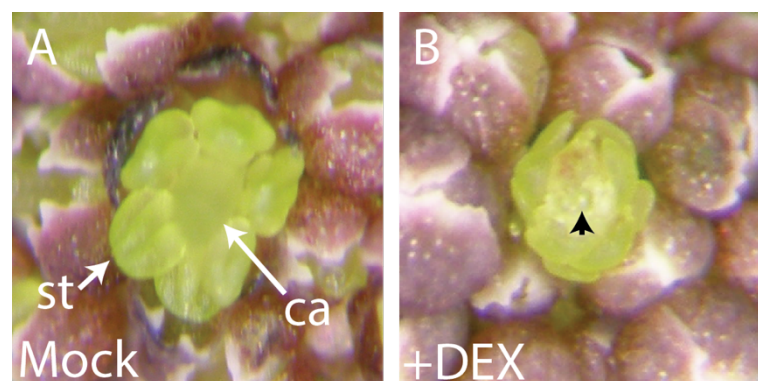


Figure 3.13 – Results of *AG* knockdown at the onset of floral induction. 7 day old flowers treated with a mock solution (A) or 10 μ M DEX (B) at the time of DHT treatment. Sepals have been removed to aid visualisation. Stamen (st) and carpel (ca) primordia are clearly visible in mock-treated plants, whereas the floral meristem is present in the presumptive 4th whorl of those plants treated with DEX (black arrowhead). Petal primordia are present in the 3rd whorl

Once the floral meristem became determinate, additional flowers were produced that possessed supernumerary stamens. Petals were frequently substituted with further developing flowers. The presence of floral meristems within the DEX-treated flowers 7 days post induction is likely the cause of ectopic flower formation. As *AG* mRNA levels

progressively return to wild-type levels the meristematic tissue begins to differentiate and produce determinate floral primordia.

Flowers treated with DEX at 4 days post DHT treatment developed 3rd whorl organs that were often chimeric for petal and stamen tissues (**Fig. 3.14**), as well as 4th whorl structures that resembled bulbous gynoecial tissues (**Fig. 3.12 D** and **Fig. 3.14 A**), with the epidermis possessing sepal-like characteristics. Upon dissection, both stamen and carpel tissues could be observed inside the structure. Finally, carpels of plants treated at 7 days remained similar to mock-treated plants, though most remained un-pollinated due to the stamen defects described earlier (**Fig. 3.12 E**). These results suggest that the gene regulatory networks *AG* directs, or is at least involved in, are susceptible to perturbation at multiple stages in flower development.

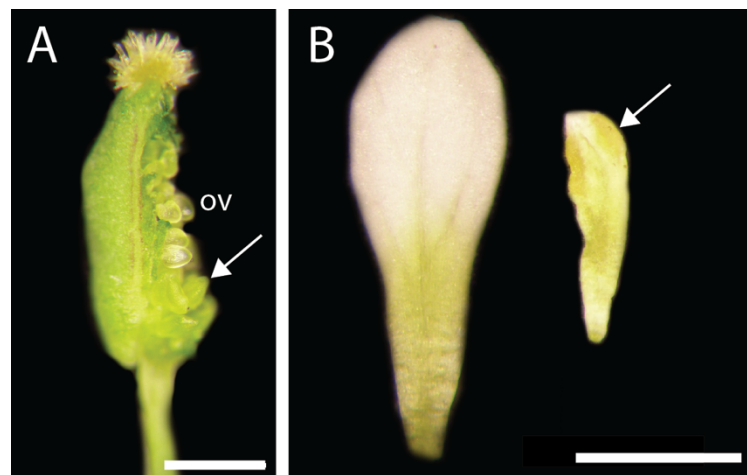


Figure 3.14 – Results of *AG* knockdown at 4 days post floral induction. (A) Carpel of a mature flower treated with DEX. The epidermis on the right side has been removed to visualise the inner tissues. Both ovules (ov) and stamen primordia (arrow) are visible. (B) 2nd whorl petal (left) and a 3rd whorl petal-stamen chimera from a mature

flower that had been treated with DEX. Anther tissue is present around the apical region of the petal blade (arrow). Scale bars: 1 mm.

3.2.13. Phenotypic analysis of stage-specific AP3 knockdowns during flower development

Inflorescences of FIS-AR AP3amiR^{DEX} plants were treated with DEX in the same manner as the knockdowns performed on FIS-AR AGamiR^{DEX} plants, and the phenotypic consequences of reducing *AP3* activity at different stages in flower development were observed. Plants treated with 10 μ M DEX at the onset of flower development experienced the substantial phenotypic changes to the 2nd, 3rd, and 4th whorls (**Fig. 3.15 B & D**). While sepal development remained largely unaffected, complete petal-to-sepal transformations were observed. Furthermore, DEX-treated flowers did not develop any distinct 3rd whorl organs. Instead, the growth of ectopic carpels originating from the presumptive 3rd whorl, and fusing with the 4th whorl tissues, resulted in gynoecia with more than 2 carpels (**Fig. 3.15 B**). This phenotype did not occur in all flowers treated at this early stage, as can be seen in **Fig. 3.15 D**.

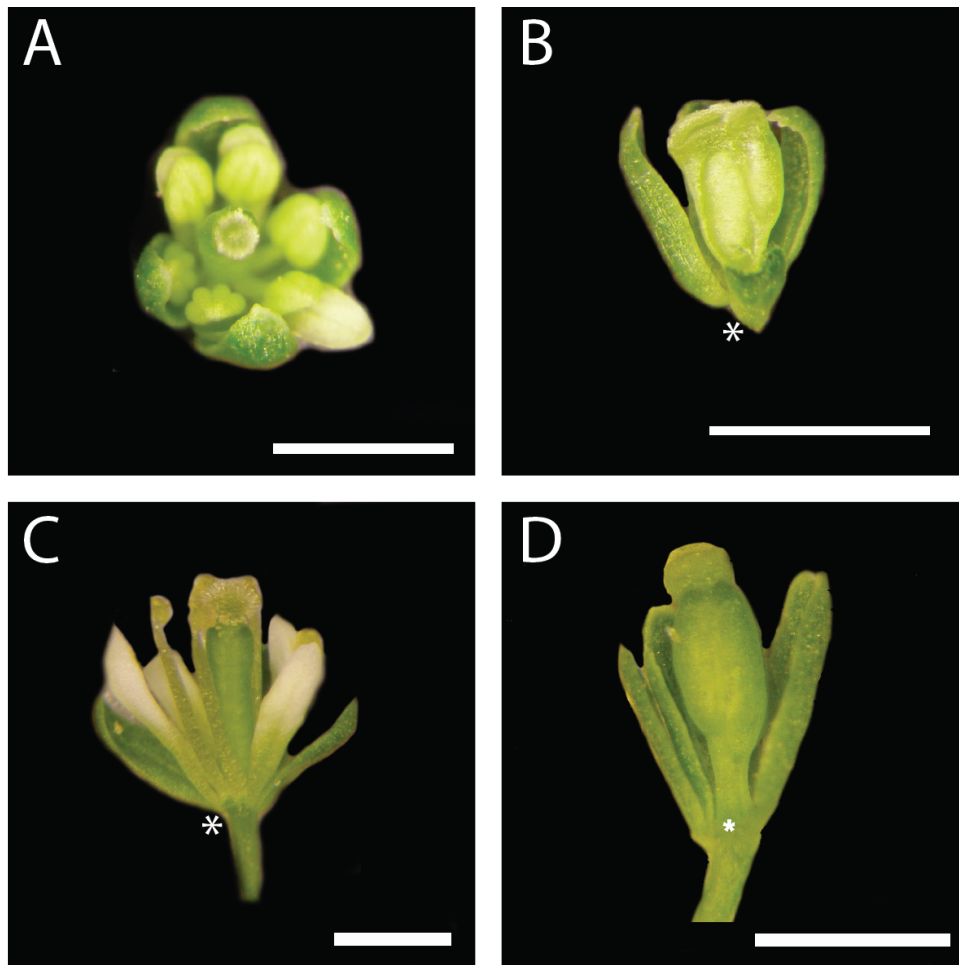


Figure 3.15 – Results of *AP3* knockdown at the onset of floral induction. (A) Flower treated solely with 500 μ M DHT (mock-treated), 9 days after induction. The full complement of floral organs is present. (B) Flower treated simultaneously with 500 μ M DHT and 10 μ M DEX, 9 days after induction. Petals and stamens are absent, and carpel development defects are apparent. Carpel tissue, apparently arising from the presumptive 3rd whorl, can be seen to be fused to the gynoecium. (C) Mock-treated flower 12 days after induction. (D) Flower treated simultaneously with 500 μ M DHT and 10 μ M DEX, 12 days after induction. Petals are replaced with sepals and 3rd whorl organs are absent. Asterisks denote sepals that have been removed to aid visualisation of the inner whorls. Scale bars: 1 mm.

Flowers treated at 2 days post floral induction had a similar transformation of sepals to petals (**Fig. 3.16**), though distinct 3rd whorl organs were more apparent than plants treated with DHT and DEX simultaneously. In place of stamens, there was an abundance of

ectopic gynoecial structures, such as visible ovules and stigmatic tissues. However, these transformations were generally not fused to the 4th whorl tissues and overall carpel development proceeded in a manner similar to mock-treated plants. In addition, some filaments were occasionally present.

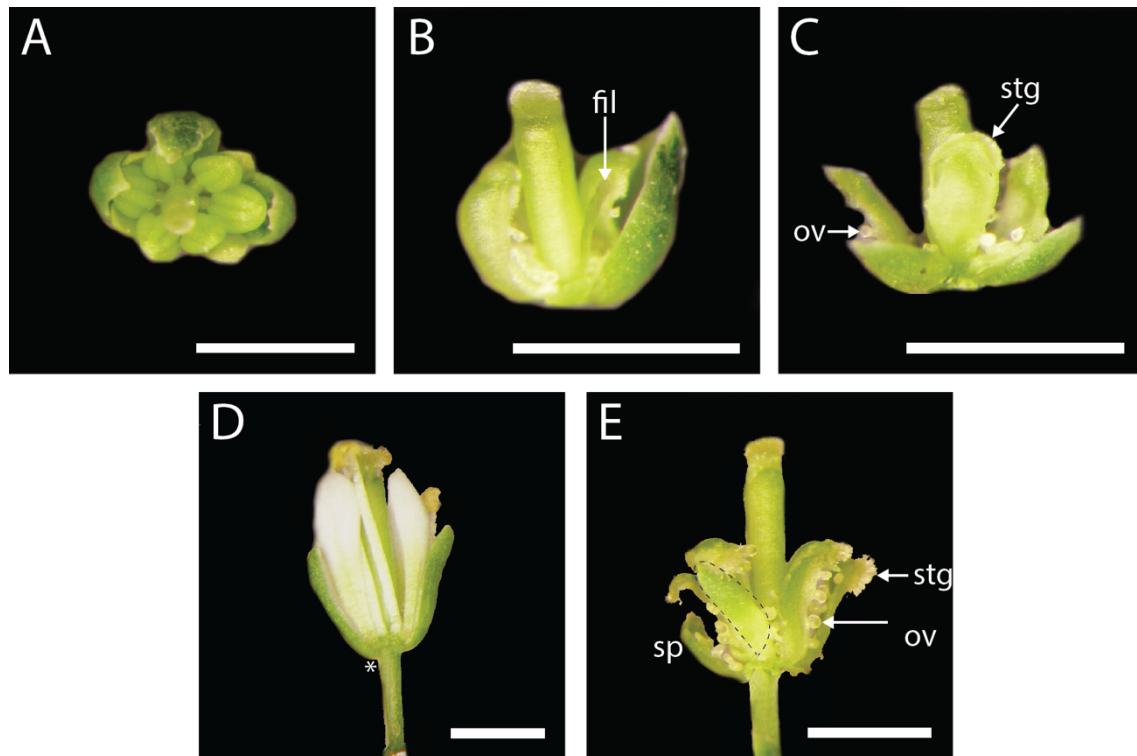


Figure 3.16 – Results of *AP3* knockdown at 2 days post floral induction. (A) Flower treated solely with 500 μ M DHT (mock-treated), 8 days after induction. The full complement of floral organs is present. One sepal is not visible. (B & C) 8-Day-old flowers treated with 10 μ M DEX, 2 days after induction. 3rd whorl organs are largely composed of gynoecial tissues or filaments (fil). Ovules (ov) and stigmatic (stg) tissue are clearly visible. Carpel formation in the 4th whorl is largely normal. (D) Mock-treated flower 14 days after induction. The full complement of floral organs is present. One sepal is not visible has been removed to aid visualisation of the inner whorls (*). (E) 14-Day-old flower treated with 10 μ M DEX, 2 days after induction. All but one of the 1st whorl sepals have been removed (sp). All but one of the 2nd whorl petal-to-sepal conversions have been removed (outlined), and the development of ectopic stigmatic and ovule tissues is clear. Scale bars: 1 mm.

Flowers treated with DEX at 4 (**Fig. 3.17**) or 7 (**Fig. 3.18**) days after floral induction had less severe homeotic transformations than those treated at earlier stages, insomuch that 3rd whorl organ identity was established to a greater extent, although treatment at both of these time-points still resulted in flowers with incorrectly specified stamens, and were therefore sterile despite normal development of the gynoecium. Additionally, in both cases petal-to-sepal transformations were still apparent. (**Fig. 3.17 B** and **Fig. 3.18 B**).

Anther epidermal tissue was observed in the stamens of flowers treated at 4 days (**Fig. 3.17B**), though it was part of mosaic organs that also contained ectopic gynoecial tissues (**Fig. 3.17 B & D**). Flowers treated at 7 days displayed the least severe defects, as was the case with the *AG* knockdowns previously described. Despite the persistent loss of petal identity (**Fig. 3.18 B & D**), the 3rd whorl organs visually appeared to be fully composed of only stamen tissues. However, the loss of *AP3* activity at this stage was still sufficient to halt stamen maturation. To this end, in contrast with mature mock-treated flowers (**Fig. 3.18 C**), filament extension did not occur and the anthers did not produce pollen (**Fig. 3.18 D**).

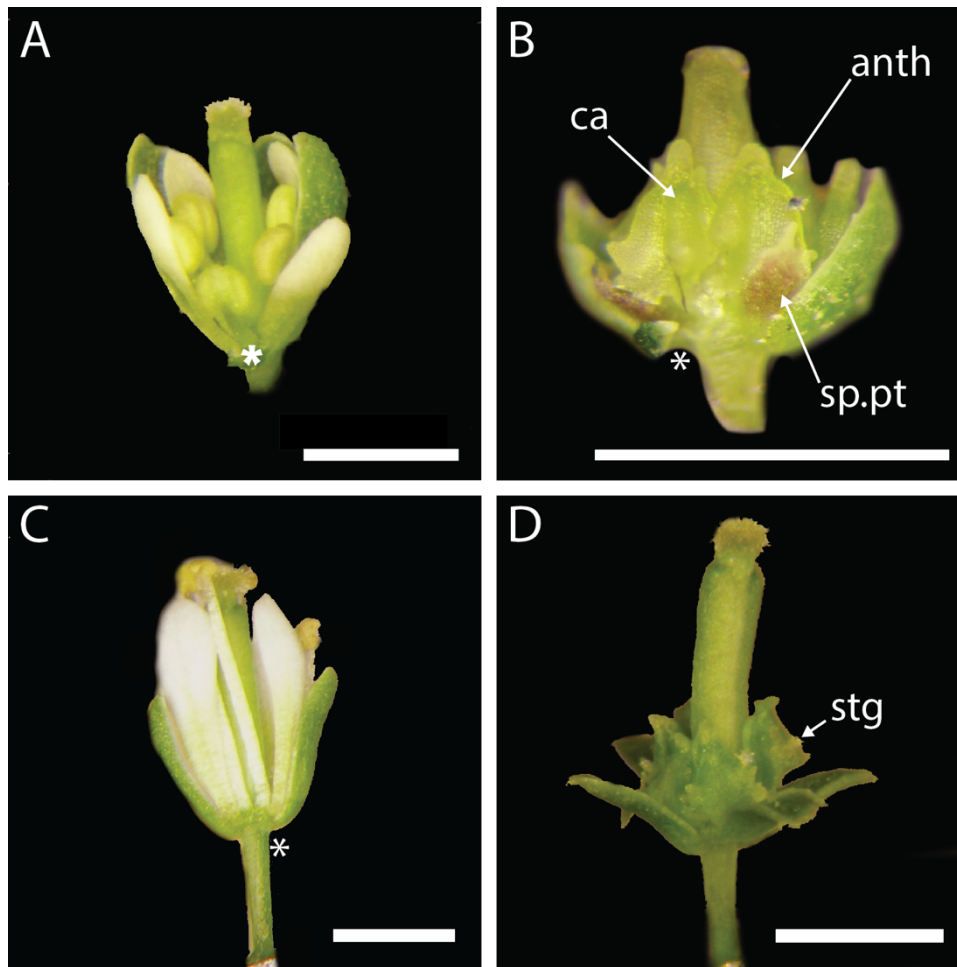


Figure 3.17 – Results of *AP3* knockdown at 4 days post floral induction. (A) Flower treated solely with 500 μ M DHT (mock-treated), 12 days after induction. The full complement of floral organs is present. (B) 12-day-old flower treated with DEX 4 days after floral induction. Petal-to-sepal conversions (sp.pt) are apparent in the 2nd whorl, and tissues characteristic of anther (anth) and carpel (ca) epidermises are both visible on 3rd whorl organs. (C) Mock-treated flower 14 days after induction. The full complement of floral organs is present. (D) 14-day-old flower treated with DEX 4 days after floral induction. Stigmatic tissue (stg) can be seen on developing 3rd whorl stamen/carpel mosaic organs. Asterisks denote sepals that have been removed to aid visualisation of the inner whorls. Scale bars: 1 mm.

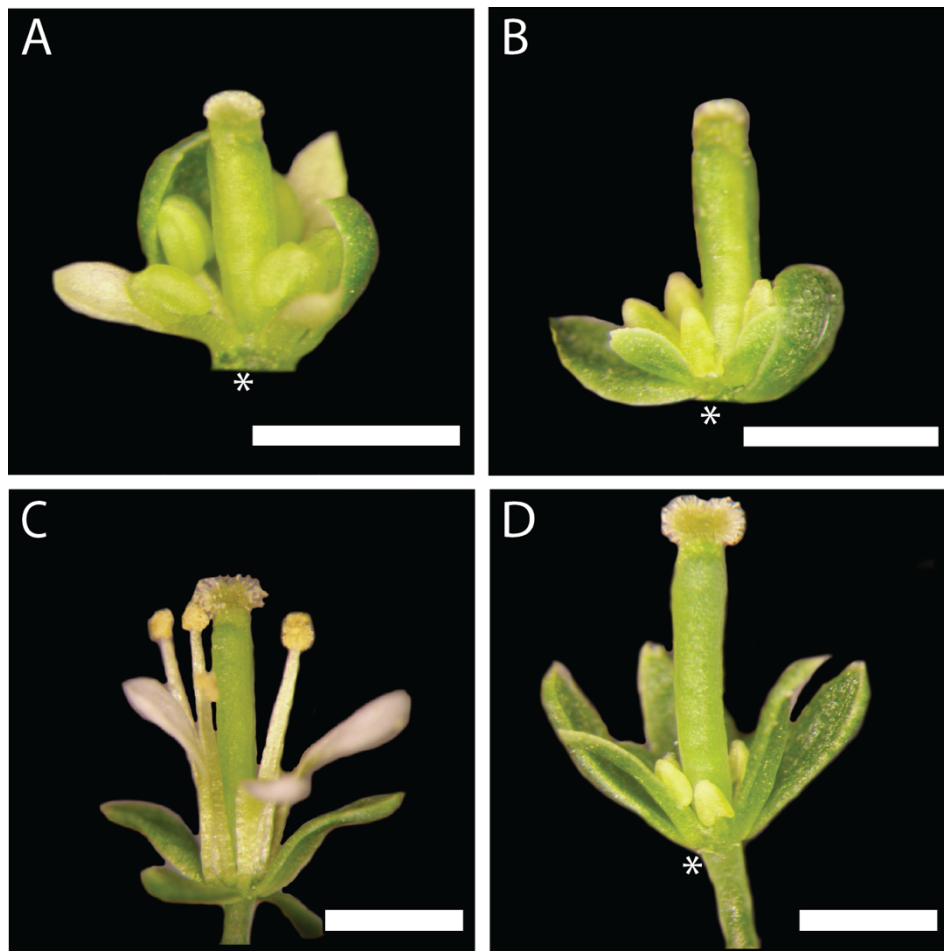


Figure 3.18 – Results of *AP3* knockdown 7 days post floral induction. (A) Flower treated solely with 500 μ M DHT (mock-treated), 12 days after induction. The full complement of floral organs is present. (B) 12-day-old flower treated with DEX 7 days after floral induction. Petal-to-sepal conversion are apparent. Stamens are present within the third whorl. (C) Mock-treated flower 15 day post floral induction. The full complement of floral organs is present. (D) 15-day-old flower treated with DEX 7 days after floral induction. Stamen filaments have not extended and no anthesis is visible. Asterisks denote sepals that have been removed to aid visualisation of the inner whorls. 2nd whorl sepals have been removed in panels A and D. Scale bars: 1 mm.

3.3. Discussion

The main objectives of this chapter were 1) to characterise and optimise novel floral induction systems that would more accurately reflect the endogenous expression pattern of *API*, in contrast to the original floral induction system that constitutively expressed *API-GR*, and 2) combine one of these systems with a second inducible amiRNA system in order to be able to independently control both the onset of flower development, and the down-regulation of floral regulators under examination in a temporally-controlled manner. To this end, after assessing the responses of the androgen-responsive, ethanol-responsive, or estradiol-responsive systems, the FIS-AR construct was selected for use due to its more reliable response to its inducer over the other two systems. While it has been shown that the latter two systems are capable of being reliably activated in other developmental studies, the same result was not achieved in the experiments described here. In contrast to the FIS-AR system, the ethanol-responsive system responded to its treatment at a lower frequency, and the estradiol-responsive system was unresponsive to 17- β -estradiol treatment.

The use of the ACLR/ALCA ethanol-responsive system to drive *API* expression did not result in a satisfactory induction of flower formation when the 35S>> *API*^{EtOH} transgene was present in the *ap1-1 cal-1* background, despite the high level of induction of *API* mRNA upon treatment with ethanol vapour. Taking this into consideration, along with previous reports of this system being used to successfully activate amiRNA transcription (Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013, 2015), and subsequently reduce target mRNA levels, it is possible that synchronous floral induction was not achieved at a higher frequency because of some factor(s) other than the level of *API* transcription. Those flowers that did form often had fewer than four petals, a phenotype reminiscent of the

original floral induction system in which *API* was constitutively expressed (Wellmer *et al.*, 2006). While the causes of this poor induction of flower formation are not known, the possibility exists that the combination of the ALCR transcription factor with ethanol could be causing undue stress to the plants, resulting in a block to flower formation despite the presence of *API*. We have shown through GO term analysis, determined using microarrays, that differentially expressed genes identified in plants expressing an *AG*-targeting amiRNA driven by the ethanol responsive system (*L-er* 35S::ALCR ALCA::AGamiR) were highly enriched for terms associated with stress response when compared to plants expressing the same amiRNA using the DEX-inducible LhG4-GR/6xOP system (O'Maoileidigh *et al.*, 2015). Furthermore, it was demonstrated that some differentially expressed genes identified from the aforementioned microarray experiment also responded to ethanol treatment in plants harbouring an ethanol-inducible control amiRNA (not predicted to target any sequence in the *Arabidopsis* genome), indicating that use of ALCR/ALCA system could introduce a systematic bias to global analyses of gene perturbations. For these reasons, the DHT-inducible floral induction system, and the DEX-inducible amiRNA constructs, were favoured over any of the ethanol-inducible systems.

To optimise the response of the DHT-responsive system, a series of concentrations were assessed to determine at what point the response would resemble that of the previously-established floral induction systems (35S::AP1-GR, and to a greater extent, AP1::AP1-GR). To achieve a satisfactory response from this line, the cyclic polysaccharide (2-Hydroxypropyl)- β -cyclodextrin was used to improve the solubility of DHT at concentrations beyond 50 μ M, by providing a hydrophobic cavity with which it could complex and therefore achieve greater activation of the AP1-AR fusion protein. This is

in contrast with the AP1-GR protein, which is responsive to a much lower concentration of DEX (10 μ M). The mechanism through which a greater activation of AP1-AR occurs has not been determined, although one possibility is that addition of cyclodextrin prevents the aggregation of DHT molecules in aqueous solution, due to their hydrophobicity, and subsequently increases the available quantity of DHT to pass into the meristematic cells. Considering the structural similarity between DEX and DHT, and the GR and AR ligand binding domains in use, the question remains as to why the AP1::AP1-GR system requires a much more dilute concentration of its inducer to consistently activate flower development in a synchronous manner.

Employing two independent hormone-inducible systems in a single plant line requires that each system is reliably activated solely with its specific inducer, and that there is no appreciable cross-activation. To be able to confidently activate amiRNA transcription in a stage-specific manner, it was necessary to demonstrate that DHT does not activate LhG4-GR or AP1-GR, and DEX does not activate AP1-AR. Treatment of FIS-GR plants, and AP1-AR 35S::LhG4-GR amiR^{DEX} plants, with 500 μ M DHT did not result in synchronous floral induction, or transcription of the amiRNA, respectively.

DHT-treated AP1::AP1-GR plants developed in the same manner as mock-treated plants, insomuch that they began to spontaneously form non-synchronous flowers that lacked perianth organs, characteristic of autonomously transitioning plants. Furthermore, qPCR analysis of DHT-treated AP1-AR AP3amiR^{DEX} plants did not result in an upregulation of amiRNA transcription at any tested DHT concentration. These results strongly suggest that, despite belonging to the same family of nuclear receptors, the presence of DHT is not sufficient to activate the GR ligand binding domain.

In the same vein, FIS-AR AP3amiR^{DEX} plants treated solely with DEX did not form flowers in a synchronous manner, and those flowers that did form as a result of spontaneous transition of inflorescence meristem cells to floral meristems, characteristic of *ap1-1 cal-1* plants displayed 3rd whorl organ identity defects associated with a reduction in *AP3* activity. This is in contrast with mock-treated plants, that developed flowers with functional stamens, and demonstrates that DEX does not activate the AP1-AR fusion protein in any visible manner.

The results of these experiments validate the simultaneous use of a DHT- and DEX-inducible system for gene perturbation experiments. Two previously validated DEX-inducible amiRNAs, targeting *AG* and *AP3* were crossed into the FIS-AR line in order to first investigate the phenotypic consequences of floral homeotic gene knockdown at different stages in flower development. A stage-specific series of these knockdowns was performed for each of the lines, resulting in mature flowers with differing phenotypes dependent on the stage at which they were treated with DEX. These results showed that, in general, earlier activation of amiRNA transcription caused more severe defects in the developing flowers, and the consequences of perturbations to the floral homeotic genes' activity are dependent on the developmental stage at which the knockdown occurs.

For instance, in the FIS-AR AGamiR^{DEX} line, floral meristem determinacy was temporarily blocked for around a week in those flowers that were treated simultaneously with DHT and DEX, eventually resulting in the formation of a second flower arising from the 4th whorl of the first. Additionally, stamens were fully transformed into petals. Flowers that received DEX treatment at a later developmental stage (7 days after DHT

treatment had less severe morphological defects, although it was apparent that the reduction in *AG* activity at this time-point was still sufficient to perturb the regulatory networks directing stamen development and maturation, even though the organs had already been specified. Similar phenotypes were observed in the FIS-AR AP3amiR^{DEX} line at later stages, while amiRNA induction in earlier stages led to the complete abolition of petal and stamen identity, and the ectopic development of tissues usually confined to the gynoecium. These analyses demonstrate the different roles that the homeotics have depending on the stage of development, the most obvious of which being the earlier roles of AP3 and AG to specify the floral organs of the three inner whorls, and later on being continually required to regulate the network(s) that ensure the maturation of the reproductive organs. A similar series of knockdowns are performed in Chapter 4, and the changes to the global expression profiles are analysed for both of the lines described here using the next generation sequencing technology, RNA-sequencing (RNA-seq), in order to investigate the genes that AP3 and AG regulate at specific stages of flower development. The information gained from these results will provide us with a greater insight into the roles of the homeotics from a temporal perspective, and therefore the data produced will begin to give us a clearer picture of which regulatory networks are influenced by these proteins at different stages in the reproductive cycle of *Arabidopsis*.

4. Chapter 4 – Transcriptomic analysis of stage specific gene perturbations during flower development

4.1. Introduction

4.1.1. Unravelling the spatio-temporal roles of floral homeotic transcription factors

One of the limitations associated with current analyses of the effects of gene perturbations on global gene expression within the developing flower is, as described in Chapter 3, that oftentimes a static mutation is used as the basis for the experiment or a collection of developmental stages are collected within the same sample. A consequence of these methods is that we are unable to deduce the effects of these lesions on the gene regulatory network in a manner that allows for the identification of differentially regulated genes at a given developmental stage. The use of static mutations also inevitably introduces systematic biases to the experiment, as large portions of the downstream network are likely never activated, making it difficult to identify targets that would respond to gene perturbations at one stage but not another.

The differing roles of the floral homeotic genes at various stages of flower development have been well documented. For instance, AP1 and LFY regulate the initiation of the floral meristem at the earliest stages before later going on to specify the floral organs by directly inducing the transcription of other homeotics, such as *AP3*, *PI*, and *AG* (Jack, Brockman and Meyerowitz, 1992; Busch, 1999; Ng and Yanofsky, 2001).

The stage-specific activities of AG, which have been so far elucidated, show that within the early stages of development it is critical to the regulation of floral meristem patterning and determinacy (Bowman, Smyth and Meyerowitz, 1989; Sun *et al.*, 2009, 2014), while also being required for the correct specification of stamens and carpels, as well as sporogenesis (Ito *et al.*, 2004). It has also been demonstrated that prolonged AG activity in later stages is required for the regulation of different transcriptional programs underlying various processes during stamen maturation (Ito *et al.*, 2007; O'Maoileidigh *et al.*, 2013). Differing roles have also been described for AP3/PI depending on the stage of development, as they are initially required for the specification of petal and stamen primordia but go on to regulate later stage targets, such as *NAC-LIKE*, *ACTIVATED BY AP3/PI (NAP)* (Sablowski and Meyerowitz, 1998), which is involved in cell division and expansion in stamens and petals, and *SQUASOMA PROMOTER BINDING PROTEIN-LIKE 8 (SPL8)*, a regulator of microsporogenesis (Unte *et al.*, 2003; Wuest *et al.*, 2012). Furthermore, the phenotypic consequences of homeotic gene perturbation at different stages during flower development have been clearly shown in amiRNA-mediated knockdown experiments against AG and AP3/PI (Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013; this study, Chapter 3), verifying the dynamic nature of the developmental roles associated with these regulators.

To examine the effects of stage-specific gene perturbations during flower development, doubly-transgenic hormone-inducible lines have been developed, which allow for the synchronous development of hundreds of floral buds as well as the user-specified down-regulation of a gene under investigation through the activation of purpose-designed artificial microRNAs. The lines developed in Chapter 3, namely *ap1-1 cal-1 AP1::AP1-AR 35S::LhG4-GR 6xOP::AGamiR* and *ap1-1 cal-1 AP1::AP1-AR 35S::LhG4-GR*

6xOP::AP3amiR (referred herein as FIS-AR AGamiR^{DEX} and FIS-AR AP3amiR^{DEX}, respectively), were used in this chapter to reduce the levels of *AG* and *AP3* mRNA transcripts present in developing floral tissues, and subsequent global analysis of the difference these knockdowns make to the transcriptome was studied.

Techniques that facilitate the analysis of the complement of transcripts in a given cell or sample, the transcriptome, are essential to understanding how the genome functions with respect to the cellular context. To this end the field of transcriptomics seeks to identify and catalogue all present transcripts, including small and non-coding RNAs in addition to mRNAs and any isoforms. Furthermore, as it is possible to quantify the expression levels of genes through their relative abundances, deeper insights can be obtained when studying developmental programs or responses to specific physiological conditions for instance.

RNA sequencing (RNA-seq) is a recently established high-throughput method of delineating transcriptomes by way of applying next-generation sequencing (NGS) technologies to cDNA libraries prepared from purified RNA. Briefly, cDNA is generated from sample RNA by reverse transcription, which is then fragmented and subjected to adaptor ligation (more detail available in the Materials and Methods). High-throughput sequencing is applied to each fragment to determine its nucleotide composition with short reads from either one end of the molecule (single-end sequencing), or both (pair-end sequencing). Files containing the raw reads can then be aligned to an annotated transcriptome, or they can be used for *de novo* transcriptome assembly which facilitates the identification of splice variants that might not be otherwise identified with annotated references. Some of the advantages of RNA-seq over other methods of transcriptome

analysis, such as microarrays or tag-based Sanger sequencing of cDNA or express sequence tag (EST) libraries, include more sensitive identification of similar sequences and transcript isoforms, greater sequence resolution, better estimations of expression levels, less reliance on pre-existing annotated genomes, and reduced cost (Wang, Gerstein and Snyder, 2009).

4.1.2. *Methods for RNA-seq analysis – genome-guided transcriptome assembly*

To assemble the transcriptome from raw reads it has typically been necessary to use bioinformatics tools that optimally align each read to the reference genome while also accounting for the presence of spliced transcripts. One particular drawback to this method is the amount of computational time required to perform all of the alignments. For instance, aligning and quantifying a set of 20 Human samples, each containing 30 million reads, to the annotated transcriptome can require up to 42 hours when using 20 cores to perform the processing. This issue has been recently circumvented, in a program called *Kallisto* (Bray *et al.*, 2016), by implementing the pseudo-alignment of transcripts to the reference transcriptome, resulting in alignment steps that have similar accuracy, but are up to two orders of magnitude faster than pre-existing methods.

Pseudo-alignment of reads requires the creation of an index consisting of a de Bruijn Graph derived from a reference transcriptome (T-DBG) (**Fig. 4.1**). The T-DBG is a representation of a sequence with respect to its k -mer composition. The term ' k -mer' refers to all of the sub-sequences of a given length k present within a sequence. Using this, an index is constructed of all present k -mers in the sequence, which the reads are then referenced against to determine the origin of the transcript. This occurs through matching the k -mers present in the transcript to the index. In the case of identifying transcripts of splice variants, *Kallisto* conserves computational requirement by assigning

hashes to each k -mer. The program identifies sequences among the transcripts that do not differ, and thus the k -mers that are identical are placed into a single compatibility class, foregoing the requirement of identifying redundant k -mers that describe the same part of a transcript. Redundant k -mers are subsequently skipped, and final identification of the transcript is determined by the combination of compatibility classes that represent the read's sequence.

Quantitative analysis of the aligned reads, using the *DESeq2* package for *R* (Love, Huber and Anders, 2014), results in normalised counts for all identified transcripts in each sample. The mean value for each transcript, in each condition, is then divided by the mean value present within the corresponding control sample. In the case of this study normalised counts of transcripts obtained from DEX treatments are compared to those of mock treatments. A $\log_2$ -transformed fold change value and a standard error is obtained for each ratio, and a statistical test is applied to determine if the gene is significantly differentially expressed. A false discovery rate (FDR) of 0.05 was applied to the results, and those with an adjusted p -value < 0.05 were considered for downstream analysis.

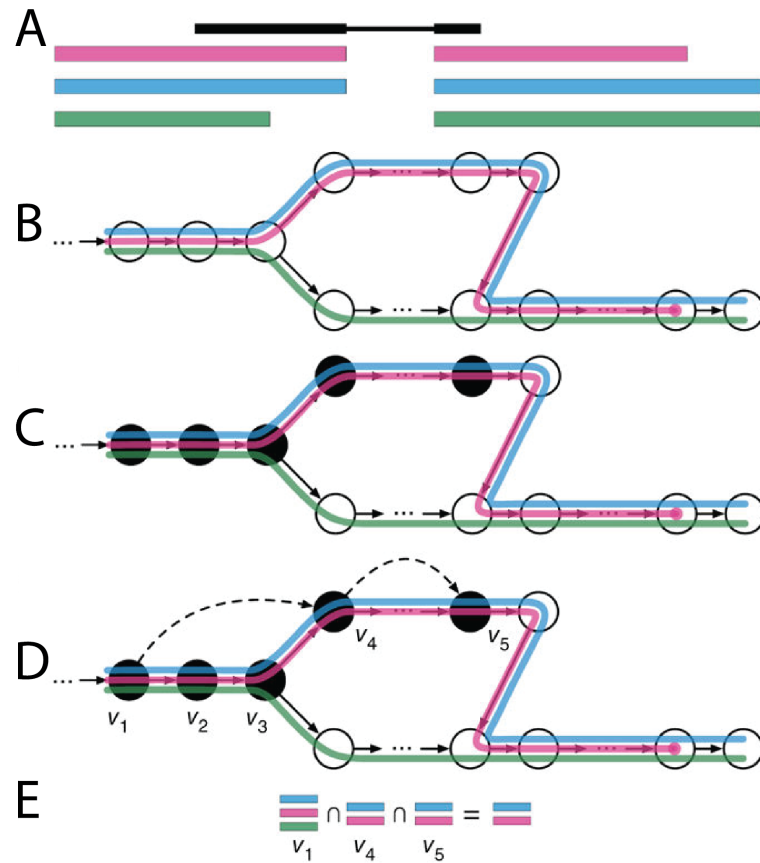


Figure 4.1 – Overview of RNA-seq data processing by the *Kallisto* software. The input consists of a reference transcriptome and reads from an RNA-seq experiment. (A) Three transcripts with exon regions that are overlapping (coloured lines), and the sequenced transcript (black) (B) A transcriptome de Bruijn Graph (T-DBG) is constructed, where each clear circle represents a k -mer node. Identical k -mers for each transcript are placed in the same compatibility class. (C) Each k -mer sequence is converted to a hash (black circles) in order to find the overall compatibility class of each read. (D) Redundant k -mers, those that belong to the same compatibility class, are skipped. That is, the k -mers present at nodes V_1 - V_3 are assigned to all of the transcripts, so there is no requirement to check each one. (E) The k -compatibility class of the read is determined by taking the intersection of the k -compatibility classes of its constituent k -mers. Figure and legend were adapted from Bray *et al.* (2016).

4.1.3. Chapter aims

As described above, while the developmental processes regulated by the floral homeotic genes at different stages of flower development have been well studied, only limited information is available about their stage-specific targets, and this chapter aims to gain more of an insight into the dynamic regulatory roles of *AP3* and *AG* as the flower develops. To achieve this, an analysis was performed using RNA-seq data from a series of stage-specific knockdowns in developing flowers, with *AP3* or *AG* as the targets. To investigate whether the data reflect the results of similar studies, comparisons are made against genes identified through genome-wide binding studies, or through differential expression analysis, to examine the overlap between datasets. Analysis of the GO terms significantly associated with the differentially expressed genes is also performed, in order to see if terms related to flower development are present. The results of these probes should provide a general consensus as to whether the data is suitable for further analysis.

4.2. Results

4.2.1. *Experimental set-up for stage-specific perturbation experiments*

Two DHT-responsive floral induction system lines, harbouring artificial microRNAs that target either *AG* or *AP3* mRNA (previously described in Chapter 3), were selected for use in stage-specific gene perturbation experiments, as it had been demonstrated that satisfactory independent induction of both synchronous flowering, amiRNA induction, and target knockdown, could be achieved. To examine the transcriptomic consequences of *AG/AP3* knockdown at different stages in flower development, the undifferentiated inflorescences-like meristems of FIS-AR AGamiR^{DEX}, or FIS-AR AP3amiR^{DEX}, plants were treated with 500 μ M solutions of DHT to induce flower development. Three knockdown time-points were selected: 3 days, 5 days, and 8 days after DHT treatment. DEX (or mock) solutions were applied to the developing buds 24 hours before the time-points for sample collection (2 days, 4 days, and 7 days, respectively) (**Fig. 4.2**). Upon reaching the designated time-point, the developing tissue was scraped from the inflorescences, in order to obtain samples enriched for the tissues under investigation, and used for total RNA extraction. Two biological replicates were used per line, and for each time-point.

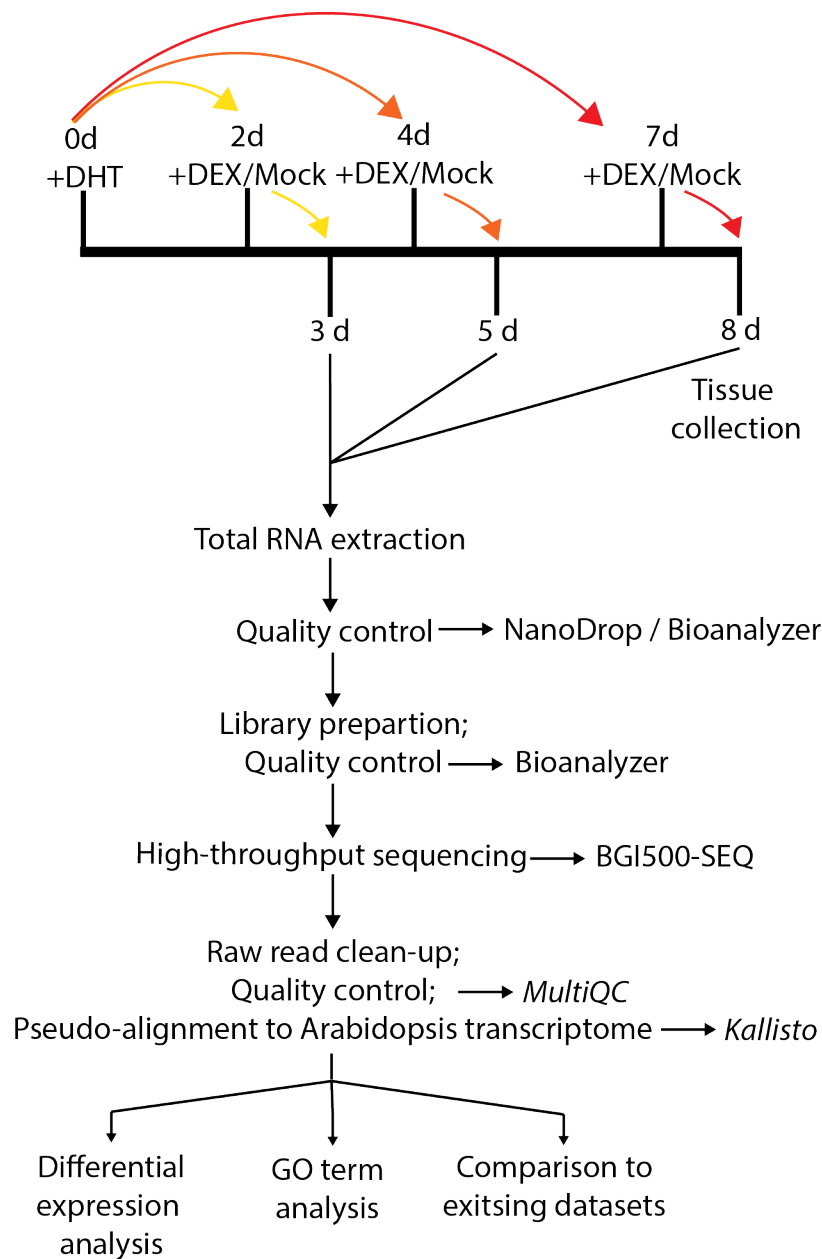


Figure 4.2 – Simplified experimental workflow for gene perturbation experiments and data analysis. Inflorescences of FIS-AR AGamiR^{DEX}/ FIS-AR AP3amiR^{DEX} plants are treated with 500 μ M DHT, and subsequently treated for 24 hours with 10 μ M DEX before tissue collection at the given time-points. After total RNA extraction and quality control, cDNA library preparation and high-throughput sequencing is performed. Raw reads are processed to remove adaptor sequences and reads of poor quality, and pseudo-alignment of the reads is performed on the *Arabidopsis* transcriptome. Downstream analyses are performed using the normalised counts of all genes present from the pseudo-alignment.

4.2.2. Preliminary validation of target knockdowns

The plant lines used in these experiments have been previously shown to respond to DEX treatment in a predictable manner, such that amiRNA induction occurs to a sufficient level after a single treatment with 10 μ M DEX (Discussed in Chapter 3). To verify that the DEX treatments performed on the collected tissue samples responded as intended, and conversely the mock treatments did not result in any unintended downregulation of the target mRNAs, RT-qPCR was performed on cDNA that had been synthesised from total RNA, purified from the collected tissue. The relative levels of *AG* and *AP3* mRNA were determined across all time-points and conditions, showing that both lines exhibited high levels of target knockdown (**Fig. 4.3**).

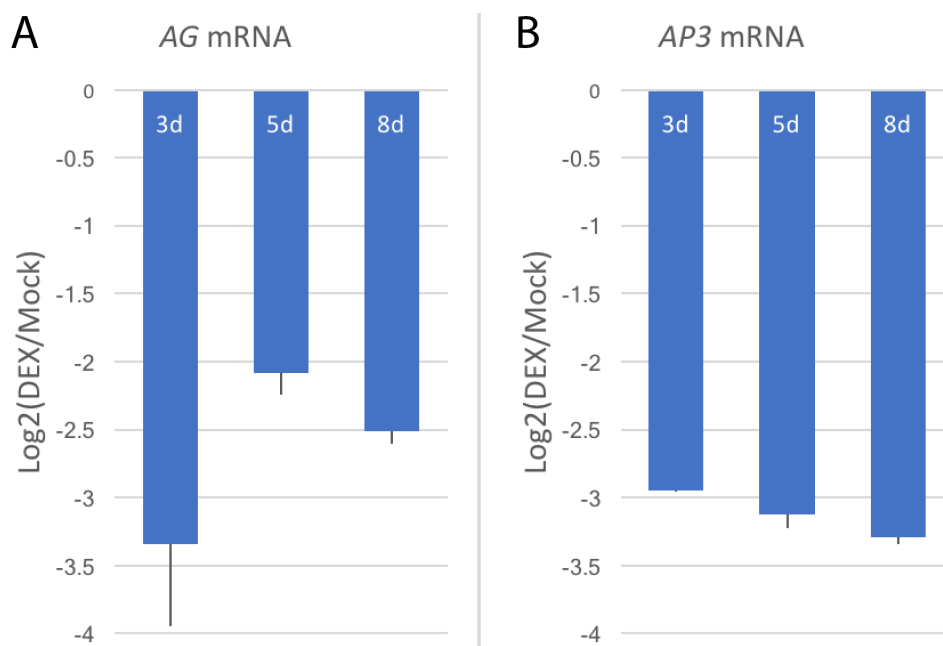


Figure 4.3 – Inducible knockdown of *AG* and *AP3* at different stages of flower development. Results of RT-qPCR experiments to determine the relative levels of mRNA of *AG* (A), or *AP3* (B), in FIS-AR *AG*amiR^{DEX} and FIS-AR *AP3*amiR^{DEX} plants, respectively, at different time-points after activation of flower development (as indicated). Results are relative to the reference gene (REF1), and displayed as log₂-

transformed fold changes (DEX/mock) values. Two biological and two technical replicates were used for each time-point. Error bars represent standard deviations.

4.2.3. *Quality assessment of total RNA for use in RNA-seq experiments*

Total RNA purified from FIS-AR AGamiR^{DEX} / FIS-AR AP3amiR^{DEX} plants was subjected to spectrophotometric and electrophoretic quality control measures before being used for next-generation sequencing library construction. Samples were first analysed on a Nanodrop1000 spectrophotometer to assess RNA purity, and an Agilent Bioanalyzer 2100 to assess RNA integrity. A value of >1.8 was required for the OD_{260/230} and OD_{260/280} readings obtained using the spectrophotometer to indicate RNA purity, and a value of >6.5 was required for the RNA Integrity Number (RIN) readings obtained with the Bioanalyzer. All samples had OD_{260/230}/OD_{260/280} values between 1.98 and 2.39, and RIN values between 8.4 and 9.6, indicating the RNA was pure and of sufficiently high quality for successful library construction.

4.2.4. *Quality assessment of the RNA-seq data*

The libraries generated from extracted total RNA of FIS-AR AGamiR^{DEX} / FIS-AR AP3amiR^{DEX} plants were subjected to next-generation sequencing on a BGI500-SEQ high-throughput sequencing machine. The average read length of the libraries was 50bp and all libraries were subject to single-end sequencing. All libraries returned approximately 23.72 million clean reads (adaptor sequences and low quality reads removed), estimated to provide coverage of approximately 19,000 genes with more than 10 reads (Lei *et al.*, 2015), and 2 biological replicates were sequenced for each experiment. The resulting reads were first analysed using the *FastQC* software (www.bioinformatics.bbsrc.ac.uk) to determine the overall sequencing quality of the

libraries and to investigate whether any issues were apparent with the sequencing as a consequence of the library construction process. All libraries had a sequence quality score of above Phred 30 at any given base (**Fig. 4.4**). This is determined using the base-calling program, *Phred*, which estimates the probability of a base call being incorrect (Ewing *et al.*, 2005). The quality score (q) is defined as the $\log_{10}$ -transformed error probability of an incorrect base-call, where p is the estimated error probability for the given base:

$$q = -10 \times \log_{10}(p)$$

Therefore, a quality score of Phred 30 indicates that the average probability of a base-call error is 1/1000.

The amount of duplicated sequences for the majority of the libraries resulted in a failure warning (orange and red lines) from *FastQC* (**Fig. 4.4 B**). The graph shows that the majority of sequences for the libraries have low duplication levels of 2-9 copies, and peaks for moderately duplicated sequences (10-100 copies) are present for all samples, representing up to 20% of the library in some instances. However, the software makes the assumption that any NGS library being analysed is expected to be diverse to the point where the frequencies of all represented sequences are approximately even, and therefore returns ‘fail’ results when this is not the case. This could be partially caused by duplicate sequences present in the *Arabidopsis* genome, leading to an over-representation of moderately duplicated sequences. A lack of enrichment of highly duplicated sequences beyond the 10-100 copies level suggests that the RNA-seq library construction was not subject to contamination or PCR duplication of unwanted sequences.

Finally, a bias for particular bases is present in the reads for all of the libraries around the first 11 bases of each read. Considering that the fragment generation for the library is

meant to be random, it would be expected that the frequency of each base would be reflective of the frequencies present in the *Arabidopsis* genome. However, as the libraries were produced using random hexamer primers, whose priming efficiencies are not all equal, the result is an enrichment of particular bases at the starts of the reads, though in practice this has not caused downstream analysis issues concerning quantitation of the RNA populations as the bias is not frequent enough (Hansen, Brenner and Dudoit, 2010). Overall, the quality control assessment of the RNA-seq reads is indicative of high quality sequences suitable for downstream differential gene expression analysis.

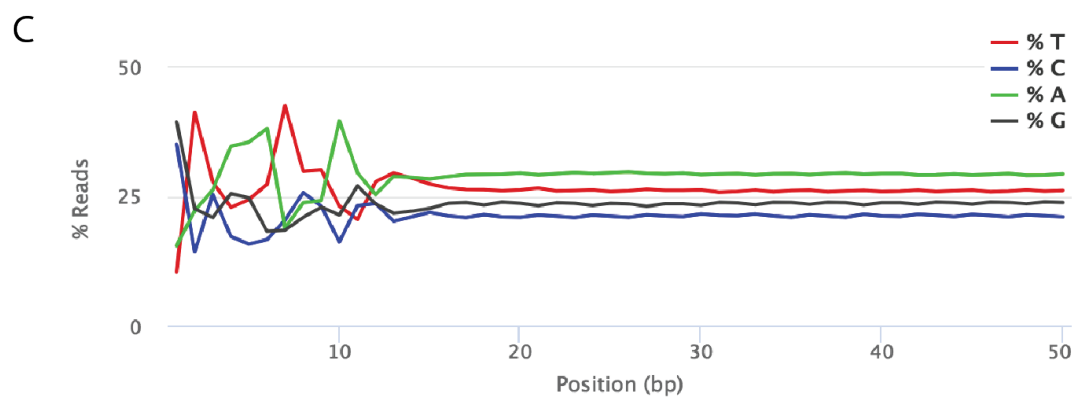
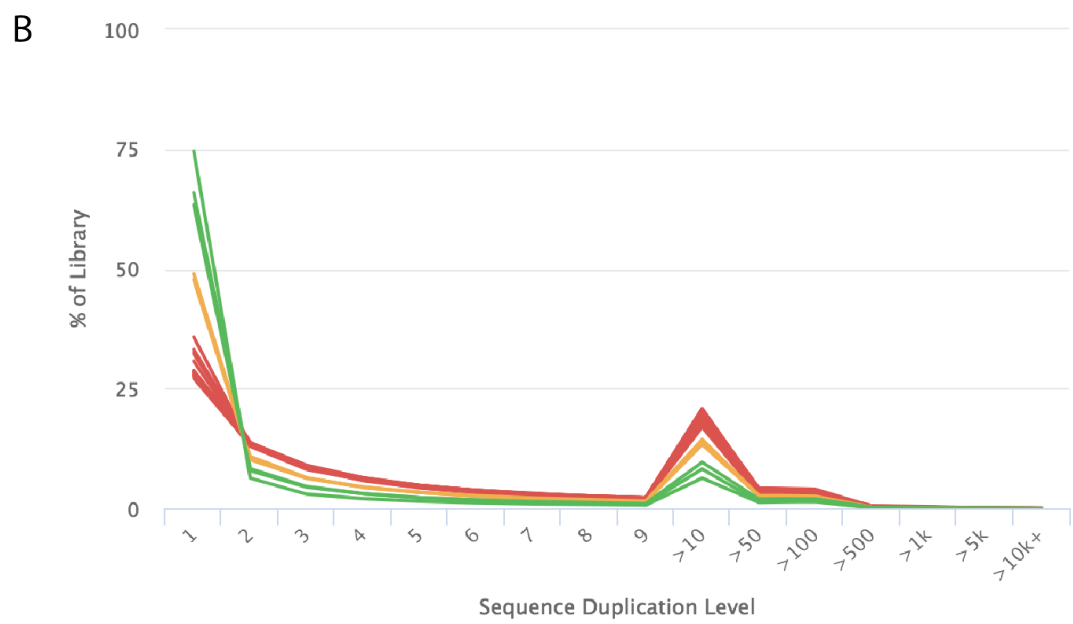
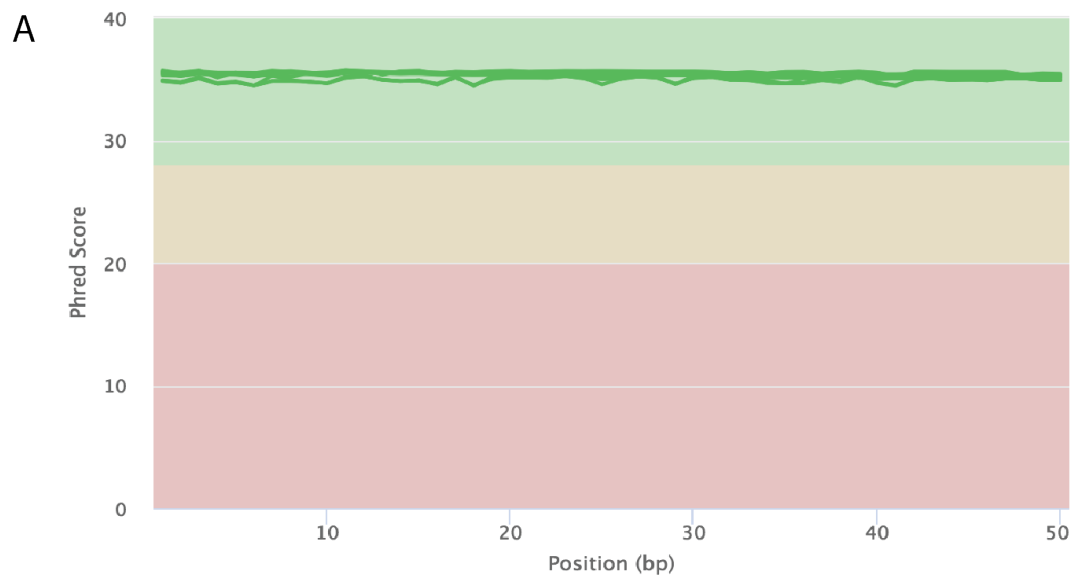


Figure 4.4 – Results of RNA-seq quality controls. (A) Phred quality scores (green lines) for read sequencing at each given position within for all RNA-seq experiments. All scores within the green margins are considered to be of high quality. The average quality of all reads is high for the entire length of the read (x -axis). (B) Duplication levels of the reads present in the RNA-seq data from 24 libraries. All of the sequence data contains a significant number of multiple reads, especially at the >10 level. (C) Per base sequence content of sequence reads from a representative library. All sequenced libraries possess a similar level of base content bias from positions 1-11. These nucleotides were trimmed before alignment to the *Arabidopsis* transcriptome. All graphs were generated using the *MultiQC* software (Ewels *et al.*, 2016).

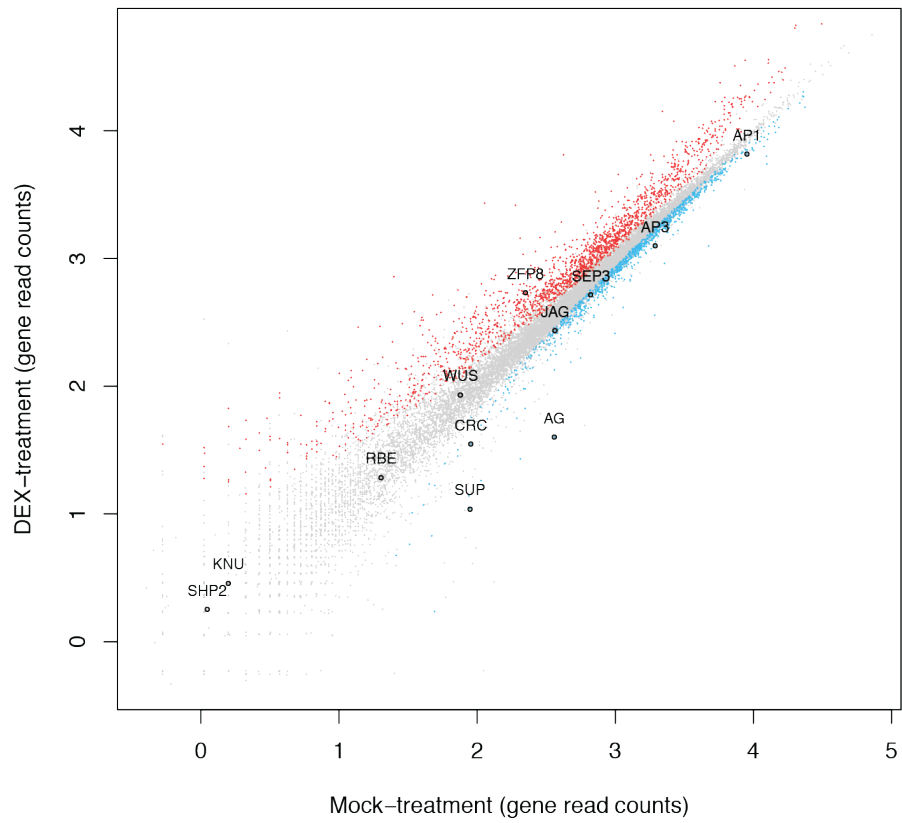
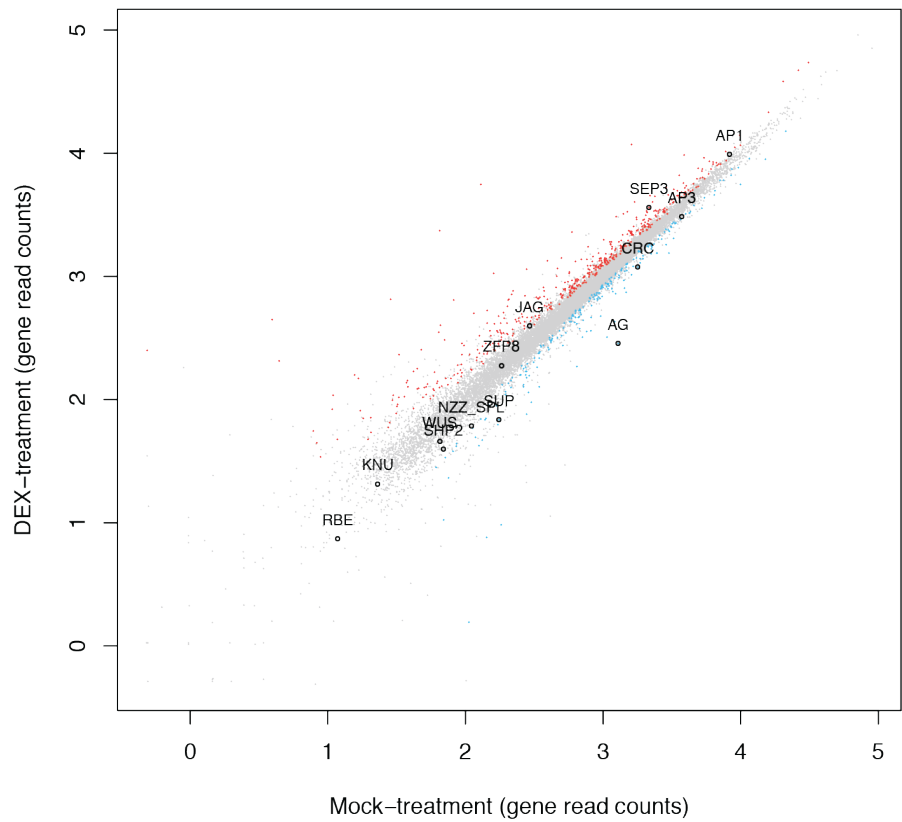
4.2.5. Identification of differentially expressed genes

Pseudo-alignment of the raw reads to the *Arabidopsis* transcriptome returned expression values for 23,004 genes for all samples, whereas the total number of coding genes in the *Arabidopsis* genome is currently estimated to be between 27,411 (TAIR10) and 27,655 (Araport11) (Swarbreck *et al.*, 2008; Lamesch *et al.*, 2012; Cheng *et al.*, 2017). To identify differentially expressed genes present within each time-point examined, the $\log_2$ -fold change was calculated for each identified gene using the *DESeq2* package in *R* (Love, Huber and Anders, 2014) (analysis performed with the help of Dr. Darren Fitzpatrick, Bioinformatics Core Unit, Trinity College Dublin). The average mean expression of each time-point was divided by the average value obtained from its respective mock treatment. Genes that had been identified as upregulated or downregulated were then further selected by applying an FDR cut-off of < 0.05 , and those which did not satisfy this criterion were judged to be not differentially expressed. Table 4.1 lists the number of differentially expressed genes, for each time-point, identified using this method, and **Figures 4.5 & 4.6** show the gene read counts for all tested genes.

Table 4.1 – Differentially expressed genes identified in FIS-AR AGamiR^{DEX} (AG) and FIS-AR AP3amiR^{DEX} (AP3) knockdown experiments. The number of up and downregulated genes is shown for each time-point.

Time-point	AG – Downregulated	AG – Upregulated
3d	995	1762
5d	221	465
8d	197	211
	AP3 – Downregulated	AP3 - Upregulated
3d	29	157
5d	65	143
8d	157	145

To begin to validate whether the differentially expressed genes identified across the *AG* and *AP3* knockdown experiments are consistent with previous findings, a few different methods were used. First, manual searches of the datasets for differentially expressed candidate genes, already known to be regulated by either of the transcription factors, were performed to initially corroborate that the knockdowns had the intended effects. Second, GO term analysis of the DEGs was used to investigate the functional terms associated with significantly over-represented genes, and also to begin to provide some possible explanations for the occurrence of any hitherto unexpected DEGs. Third, comparisons to existing datasets, studying aspects of flower development related to AP3 and AG, were made to determine the amount of overlap between the results. The combination of these examinations should provide an overall indication as to how much these data agree with previously published studies.

A**AGamiRNA – 3d****B****AGamiRNA – 5d**

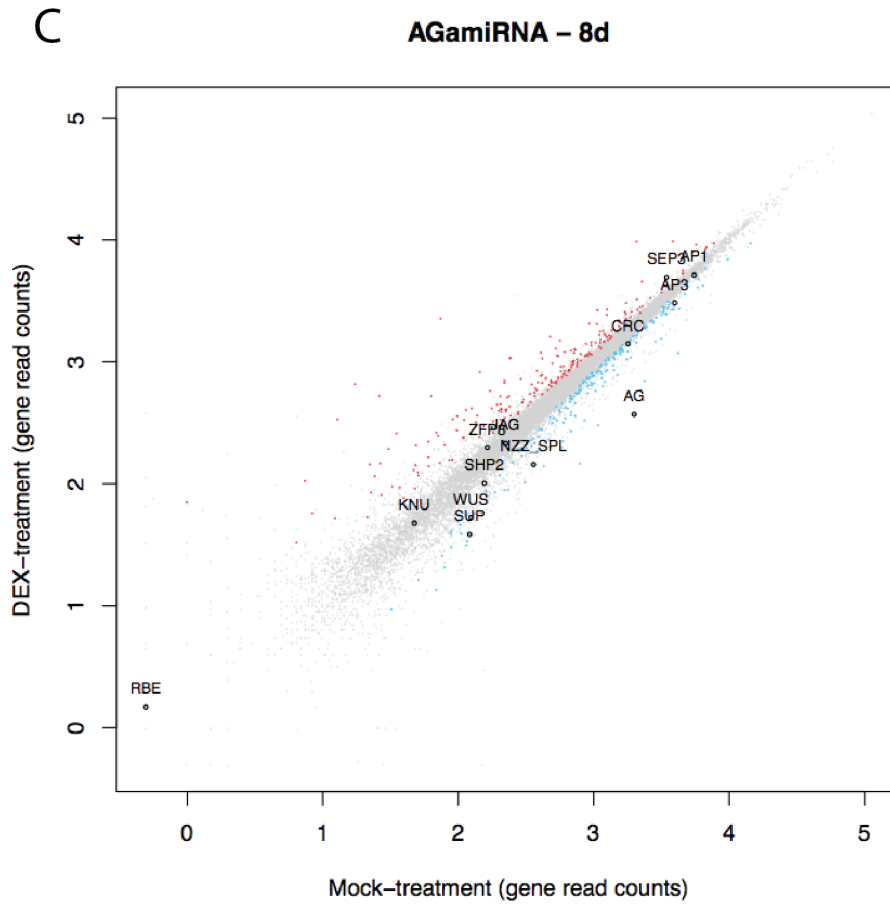
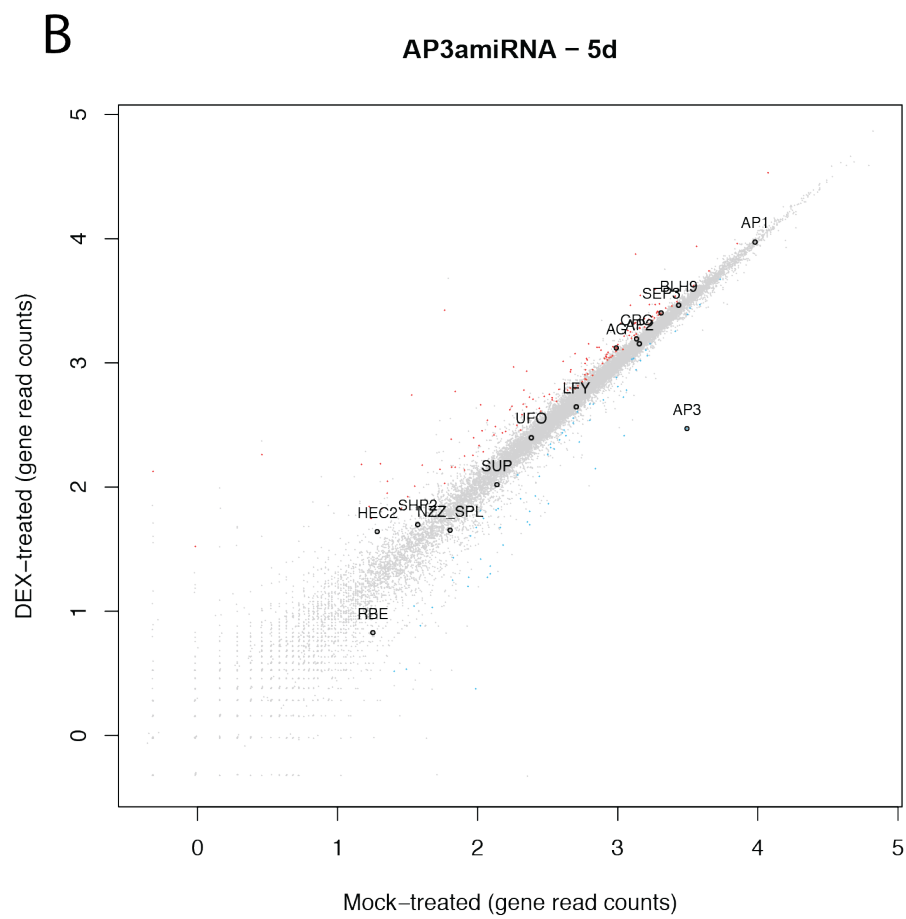
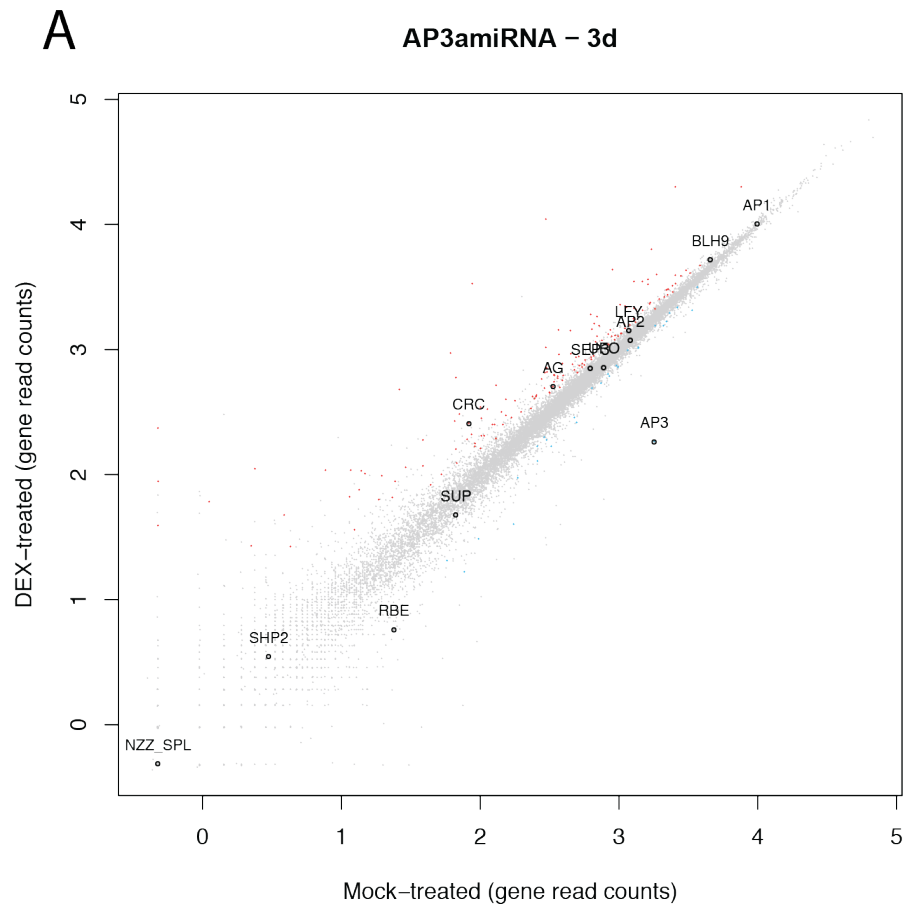


Figure 4.5 – Differentially expressed genes identified in *AG* knockdown experiments. Log₁₀ scatter plots of normalised transcript abundances, from flowers collected 24 hours after AGamiR^{DEX} induction, at 3 (**A**), 5 (**B**), and 8 (**C**) days. Red dots represent significantly upregulated genes, blue dots represent significantly downregulated genes. Grey dots represent genes that are not differentially expressed. Selected genes, known to be regulated directly or indirectly by AG are labelled.



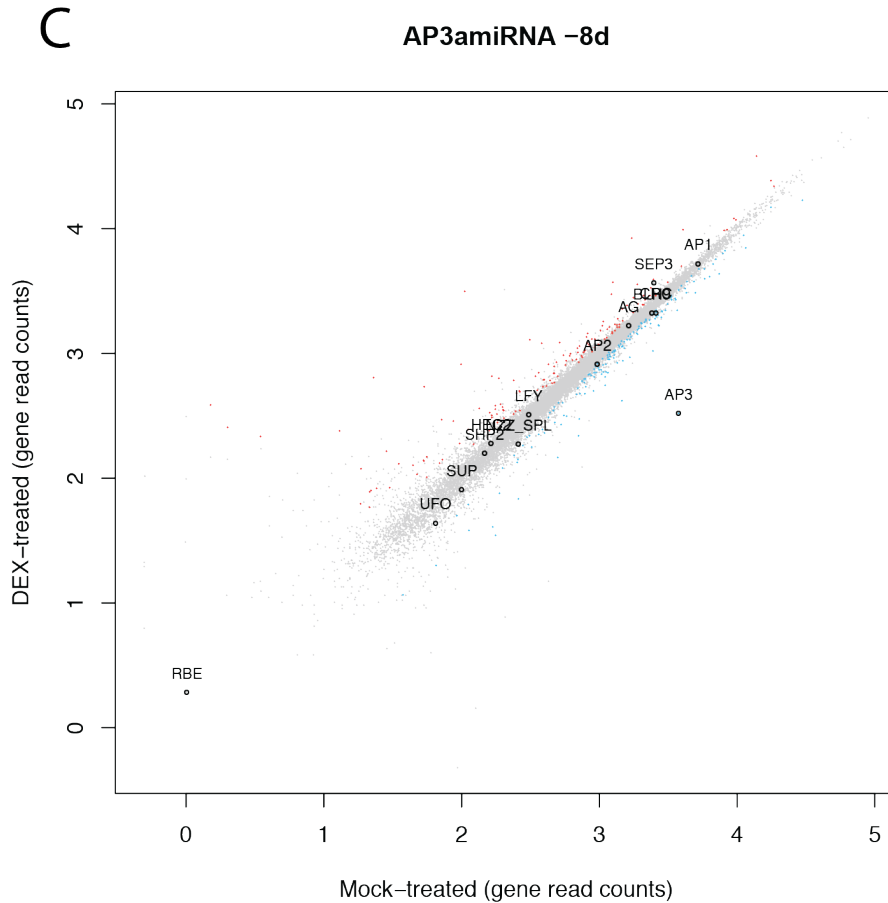


Figure 4.6 – Differentially expressed genes identified in the *AP3* knockdown experiments. Log₁₀ scatter plots of normalised transcript abundances, from flowers collected 24 hours after AP3amiR^{DEX} induction, at 3 (A), 5 (B), and 8 (C) days. Red dots represent significantly upregulated genes, blue dots represent significantly downregulated genes. Grey dots represent genes that are not differentially expressed. Selected genes, known to be regulated directly or indirectly by AP3 are labelled.

4.2.6. Differentially expressed genes identified in the perturbation experiments

Across all knockdown samples, the number of downregulated and upregulated genes totals 1348 and 2248 for *AG*, and 241 and 318 for *AP3*. Initial screening of the lists of DEGs for genes known to be involved in flower development returned numerous hits. For the *AG* knockdowns, multiple regulators of floral organ identity and morphogenesis, such as *CRABS CLAW* (*CRC*) (Bowman and Smyth, 1999), *SEPALLATA1 & 2* (*SEP1 & SEP2*) (Pelaz *et al.*, 2000), *SUPERMAN* (*SUP*) (Sakai, Medrano and Meyerowitz, 1995), *APETALA1 & 2 & 3* (*AP1, AP2, and AP3*) (Bowman, Smyth and Meyerowitz, 1989; Bowman *et al.*, 1993), *KANADI* (*KAN*) (Kerstetter *et al.*, 2001), *SHATTERPROOF1* (*SHP1*) (Ma, Yanofsky and Meyerowitz, 1991), and *NUBBIN* (*NUB*) (Dinneny, Weigel and Yanofsky, 2006), were all observed to be downregulated in at least one time-point. Furthermore, genes known to be involved in microsporogenesis were downregulated, such as *SPL8* (Unte *et al.*, 2003) and *NOZZLE/SPOROCTELESS* (*NZZ/SPL*) (Yang *et al.*, 1999).

Upregulated genes involved in flower development included *SEP3 & 4*, *ROXY1* (Xing, 2005) and *ASYMMETRIC LEAVES 2* (*AS2*) (Xu *et al.*, 2008). Tables 4.2 and 4.3 summarise previous evidences for *AG*-dependent regulation of these genes, obtained through ChIP-Seq against *AG* or *SEP3* (O'Maoileidigh *et al.*, 2013; Pajoro *et al.*, 2014), and microarray analysis of genes that are differentially expressed in response to *AG* activation (in an *apl-1 cal-1* background) through its fusion to the GR ligand binding domain (Gómez-Mena *et al.*, 2005).

Floral regulators downregulated in at least one of the *AP3* knockdown time-points included stamen development genes such as *SPL8* (Unte *et al.*, 2003) and *AtSWEET13*

(Kanno *et al.*, 2016), and genes involved in integument and ovule formation like *ABERRANT TESTA SHAPE (ATS)* (McAbee *et al.*, 2006) and *TRANSPARENT TESTA GLABRA2 (TTG2)* (Johnson, 2002). Upregulated genes included *AG*, *CRC*, and *NUB* (summarised in Table 4.4), the latter two upregulated in response to AG activation as well (Gomez-Mena, 2005). Overall, the presence of numerous differentially expressed genes, already known to regulate flower development, indicates at least in part, that the knockdowns of *AG* and *AP3* resulted in changes to the expression levels of genes involved in flower development.

Table 4.2 – Summary of evidence for AG knockdown-dependent regulation for downregulated genes identified in this study.

		Differentially expressed?			Binding evidence? (ChIP-Seq)		Differential expression evidence?
Alias	AGI	3d	5d	8d	AG - O'Maoileidigh <i>et al.</i> (2013)	SEP3 - Pajoro <i>et al.</i> (2014)	Gomez-Mena <i>et al.</i> (2005)
<i>CRC</i>	AT1G69180	Yes	Yes	No	Yes	Yes	Yes
<i>SEP1</i>	AT5G15800	Yes	No	No	No	Yes	Yes
<i>SEP2</i>	AT3G02310	Yes	No	No	Yes	Yes	Yes
<i>SUP</i>	AT3G23130	Yes	Yes	Yes	Yes	Yes	Yes
<i>API</i>	AT1G69120	Yes	No	No	Yes	Yes	No
<i>AP2</i>	AT4G36920	Yes	No	No	Yes	Yes	No
<i>AP3</i>	AT3G54340	Yes	No	No	Yes	Yes	Yes
<i>KAN</i>	AT5G16560	Yes	No	No	No	Yes	No
<i>SHPI</i>	AT3G58780	No	Yes	No	Yes	Yes	Yes
<i>NUB</i>	AT1G13400	No	Yes	No	No	Yes	Yes
<i>NZZ/SPL</i>	AT4G27330	No	No	Yes	Yes	Yes	No
<i>SPL8</i>	AT1G02065	No	No	Yes	Yes	Yes	No

Cells are colour-coded to aid visualisation. Yellow cells represent negative results

Table 4.3 - Summary of evidence for AG knockdown-dependent regulation for upregulated genes identified in this study.

		Differentially expressed?			Binding evidence? (ChIP-Seq)		Differential expression evidence?
Alias	AGI	3d	5d	8d	AG - O'Maoileidigh et al. (2013)	SEP3 - Pajoro et al. (2014)	Gomez-Mena et al. (2005)
<i>ROXY1</i>	AT3G02000	No	No	Yes	Yes	Yes	No
<i>SEP3</i>	AT1G24260	No	Yes	No	Yes	Yes	Yes*
<i>SEP4</i>	AT2G03710	No	Yes	No	No	Yes	No
<i>AS2</i>	AT1G65620	No	Yes	No	No	Yes	No

Cells are colour-coded to aid visualisation. Yellow cells represent negative results

*Gene identified as differentially expressed, but in the opposite manner

Table 4.4 – Summary of evidence for AP3 knockdown-dependent downregulated and upregulated genes identified in this study.

		Differentially expressed?			Binding evidence? (ChIP-Seq)	Differential expression evidence?
Alias	AGI	3d	5d	8d	Wuest <i>et al.</i> (2012)	

Downregulated

<i>SPL8</i>	AT1G02065	No	No	Yes	Yes	No
<i>ATSWEET13</i>	AT5G50800	No	No	Yes	No	No
<i>ATS</i>	AT5G42630	No	No	Yes	Yes	No
<i>TTG2</i>	AT2G37260	No	No	Yes	Yes	No

Upregulated

<i>AG</i>	AT4G18960	Yes	Yes	No	Yes	No
<i>CRC</i>	AT1G69180	Yes	No	No	Yes	Yes
<i>NUB</i>	AT1G13400	Yes	No	No	No	Yes
<i>GA3OX1</i>	AT1G15550	Yes	Yes	No	No	No

Cells are colour-coded to aid visualisation. Yellow cells represent negative results

4.2.7. GO terms associated with down-regulated genes

To gain some insight into the biological terms associated with the DEGs identified in both knockdowns, lists were assembled that contained all downregulated or upregulated genes across each of the time-points (i.e., all DEGs present within the 3d, 5d, and 8d, time-points of each knockdown series were identified and grouped as downregulated or upregulated). The *AgriGO* software (Du *et al.*, 2010; Tian *et al.*, 2017) was used to

perform Gene Ontology (GO) analyses, identifying functional terms significantly over-represented in the queried gene lists (FDR-adjusted $p < 0.05$). Terms related to flower development, reproductive development, organ morphogenesis/development, and meristem-related processes were found to be enriched, compared to the number of terms expected to arise by chance, in the downregulated genes of both knockdown series (**Fig. 4.7**).

The most highly enriched GO terms returned for the *AG* gene set also contained terms for genes related to control of the cell cycle, whereas this was not the case for the *AP3* gene set, agreeing with the findings of O'Maoileidigh *et al.* (2013) that *AG* activity has some influence over this process independently of the B function genes (**Fig. 4.8 A**). The most highly enriched terms for the *AP3* gene set included processes related to anthocyanin biosynthesis, flavonoid biosynthesis, and cell fate specification (**Fig. 4.8 B**). Tables containing enriched GO terms for each time-point are available in the Appendix.

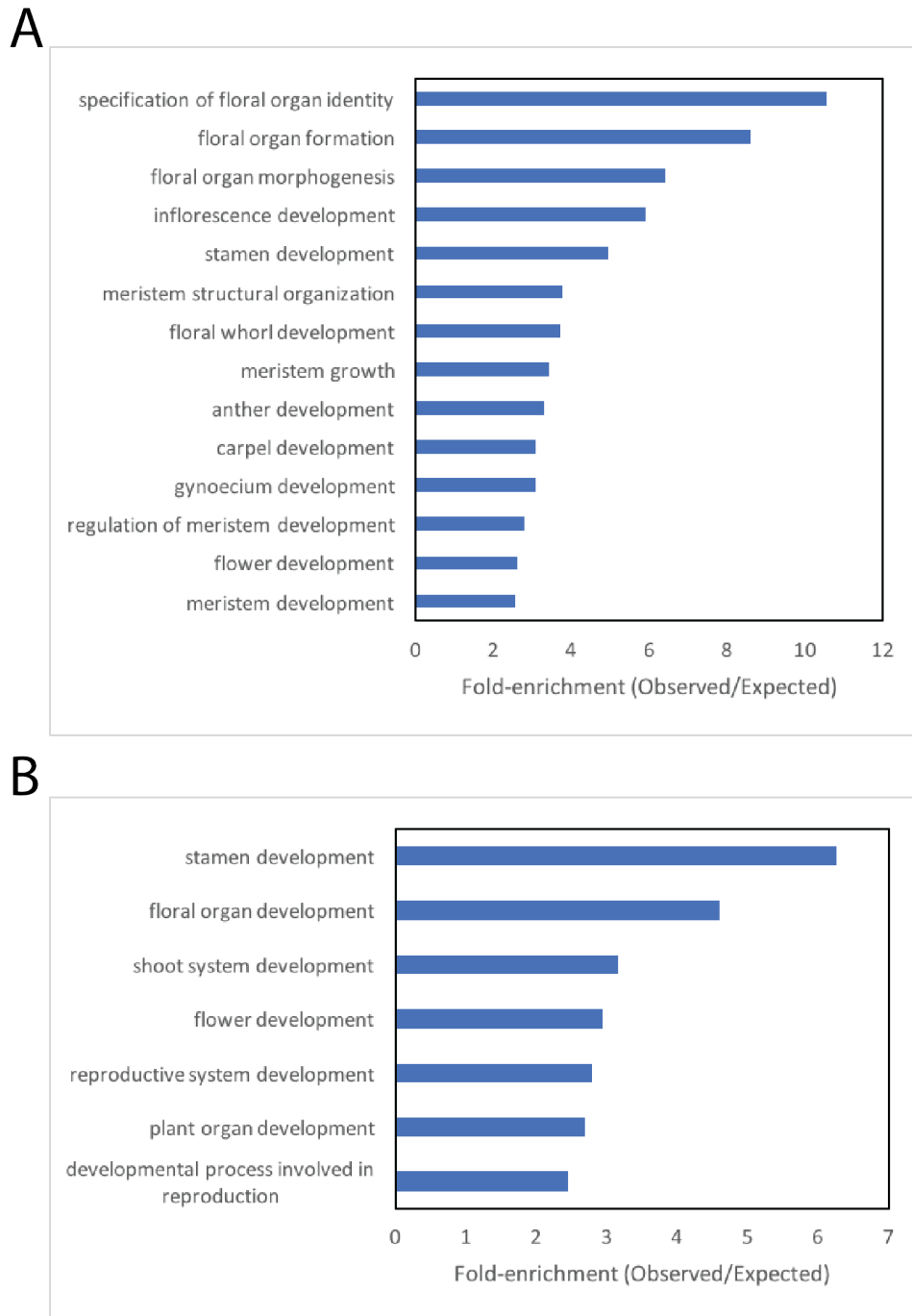


Figure 4.7 – Enriched GO terms related to flower development among the differentially expressed genes of the *AG* and *AP3* knockdown experiments. Results of significantly over-represented GO terms associated with reproductive development in downregulated genes of the combined *AG* (A) and *AP3* (B) knockdown samples. Results are displayed as fold-enrichment over the number of genes expected to arise by chance. Significant GO terms were determined using the Fisher’s exact test. FDR adjusted *p*-value cut-off: 0.05.

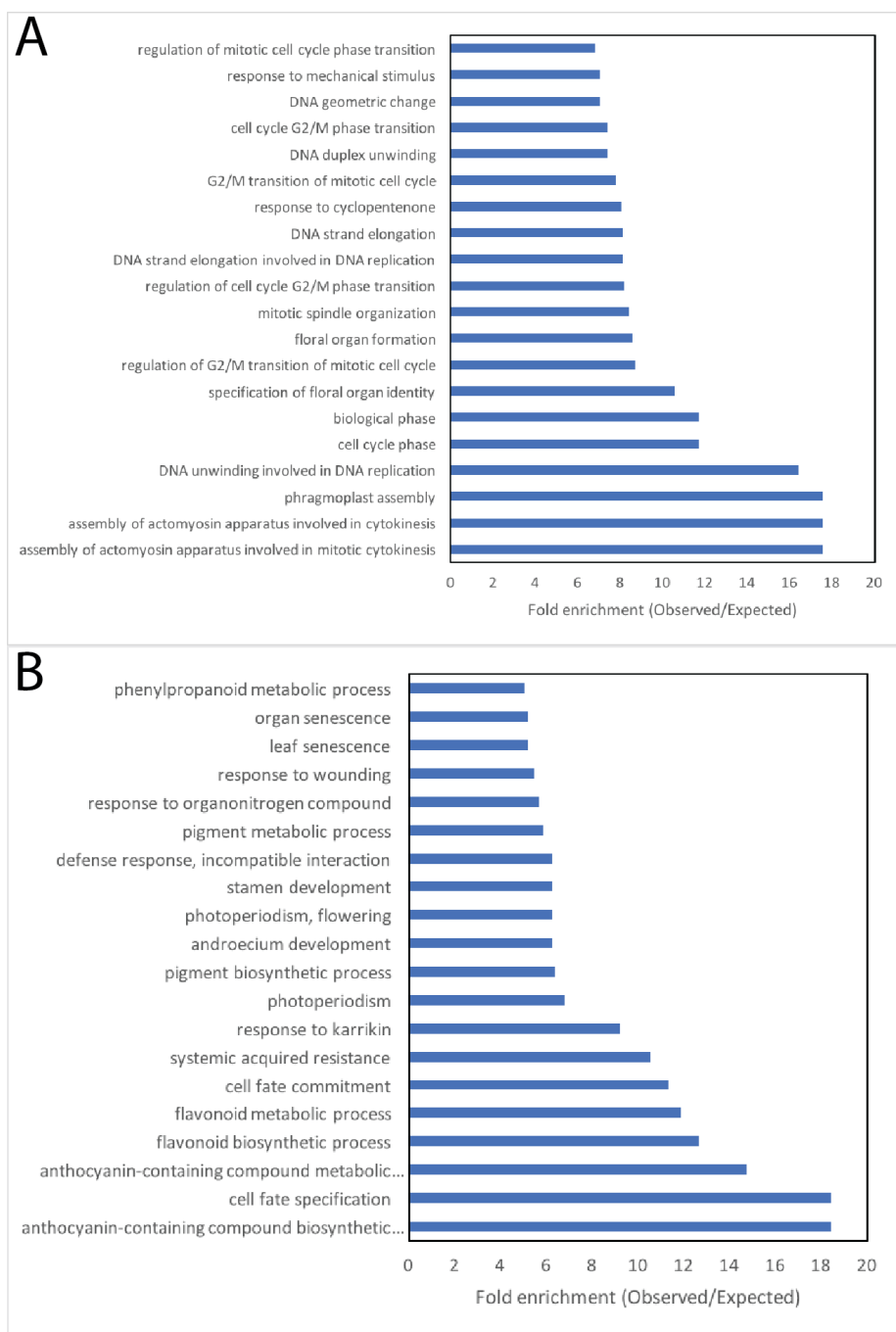


Figure 4.8 – Enriched GO terms associated with downregulated genes of the *AG* and *AP3* knockdown experiments. Results of significantly over-represented GO terms associated with downregulated genes of the combined *AG* (**A**) and *AP3* (**B**) knockdown samples. Results are displayed as fold-enrichment over the number of genes expected to arise by chance. Significant GO terms were determined using the Fisher's exact test. FDR adjusted *p*-value cut-off: 0.05. The 20 most enriched terms are shown.

4.2.8. *GO terms associated with upregulated genes*

In the same manner as above, enriched GO terms were identified in the upregulated genes of both gene sets. The most enriched terms returned for upregulated genes in the *AG* knockdown series were predominantly related to chloroplast and photosynthetic properties (**Fig. 4.9 A**), which is in agreement with the results obtained from microarray experiments using *ag-1*, suggesting that AG activity serves to repress genes involved in the leaf development program (O'Maoileidigh *et al.*, 2013). Terms related to organic compound biosynthesis and gibberellic acid synthesis were prevalent in the *AP3* gene set, along with genes related to sexual reproduction and meristem maintenance (**Fig. 4.9 B**). The significance of the majority of these terms is presently unclear, although the lack of similarity between the two knockdowns is in favour of the notion that the genes represented here are the result of a reduction in *AG* and *AP3* activity, respectively, and not necessarily caused by some common factor related to the use of the transgenic systems and hormone treatments used to obtain the tissue samples. Additionally, the presence of terms related to Gibberellic Acid (GA) biosynthesis could be an indirect response to the upregulation of *AG*. For instance, transcription of *GIBBERELLIN 3-OXIDASE-1* (*GA3OX1*) (log₂-transformed fold change value: 1.8) is known to be upregulated in response to AG activation (Gomez-Mena, 2005).

4.2.9. *Examination of co-expressed genes between the AG and AP3*

knockdowns

The number of identified differentially expressed genes between the time-points, for each line, varies in most cases. Examination of the overlap between differentially expressed genes, among the time-points for each condition, reveals that more genes are consistently upregulated, rather than downregulated (**Fig. 4.10**), and that the number of significantly

upregulated genes found in all FIS-AR AGamiR^{DEX}/AP3amiR^{DEX} samples is identical (**Fig. 4.10 A & C**). To investigate the functions of these genes, and whether they are related to flower development or some other process, gene lists were compiled for both datasets and compared. First, both gene sets were tested separately for significantly over-represented GO terms using the Fisher's exact test with an FDR cut-off of 0.05. Neither set returned terms related to flower development under these conditions. 31 Genes were present in both lists, whereas the expected number of genes to be present at random in both is <1. GO term analysis of the shared genes results in a significant over-representation of terms related to stimulus response and transporter activity (**Fig. 4.11**). Considering the GO terms, the high proportion of genes (64%) present in both lists, and consistent upregulation of the genes across all samples, it is likely that these genes have, at least in part, been differentially regulated in response to the DEX treatments.

The genes identified above were queried in two independent differential gene expression datasets, in which an amiRNA targeting *AP3* was driven by either the DEX-inducible LhG4-GR system or the ethanol-inducible ALCR/ALCA system (O'Maoileidigh *et al.*, 2015), in order to further determine whether they were differentially expressed as a result of some systemic property of the inducible system used. All 31 genes were significantly upregulated ($p < 0.05$) in the LhG4-GR dataset, whereas 16 were determined to be differentially expressed in the ALCR/ALCA dataset. Of these DEGs, 10 were upregulated.

These results indicate that the majority of significantly-upregulated genes shared between all of the *AG* and *AP3* knockdown experiments in this study are likely to be a consequence of the activation of the LhG4-GR transcription factor. To further confirm this it would be

necessary to examine the transcriptomes of *apl-1 cal-1* and *apl-1 cal-1* 35S::LhG4-GR plants, lacking a functional amiRNA, after treatment with DEX. If the same changes were not seen in the *apl-1 cal-1* plants, but were in *apl-1 cal-1* 35S::LhG4-GR, this would provide strong support for this hypothesis.

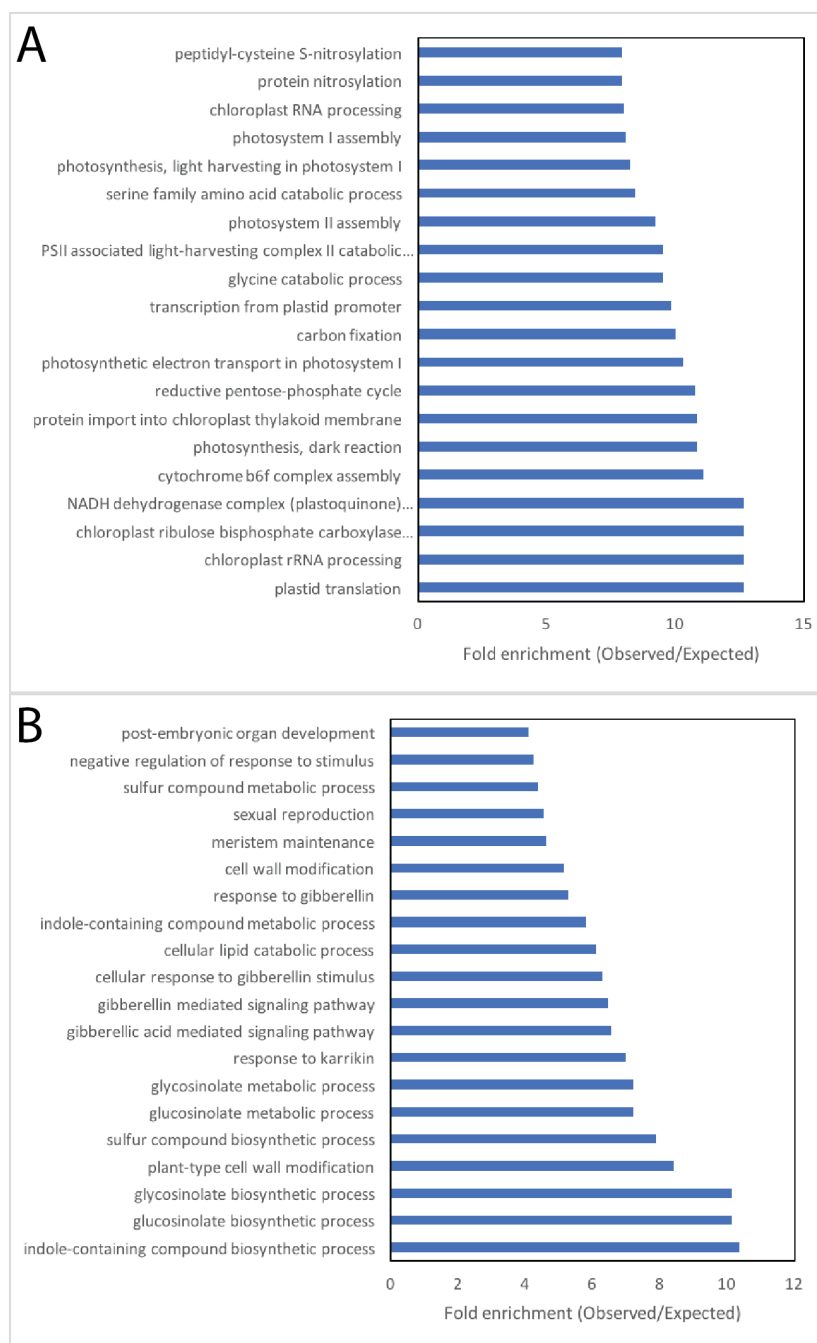


Figure 4.9 – Enriched GO terms associated with upregulated genes of the *AG* and *AP3* knockdown experiments. Results of significantly over-represented GO terms associated with upregulated genes of the combined *AG* (A) and *AP3* (B) knockdown samples. Results are displayed as fold-enrichment over the number of genes expected to arise by chance. Significant GO terms were determined using the Fisher's exact test. FDR adjusted *p*-value cut-off: 0.05. The twenty most enriched terms are displayed.

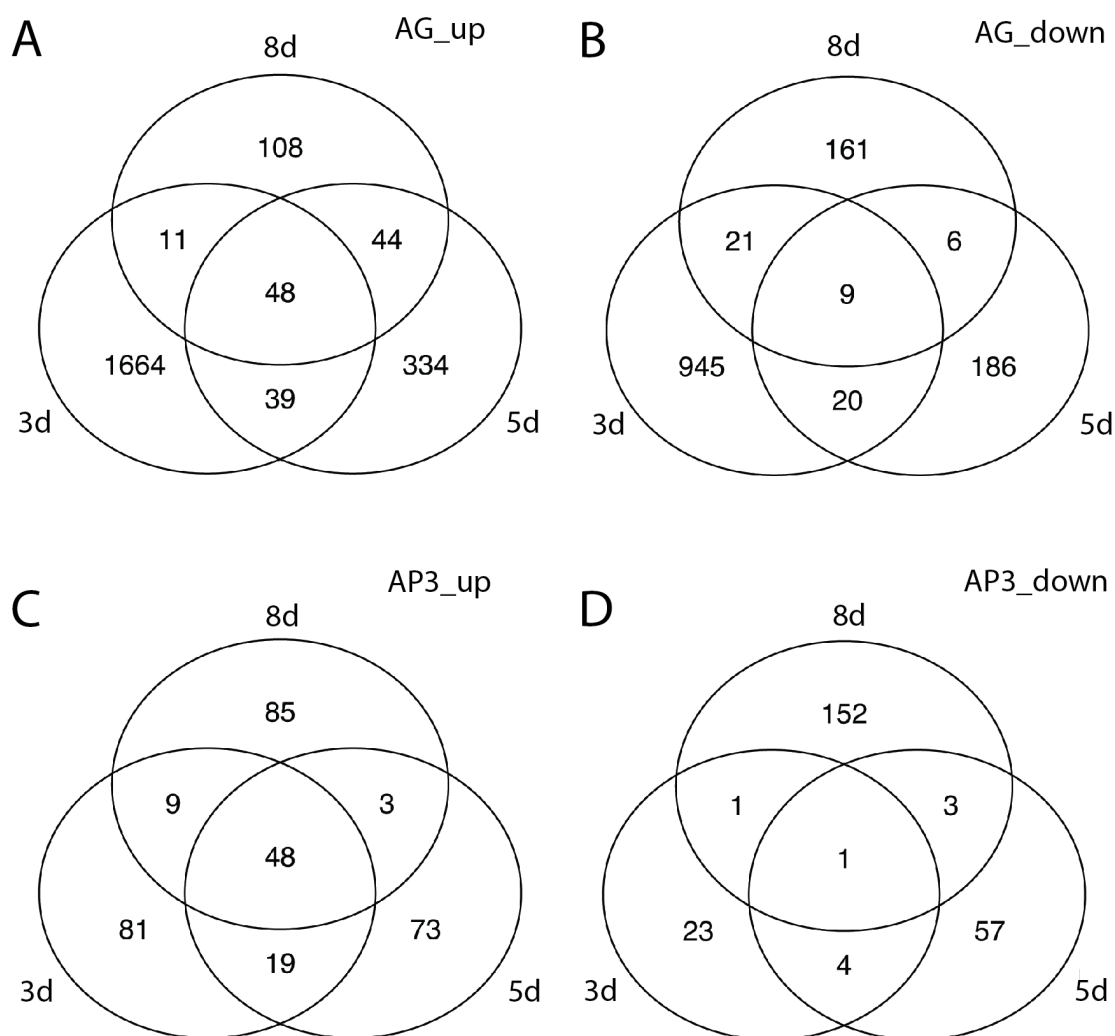


Figure 4.10 – Co-expressed genes present in the *AG* and *AP3* knockdown experiments. Overlap between differentially expressed genes in the FIS-AR AGamiR^{DEX} (**A & B**) and FIS-AR AP3amiR^{DEX} (**C & D**) time-course experiments, where amiRNA transcription was activated 24 hours before tissue collection. Panels **A** and **C** show the overlap between upregulated genes, and panels **B** and **D** show the overlap between downregulated genes. The time-points (3d; 5d; 8d) denote the amount of time that had passed since induction of flower formation with DHT prior to tissue collection.

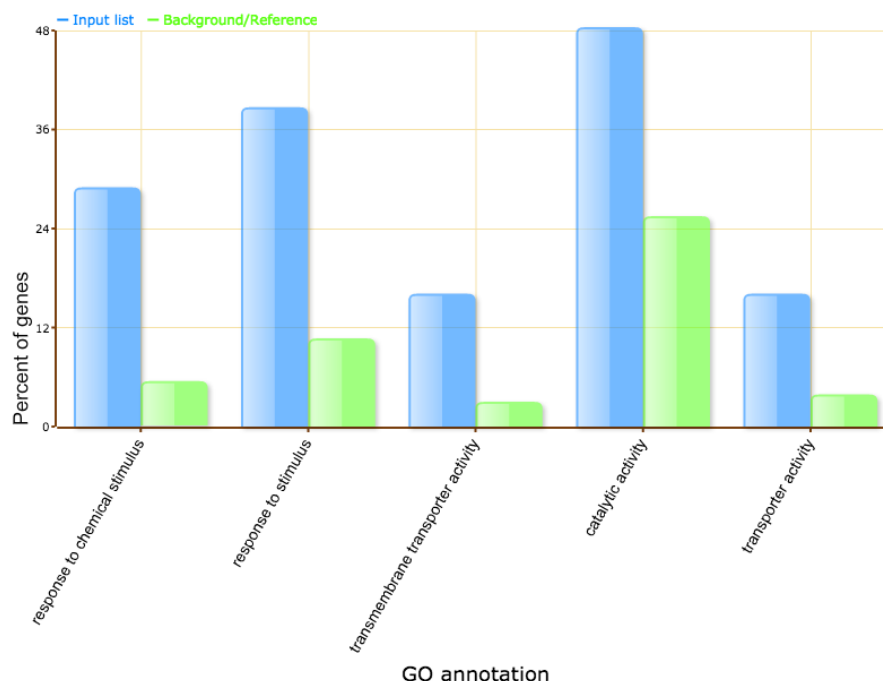


Figure 4.11 – GO terms associated with the co-expressed upregulated genes common to the FIS-AR AGamiR^{DEX} and FIS-AR AP3amiR^{DEX} experiments. Results of GO terms obtained by identifying genes over-expressed in all AG samples (48) and all AP3 samples (48). The genes common to both lists were subsequently identified (31). Blue bars signify the percentage of genes queried that had the GO term associated with them, and green bars represent the percentage of genes expected to be associated with the terms by chance. Graph obtained using the *AgriGO* software (Du *et al.*, 2010). *P*-values were determined used the Fisher’s exact test. A FDR adjusted *p*-value cut-off of 0.05 was used.

4.2.10. Comparisons to existing datasets

The lists of differentially expressed genes obtained for each knockdown time-point range from around 30 to over 1500 when using an adjusted *p*-value cut-off of 0.05. Several gene-list comparisons were made against publically available datasets, focusing on similar subjects, to give an idea of whether or not these results are in agreement with the literature. The list of DEGs for each knockdown dataset was compared to the other datasets, and the number of overlapping genes between the lists was, in turn, compared to the number of genes expected to be present by chance, according to following formula:

$$\text{Expected matches} = \frac{N^{\circ} \text{ genes in dataset 1} \times N^{\circ} \text{ genes in dataset 2}}{N^{\circ} \text{ genes in Arabidopsis genome (27655)}}$$

An over-representation of genes in the queried RNA-seq datasets, determined if the number of observed matches was higher than the number of expected matches, provides a suggestion that the data describe perturbations to the flowering gene regulatory network as a result of *AG* or *AP3* downregulation. The hypergeometric test was subsequently applied to measure the statistical significance of the overlap between datasets.

4.2.10.1. Comparison of DEGs from perturbation experiments to other genome-wide datasets for flower development

Datasets from genome-wide localisation studies

Previous work in the Wellmer laboratory has focused on identifying the genome-wide DNA-binding profiles of AG and AP3 using next generation sequencing of genomic fragments, obtained through chromatin immunoprecipitation (ChIP-Seq), from 4-day-old flowers of induced 35S::AP1-GR AP3::AP3-GFP *ap1-1 cal-1 ap3-3* or AP1::AP1-GR

AG::AG-GFP *ap1-1 cal-1 ag-1* plants (Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013). All unique genes, which were either upregulated or downregulated in the combined AG or AP3 knockdown data described here, were referenced against genes that were identified as high-confidence targets for AG or AP3 binding, summarised in Table 4.5. In all comparisons, more genes were present than expected by chance. While the *p*-values returned for overlap between the upregulated genes were in both cases above the threshold for statistical significance, the overlaps between downregulated genes were highly significant.

Table 4.5 – Comparison of DEGs identified in this study to genes identified in ChIP-Seq datasets examining genome-wide AG and AP3 binding profiles

AG-GFP (O'Maoileidigh <i>et al.</i> , 2013)		AP3-GFP (Wuest <i>et al.</i> , 2012)	
Genes bound	1958	Genes bound	2146
AG Upregulated (this study)	2438	AP3 Upregulated (this study)	445
Overlap between datasets	185	Overlap between datasets	41
Overlap expected	173	Overlap expected	35
<i>p</i> -value	0.163	<i>p</i> -value	0.144

AG Downregulated (this study)	1413	AP3 Downregulated (this study)	251
Overlap between datasets	158	Overlap between datasets	36
Overlap expected	100	Overlap expected	19
<i>p</i> -value	5.4×10^{-9}	<i>p</i> -value	2.6×10^{-4}

4.2.11. Comparisons of DEGs to temporal gene expression data in flowers

Ryan *et al.* (2015) studied the global differential expression of genes during flower development, from the time of initiation until maturation, using the AP1::AP1-GR floral induction system. *K*-means clustering was applied to the 7405 DEGs obtained from the analysis, grouping genes according to their temporal expression patterns, resulting in 15 clusters (**Fig. 4.12**) The overlap between the DEGs identified in the knockdown

experiments, and the DEGs present in each of the clusters, was determined for the upregulated and downregulated genes for each time-point, and the resulting ratios were plotted on heat maps (**Fig. 4.13**). Differentially expressed genes across both knockdowns appeared to have the greatest overlap with clusters 5-7 and 11-13, with the highest average ratio in cluster 11 for both knockdowns. Genes assigned to clusters 5-7 tend to be expressed at earlier stages in flower development, and genes assigned to cluster 11 show the greatest increase in their expression from around 3 to 7 days after the onset of flower development (**Fig. 4.12**), coinciding with the time-points under examination in this study. The clusters with the least overlap were 2, 3, 9, and 14, with cluster 14 having the lowest average overlap. These clusters all show low levels of expression up until around 9-13 days after the onset of flowering, indicating that they are involved in processes that take place at later stages during flower development.

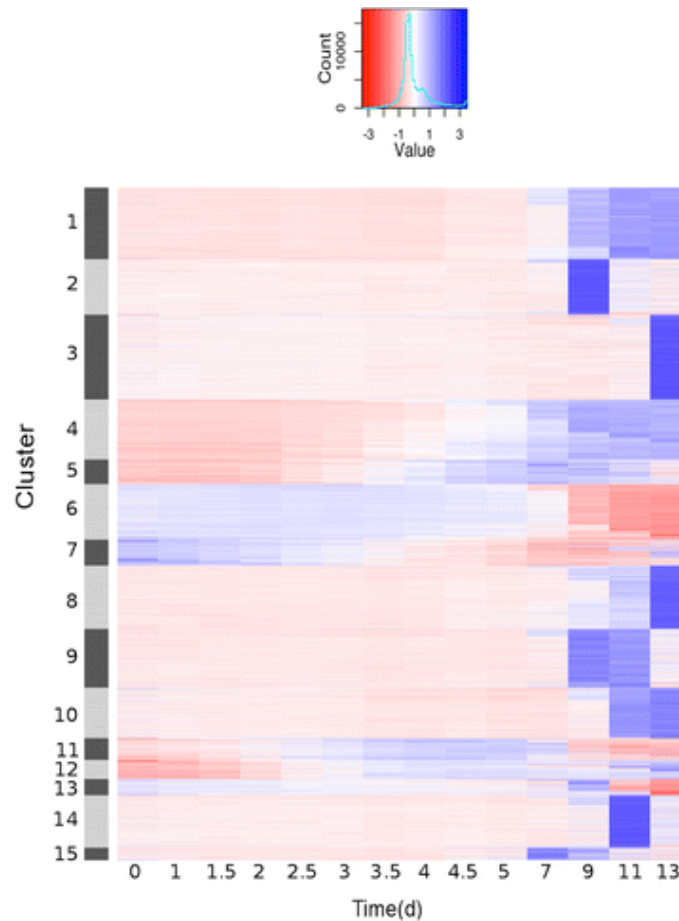


Figure 4.12 - Genes showing differential expression during flower development.

Groups of co-expressed genes were identified among 7,405 differentially expressed genes detected in the time-course experiment where tissue samples were taken and analysed by microarray analysis at different time-points after activation of flower development (as indicated). The heat map shows the results of k -means clustering ($k = 15$) used to group genes based on the similarity of their z -scores (colour-coded as per diagram at the top). The final time-point roughly corresponds to the stage at which flowers reach maturity. Figure from Ryan *et al.* (2015).

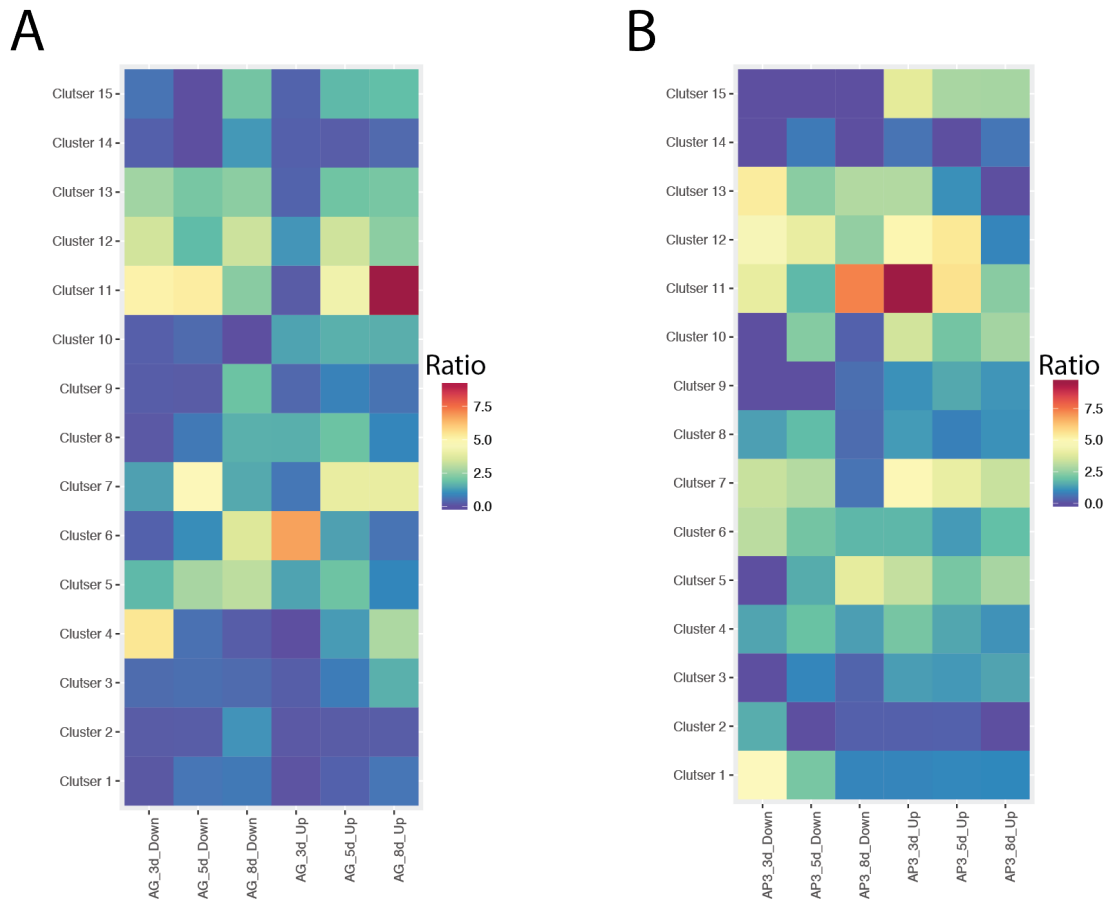


Figure 4.13 – Comparison of genes identified as differentially expressed in the perturbation experiments during flower development. Heat maps of down and upregulated genes for *AG* (A) and *AP3* (B) knockdown time-points (x-axis) compared to the genes present in each cluster from Ryan *et al.* (2015) (y-axis). Each tile represents the ratio of the number of genes present in both categories, compared to the number of genes expected to be present by chance.

4.2.12. Comparisons with other datasets related to AP3 and AG function in flower development

In the same manner as above, the overlap between DEGs and other publically available datasets was calculated in a stage-by-stage manner as much as practicable. The datasets selected for comparison are largely derived from microarray analyses of differentially expressed genes in conditions similar to the developmental context being examined in this study. Tables 4.6 - 4.9 summarise the overlap between the datasets and the time-points listed (representation factor), as well as a brief description of the experiments. A representation factor greater than 1 indicates that the overlap of genes in any given comparison was more than expected by chance. Representation factors greater than 1 were calculated for all datasets tested against the downregulated genes identified in each of the *AG* knockdown time-points, whereas the overlap calculated for some of the comparisons against upregulated genes was less than 1 and did not pass the hypergeometric test for statistical significance.

In general, these results are favourable as they collectively suggest that the differential expression data described herein are representative of the findings of previous studies. The greatest proportional similarities could be found in the comparisons of amiRNA-mediated knockdowns in this study and those of O'Maoileidigh *et al.* (2013), alluding to the reproducibility of the experiments.

Table 4.6 – Summary of *AG* knockdown dataset overlap comparisons – Upregulated genes

Upregulated genes		Representation factor (Observed/Expected)			
Dataset	Experiment	3d	5d	8d	
O'Maoileidigh <i>et al</i> 2013	<i>AG</i> knock-down (amiRNA ^{DEX} , S1-10 inflorescences) (Log ₂ -fold change > 0)	2.69	9.45	16.98	
O'Maoileidigh <i>et al</i> 2015	<i>AG</i> knock-down (amiRNA ^{EtoH} , S1-10 inflorescences) (Log ₂ -fold change > 0)	0.95*	1.56	1.52	
O'Maoileidigh <i>et al</i> 2013	<i>ag-1</i> differential expression (35S::AP1-GR <i>ap1-1 cal-1 ag-1</i>) (Log ₂ -fold change > 0)	0.87*	1.04*	N/A	
Pajoro <i>et al</i> 2014	ChIP-Seq (SEP3)	0.93*	2.23	2.28	
Gomez-Mena <i>et al</i> 2005	Activation of AG-GR (<i>ap1-1 cal-1</i>) (Log ₂ -fold change < 0)	2.37	5.45	5.66	
Jiao & Meyerowitz 2010	Whorl-specific expression levels (AP1 vs AG, S4)	1.15	1.48	1.95	
Jiao & Meyerowitz 2010	Whorl-specific expression levels (AP3 vs AG, S4)	0.63*	1.51	1.9	

p-values: * > 0.05, otherwise p < 0.05

Table 4.7 – Summary of *AG* knockdown dataset overlap comparisons – Downregulated genes

Downregulated genes		Representation factor (Observed/Expected)			
Dataset	Experiment	3d	5d	8d	
O'Maoileidigh <i>et al</i> (2013)	AG knock-down (amiRNA ^{DEX} , S1-10 inflorescences) (Log ₂ -fold change < 0)	1.61	4.13	3.68	
O'Maoileidigh <i>et al</i> 2015	<i>AG</i> knock-down (amiRNA ^{EtoH} , S1-10 inflorescences) (Log ₂ -fold change < 0)	2.47	1.43	2.16	
O'Maoileidigh <i>et al</i> (2013)	<i>ag-1</i> differential expression (35S::AP1-GR <i>ap1-1 cal-1 ag-1</i>) (Log ₂ -fold change < 0)	2.44	3.36	N/A	
Pajoro <i>et al.</i> (2014)	ChIP-Seq (SEP3)	2.02	1.45	1.83	
Gomez-Mena <i>et al.</i> (2005)	Activation of AG-GR (<i>ap1-1 cal-1</i>) (Log ₂ -fold change > 0)	4.08	7.9	6.93	
Jiao & Meyerowitz (2010)	Whorl-specific expression levels (AP1 vs AG, S4)	1.63	1.36	1.35	
Jiao & Meyerowitz (2010)	Whorl-specific expression levels (AP3 vs AG, S4)	1.11	1.52	1.45	

p < 0.05 in all cases

Table 4.8 – Summary of *AP3* knockdown dataset overlap comparisons – Upregulated genes

Upregulated genes		Representation factor (Observed/Expected)			
Dataset	Experiment	3d	5d	8d	
O'Maoileidigh <i>et al</i> 2015	<i>AP3</i> knockdown (amiRNA ^{EtOH} , S1-10 inflorescences) (Log ₂ -fold change > 0)	1.62*	1.58	1.71	
O'Maoileidigh <i>et al</i> 2015	<i>AP3</i> knockdown (amiRNA ^{DEX} , S1-10 inflorescences) (Log ₂ -fold change > 0)	14.2	13.26	15.24	
Wuest <i>et al.</i> (2012)	<i>ap3-1</i> differential expression (whole inflorescences) (Log ₂ -fold change > 0)	3.3	0	N/A	
Wuest <i>et al.</i> (2012)	<i>ap3-3</i> differential expression (whole inflorescences) (Log ₂ -fold change > 0)	7.5	7.64	N/A	
Jiao & Meyerowitz (2010)	Whorl-specific expression levels (<i>AP3</i> vs <i>AG</i> , <i>S4</i>)	1.03*	1.08*	3.32	
Jiao & Meyerowitz (2010)	Whorl-specific expression levels (<i>AP1</i> vs <i>AP3</i> , <i>S4</i>)	2.31	1.62	1.76	

p-values: * > 0.05, otherwise p < 0.05

Table 4.9 – Summary of *AG* knockdown dataset overlap comparisons – Downregulated genes

Downregulated genes		Representation factor (Observed/Expected)			
Dataset	Experiment	3d	5d	8d	
O'Maoileidigh <i>et al</i> (2015)	<i>AP3</i> knockdown (amiRNA ^{EtOH} , S1-10 inflorescences) (Log ₂ -fold change > 0)	1.63	0.9	1.62	
O'Maoileidigh <i>et al</i> (2015)	<i>AP3</i> knockdown (amiRNA ^{DEX} , S1-10 inflorescences) (Log ₂ -fold change > 0)	7	7.50	4.80	
Wuest <i>et al.</i> (2012)	<i>ap3-1</i> differential expression (whole inflorescences) (Log ₂ -fold change < 0)	4.76	0	N/A	
Wuest <i>et al.</i> (2012)	<i>ap3-3</i> differential expression (whole inflorescences) (Log ₂ -fold change < 0)	3.44	3.36	N/A	
Jiao & Meyerowitz (2010)	Whorl-specific expression levels (<i>AP3</i> vs <i>AG</i> , <i>S4</i>)	2.14	1.24*	1.58	
Jiao & Meyerowitz (2010)	Whorl-specific expression levels (<i>AP1</i> vs <i>A3</i> , <i>S4</i>)	1.86*	1.25*	2.96	

p-values: * > 0.05, otherwise p < 0.05

4.3. Discussion and future work

The purpose of these experiments was to identify genes that are controlled by *AP3* and *AG*, at different stages of flower development, using inducible artificial microRNAs and a floral induction system that facilitates the collection of synchronously developing tissue. In doing so, it should be possible to delineate the developmental stages at which these knockdowns affect certain genes, and subsequently this would ideally result in a greater understanding of aspects of the temporal regulation of genes targeted by the homeotic transcription factors. To this end, the floral regulators *AP3* and *AG* were chosen, as previous studies in the laboratory had already examined the differential expression of genes in static mutant lines, providing a base against which comparisons could be made. While some work has been performed using inducible amiRNA lines and tissue derived from multiple stages of flower development in the Wellmer laboratory, the experiments were focused on examining the transcriptional responses of specific genes rather than a global analysis. Therefore, the data obtained in this study provides new insights into the temporal aspects of homeotic regulation by examining the genes that are differentially expressed as a result of *AG* or *AP3* knockdown at different stages in flower development.

The results of the GO analysis of the DEGs identified in the perturbation experiments indicate that genes involved in flower development have responded to amiRNA-mediated knockdowns of *AP3* and *AG*. Furthermore, in the *AG* knockdowns, some of the seeming non-flower development related terms that have arisen, such as the downregulation of genes associated with the cell cycle and upregulation of genes with known roles in photosynthesis, agree with previously published data (O'Maoileidigh *et al.*, 2013). It also appears that the DEGs involved in the cell cycle processes are indirectly regulated by *AG*, as the identified genes are not represented in the genome-wide binding data for *AG*-GFP.

In the same vein, genes belonging to the most over-represented terms present in upregulated genes, associated with photosynthesis, are not present in the ChIP-seq data. This largely agrees with the findings O'Maoileidigh *et al.* (2013), that reduced AG activity leads to the derepression of the leaf development program. When the GO term analysis is performed on each time-point individually, both of the sets of terms described above are present only when *AG* is knocked down at 3d. An interesting line of enquiry at this stage would be to determine if the derepression of these photosynthesis genes is dependent on the downregulation of genes involved with the cell cycle.

The GO terms associated with DEGs in the *AP3* knockdowns do not seem to have as much corroboration with previous results outside of the terms directly related to flower development (Wuest *et al.*, 2012), and many of the terms associated with both upregulated and downregulated genes are unexpected, making confident interpretation at this stage difficult. However, they could also be indicative of novel roles that were previously unknown, and so further analysis is required to establish the significance of these potentially interesting findings.

Examination of the GO terms associated with downregulated genes at each time-point, for both experiments, also demonstrates the stage-specific roles of the homeotics. For instance, high enrichments are obtained for terms related to stamen development at the 8d time-points in both knockdowns, in addition to terms related to floral organ development (see Appendix). Moreover, both of the knockdown series possess genes with known roles in stamen development that are only differentially expressed during the 8d time-point. As AG and AP3 are known to be present in higher-order complexes within the 3rd whorl (Smaczniak *et al.*, 2012), it is likely that some overlap occurs with respect

to the DEGs present in both knockdowns. Indeed, inspection of the genes associated with stamen development in the two experiments reveals *SPL8* and *ENDONUCLEASE3* (*ENDO3*) to be present in both sets. While the biological relevance of *ENDO3* has not yet been elucidated, it was identified as being upregulated upon AG activation (Gomez-Mena, 2005), providing support for some role in stamen development. Further investigation of the genes associated with stamen development, that are unique to each gene's knockdown, could provide some new insights into the separable roles of AP3 and AG during the later stages of flower development. The identification of genes that are differentially regulated by AG or AP3 knockdown, in a stage-specific manner, is therefore particularly highlighted with results such as these.

In examining the genes that were upregulated, it was noticed that a consistent upregulation of 31 genes occurred throughout all of the samples. Analysis of the GO terms associated with these genes showed enrichments for genes involved in 'response to stimulus', and 'transport membrane activity', among others. As these genes were all upregulated after the application of DEX to the developing flowers, and the amiRNAs used in each line were designed to singularly target one transcript, it can be hypothesised that the genes identified here are differentially regulated in response to some aspect of the activation of the LhG4-GR transcription factor by DEX. Further evidence for this could come from testing the relative expression levels of some of these genes in AP1::AP1-AR plants (lacking the 35S::LhG4-GR 6xOP::amiRNA transgene) that have been treated with DEX, in comparison to those treated with a mock solution. If this line of enquiry is valid, then it would allow us to identify and characterise genes systemically upregulated as a property of the DEX-inducible system.

The issue of off-target effects is at the fore when transgenic systems are used to achieve gene perturbations. It has been shown previously that use of the ethanol-inducible ALCR/ALCA system to drive *AG*-targeting amiRNA expression, results in the differential expression of many genes that did not respond in kind to induction of the same amiRNA using the LhG4 promoter system, and there was a significant over-representation of stimulus and stress response genes (O'Maoileidigh *et al.*, 2015). Whether this is a consequence of the extended presence of ethanol and its metabolites within the developing floral tissue, aberrant activity of the ALCR transcription factor, or a combination of the two, is not determined. While using the LhG4 system to perform these experiments is therefore favorable in comparison to other often-used systems, the prospect of unintended differential gene expression remains.

Comparisons to previously published datasets have shown, in general, that the genes identified in this study as differentially expressed have significant overlap with those identified in other expression analysis studies of flower development. Furthermore, comparisons with genome-wide binding data for SEP3, AG, and AP3, during flower development also have more common genes, than is to be expected by chance, when the downregulated genes are considered. The simplest way to interpret this is that AG and AP3 are primarily activators of transcription, and so reducing their activities results in a concomitant downregulation of the genes they target. However, previous work using global expression data for *ap3-3* or *ag-1*, compared to the bound genes identified through ChIP-seq experiments, concluded that the proportions of genes that are differentially expressed in either direction, and are direct targets of AP3 or AG, are roughly the same (Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013), suggesting that both proteins act as activators or repressors of transcription depending on the developmental context. Genes

that are downregulated in these experiments should be activated by AP3 and/or AG under endogenous conditions, likely in the organs that they help to specify. Thus, upon knockdown of the homeotic genes, the identification of DEGs occurs when genes that would have otherwise been activated are not. In contrast, genes that are usually repressed at the time of perturbation may become more broadly expressed during the knockdowns. However, because these genes might also be expressed in other areas of the flower that are not subject to regulation by AP3 or AG, the changes in expression could be more difficult to detect as their expression is already significant.

4.3.1. Limitations of the data

A substantial discrepancy exists between the number of differentially expressed genes within the *AP3* and *AG* knockdown datasets, the largest of which occurring between the 3d samples. It is therefore imperative to determine if the data correctly describe the consequences of these gene perturbations, or if some part of the experiment is a causative factor. Examination of the scatter plots (**Fig. 4.6**) for *AP3* shows that many genes that might be expected to be differentially regulated in response to *AP3* knockdown remain unchanged. Considering that the normalised counts for *AP3* show that the number of transcripts is greatly reduced in DEX-treated samples (10% of mock-treated levels), and the *AP3* transcripts counted in the *AG* 3d mock-treated samples is similar (approximately 8% higher), it could be argued with some confidence that the tissue samples prepared were satisfactory. According to Ryan *et al.* (2015), the peak expression of *AP3* occurs at around 2-4 days after the onset of flower development, so it is a reasonable expectation that disrupting gene activity around this time would result in large changes to the regulation of downstream targets. Given that DEX-induced transcription of the amiRNA occurs for several days after application (demonstrated in Chapter 3), it would be of

interest to examine the expression levels putative target genes over time after a single application of DEX.

A likely limitation of the data and analysis produced so far is that the number of biological replicates per sample might not be sufficient to detect all differentially expressed genes. That is, the statistical tests applied to determine if any given gene is significantly up or downregulated would return more accurate results with a greater sample size. However, due to the cost of sequencing, it was decided that two biological replicates for each time-point would be used for the initial analysis. The integration of further sequencing runs in the future should provide the necessary information to determine whether the results described herein are truly reflective of the transcriptomic changes caused by induction of the amiRNAs.

Another limitation present within the initial differential expression analysis is that the normalised counts do not appear to contain information for endogenous microRNAs, and likely other transcripts. As an example, the leaf development regulator *JAGGED AND WAVY* (*JAW*; *MIR319A*) is not listed in this analysis, but it is in a second analysis performed by BGI. There is evidence that AG binds *JAW*'s promoter under endogenous conditions, in turn repressing part of the leaf development program in flowers (O'Maoileidigh *et al.*, 2013). A log₂-transformed fold change of ~4 is seen in the AG 3d knockdown samples in the BGI analysis, suggesting that the *Arabidopsis* transcriptome file used to perform the alignments in this study are somewhat out of date or lack the correct resolution in some manner. As these data were obtained recently, it was not possible to do comparisons against results obtained using both analyses, but it seems

somewhat likely revisiting the alignment step with a different annotated transcriptome will result in the identification of more differentially expressed genes.

After resolving these issues, further downstream analyses will include scanning of the promoter regions of DEGs for the presence of AG or AP3 binding sites, namely CArG boxes. In doing so, the results will provide a clearer picture of the genes that are directly or indirectly regulated in each case, and further validation could come from comparisons to the ChIP-Seq datasets available for both proteins, as a significant overlap would be expected for genes that are differentially regulated, contain putative binding sites, and are already present in the established genome-wide binding profiles. Additionally, clustering of genes that are differentially regulated at the various time-points in this study, similar to Ryan *et al.* (2015) (**Fig. 4.12**), will allow us to begin to answer the question of how the transcriptome reacts to gene perturbation at specific stages in flower development. While this could be achieved with the existing data, examination of more biological replicates and other time-points would likely lead to a greater resolution of the changes occurring.

5. Chapter 5 – Characterisation of two novel F-box genes in *Arabidopsis thaliana*

5.1. Introduction

5.1.1. UNUSUAL FLORAL ORGANS – A regulator of meristem architecture and floral patterning

The role of *UNUSUAL FLORAL ORGANS* (*UFO*) in floral development and meristem architecture has been continuously elucidated over the last 20 years. *ufo* mutants produce flowers that exhibit variable defects in all whorls, but especially in whorls 2 and 3 (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Petals are typically transformed into sepals, or sepaloid petals, and stamens are largely absent, replaced mostly by filaments and occasionally ectopic carpelloid structures. Chimeric stamen-petal organs are frequently formed, as well as supernumerary fused sepals and carpels. The phenotypes of *ufo* mutants are variable depending on the mutant allele. Weaker alleles in *Arabidopsis*, such as *ufo-6*, tend to have greater numbers of petals and stamens and fewer carpels than strong mutants like *ufo-1* and *ufo-2* (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995; Ni *et al.*, 2004).

Within the inflorescence, *UFO* expression is detected before the onset of floral organ formation, beginning in the IM at the specification of the floral anlagen, switching off at stage 1, and returning at stage 2 at the onset of the floral primordia (Samach *et al.*, 1999). Beyond this, its expression becomes restricted to a ring around the presumptive 2nd and 3rd whorls at stage 3 (Ingram *et al.*, 1995; Lee *et al.*, 1997). Laufs *et al.* (2003) demonstrated that the spatio-temporal regulation of *UFO* activity is paramount to the correct specification of the flower. Transient restoration of *UFO* expression at specific

developmental stages, using an ethanol inducible promoter system (Deveaux *et al.*, 2003), produces flowers that vary in their resulting rescue phenotype. An activation of *UFO* expression between stages 3 and 6 had the most restorative effect, around the time when expression of the endogenous *UFO* becomes restricted to the 2nd and 3rd whorl. Additionally, endogenous *UFO* expression during stage 2 is required for correct specification of the 4th whorl, signalling that it is also a regulator of C-class homeotic gene activity. Indeed, *AGAMOUS* (*AG*) expression has been shown to be modified in *ufo* mutants such that it is precociously expressed within the IM, contributing to the determinate IM phenotype whereby the apex terminates with a carpel-like structure (Wilkinson and Haughn, 1995).

Studies of genetic interactions between *ufo* mutants and other regulators of floral organ identity have shown that *UFO* is required for the correct expression of *AP3* (Levin and Meyerowitz, 1995; Hill *et al.*, 1998; Samach *et al.*, 1999), and plants overexpressing *AP3* and *PI* in a *ufo* background have their floral defects rescued (Krizek and Meyerowitz, 1996). Furthermore, *AP3* is ectopically and precociously expressed in *UFO* over-expression lines, demonstrating that *UFO* is placed upstream of *AP3* in the regulatory hierarchy governing flower development, although this is not the case in *lfy ufo* double mutants as *LFY* exhibits epistasis over *UFO* (Wilkinson and Haughn, 1995; Lee *et al.*, 1997; Zhao *et al.*, 1999; Risseuw *et al.*, 2013). Further to this, *lfy* phenotypes have been observed in plants harbouring a transgene over-expressing *UFO* fused to the dominant negative SRDX repressor domain (discussed in Section 5.2.3), indicating that *UFO* interacts with *LFY* when it is bound to DNA and acts as a transcriptional co-regulator (Chae *et al.*, 2008; Hanczaryk, 2014).

The nature of *LFY* and *UFO*'s interdependent activities is such that all of *UFO*'s known functions require *LFY* but not vice versa. *LFY* is expressed earlier and more broadly than *UFO* within the floral primordia (Weigel *et al.*, 1992; Weigel and Nilsson, 1995; Lee *et al.*, 1997), and over-expression of *LFY* is sufficient to ectopically activate *AP1* expression (Parcy *et al.*, 1998). Further to this, gain-of-function of *LFY* or *UFO* alone produces different phenotypes such that ectopic *LFY* expression causes shoots to be transformed into flowers whereas this is not the case with *UFO*, whose ectopic expression causes disruptions to the complement of floral organs, and produces leaves that are highly lobed in a *LFY*-dependent manner (Weigel and Nilsson, 1995; Lee *et al.*, 1997). Simultaneous over-expression of *LFY* and *UFO* is sufficient to induce *AP3* and *PI* expression in vegetative tissues, while over-expression of *UFO* alone does not achieve the same result, indicating that *UFO*'s regulation of flower development is dependent on the presence of a functional *LFY* allele (Lee *et al.*, 1997; Parcy *et al.*, 1998; Honma and Goto, 2000).

5.1.2. *UFO forms part of an E3 ligase complex*

The way in which *UFO* acts as a co-regulator of *LFY* was initially brought into light upon the identification of a conserved F-box domain within its amino acid sequence (Ingram *et al.*, 1995; Chang *et al.*, 1996). Chang *et al.* (1996) demonstrated that the F-box motif was essential for the interaction between certain cell-cycle regulators, and the S-phase Kinase-associated Protein1 (SKP1) protein, in yeast and humans, and that these interactions were required for the correct regulation of proteolysis of the Sic1p protein, whose degradation is necessary for the cell to enter into S phase (Schwob *et al.*, 1994).

The presence of an F-box motif within *UFO* suggests that it regulated floral organ development through the post-translational ubiquitination of a target protein. The

isolation of a mutant for the *ARABIDOPSIS SKP1-LIKE1* (*ASK1*) gene, homologous to *SKP1* in yeast and humans, revealed a floral organ defect phenotype reminiscent of *ufo* mutants inasmuch that both mutants affected the 2nd and 3rd whorls (Zhao *et al.*, 1999). A physical interaction has been confirmed between the two proteins using both *in vitro* and *in vivo* methods (Samach *et al.*, 1999; Wang *et al.*, 2003), providing evidence for the notion that UFO's regulation of *AP3* expression is through a mechanism involving protein degradation. It has also been shown that over-expression of a modified UFO possessing a mutated F-box domain conferred a dominant negative *ufo* phenotype, likely by way of competitive inhibition of either UFO-ASK1 or UFO-LFY protein-protein interactions, resulting in an abundance of non-ubiquitinated LFY (Risseuw *et al.*, 2013). Furthermore, while the addition of a translational fusion of the dominant activating VP16 domain to UFO caused dramatic alterations to the plant body plan, including ectopic floral meristem formation on leaf blades and severe leaf serration, mutation of the F-box domain in this construct was also sufficient to prevent these phenotypes from occurring (Risseuw *et al.*, 2013). This could suggest that UFO's interaction with LFY, and therefore aspects of LFY's activity, requires a functional interaction with ASK1 and the ubiquitination machinery. Experiments using proteasome inhibitors have provided further evidence for this, as ectopic *AP3* expression is reduced in seedlings, which over-express *UFO* and *LFY*, that are treated with epoxomicin, an inhibitor of the 20S component of the proteasome (Chae *et al.*, 2008).

5.1.3. SCF complexes

ASK1, and its homologs in other eukaryotes, mediates protein degradation as part of an SCF (Skp1-Cullin-F-box protein) E3 ligase complex, which facilitates the conjugation of ubiquitin moieties to substrate proteins, thereby altering the substrate's activity and

promoting its destruction through the 26S proteasome (Hershko, 1992; Hochstrasser, 1996). E3 ubiquitin ligases are the final step on a three-enzyme pathway, involving the E1 and E2 enzymes. The E1 molecule activates ubiquitin such that it becomes adenylated and bound to the available active site on the enzyme. Ubiquitin is then transferred to the E2 conjugating enzyme, which interacts with E3 ligases complexes to covalently attach the moiety to the substrate (for review see Pickart, 2001).

E3 ligases can be classified into two groups, depending on whether the complex contains a RING (Really Interesting New Gene)-like or a HECT (Homology to E6-AP C-Terminus) subunit (Mazzucotelli *et al.*, 2006), with SCF complexes belonging to the former. The overall structure of SCFs complexes has been deduced, consisting of 4 subunits: CULLIN (CUL), RING-BOX (RBX), SKP, and an F-box protein (Deshaies, 1999; Gray *et al.*, 1999, 2002; Zheng *et al.*, 2002). The *Arabidopsis* genome codes for a minimum of 5 CULLINs (AtCUL), 21 ASKs, 2 RBX homologs, and around 700 F-box proteins (Gagne *et al.*, 2002; Risseuw *et al.*, 2003; Liu *et al.*, 2004; Ni *et al.*, 2011). RBX and CUL together recruit the ubiquitinated E2 molecule and provide a scaffold for its interaction with the substrate, while the F-box protein is required for substrate recognition. The F-box protein associates with the SCF complex through its interaction with SKP1, which acts as a linker to the rest of the complex by interacting with CUL.

F-box proteins are defined by the presence of a conserved ~40-60 amino acid N-terminal F-box domain, which is what interacts with SKP proteins (Chang *et al.*, 1996; Kipreos and Pagano, 2000; Kuroda *et al.*, 2012), and a variable C-terminal region that interacts with the target protein (Skowyra *et al.*, 1997). In *Arabidopsis*, coupled with the large expansion of the F-box gene family, the C-terminal domain has diverged significantly

throughout evolution to possess multiple different types of protein interaction domains that presumably allow F-box proteins to take part in a multitude of cellular processes (Gagne *et al.*, 2002; Schumann *et al.*, 2011).

5.1.4. Dissecting the diversified functions of *UFO* between different species

The functions of *UFO* have been determined to be diverse between different species despite the high levels of amino acid sequence identity present between orthologues that have been studied (e.g. 91.48% between *Arabidopsis* and *Brassica napus*, and 56.32% between *Arabidopsis* and the more distantly related *Petunia hybrida*). *UFO* is an evolutionarily conserved gene, and several analysed species all possess orthologues which regulate FM and floral organ identity to different extents (Ingram *et al.*, 1995; Allen and Sussex, 1996; Taylor, Hofer and Murfet, 2001; Souer *et al.*, 2008). Mutations in these genes result in phenotypes of varying severity depending on the species. Perhaps the greatest departure from *Arabidopsis* is that in some species, such as *Solanum lycopersicum* (Tomato) or *Petunia hybrida*, FM identity is abolished when the *UFO* orthologues, *ANANTHA* (*AN*) and *DOUBLE-TOP* (*DOT*), are knocked out respectively (Souer *et al.*, 2008). In *Antirrhinum majus* (Snapdragon), mutations in the orthologue *FIMBRATA* (*FIM*) result in indeterminate flowers and a reduced level of C-class gene transcription, whereas such defects are not observed in *ufo* mutant flowers (Ingram *et al.*, 1995). The more severe phenotypic aspects of these mutants could suggest the presence of genes in the *Arabidopsis* genome that are active in functionally redundant pathways with *UFO*.

More recently it has been shown that the *cis*-regulatory elements governing *UFO* expression in different species can account for the observed differences in floral architecture and mutant phenotypes, suggesting the divergence in their activities is due to

species-specific promotor regions that are responsive to different sets of conserved upstream regulators (Kusters *et al.*, 2015). For instance, *UFO* and *FIM* are expressed within the floral meristem and are then restricted to a ring around the 2nd and 3rd whorl, whereas *DOT* is initially expressed in a wedge shape in the cells that are to become the floral meristem shortly before they begin to separate from the IM, then expression is confined to a ‘stripe at the base of the first sepal primordium’, later expanding in a ring-shape toward the sepal-petal boundary (Souer *et al.*, 2008). Additionally, the introduction of a *UFO::UFO* transgene into *Petunia* causes the inflorescence to terminate with a single flower during vegetative development, demonstrating how the regulatory elements present within divergent promoter sequences confer different activities on the conserved protein (Kusters *et al.*, 2015).

Considering the expansion of the F-box gene family (Gagne *et al.*, 2002), and that the phenotypes of *Arabidopsis ufo* mutants tend to be less severe than those of other species, we asked whether as-of-yet unidentified F-box genes existed that had some functional redundancy with *UFO*. The *Arabidopsis* eFP browser (Winter *et al.*, 2007), a collection of tissue-specific global expression data, was originally used to identify F-box genes that had a similar spatio-temporal expression profile to *UFO* (Hanczaryk, 2014), with the intention of generating double mutants and examining any potential modification to the established mutant phenotype. It was hypothesised that doing so could result in plants that more closely resembled the meristem identity defects found in other species.

5.1.5. Identification of F-box genes with a similar expression profile

The gene AT4G19865 (hereafter referred to as *NANNY*; *NAN*) was identified through these searches. A second gene, AT4G19870 (hereafter referred to as *GRANNY*; *GRA*) was

subsequently identified through a BLASTP search, and the proteins have 62% sequence homology. *NAN* and *GRA* are located next to each other on chromosome 4 (**Fig. 5.1 C**), suggesting that they are the result of a tandem duplication event. Both of the genes are predicted to encode F-box proteins with putative Kelch repeats present in their protein-interactive C-terminal domains (referred to as FBKs). Although UFO is currently classed as ‘unknown’ with respect to the structure of its C-terminal domain (Gagne *et al.*, 2002), analysis of the amino acid sequence using the online program *InterPro* suggests that it contains Kelch-type beta propeller elements (Finn *et al.*, 2017).

Using a dataset published by Wellmer *et al.* (2006), it was confirmed that *NAN* and *GRA* are expressed within the inflorescence meristem, floral meristems, and flowers. Data obtained from the *Arabidopsis* eFP browser agrees with this (**Fig. 5.1 A**), and has also returned the result that *NAN* is expressed within other tissues, such as leaves and seedlings. No data is available for *GRA*. As mentioned above, *NAN* and *GRA* are in all probability the result of a tandem duplication, an event which is markedly more common in *Arabidopsis* than in other analysed plant species. For instance, 35% of FBK genes in *Arabidopsis* are arranged in tandem repeats compared to 5% in rice (*Oryza sativa*) (Schumann *et al.*, 2011). 64% of *Arabidopsis* FBK genes are predicted to be lineage specific and do not have orthologues in other species (Schumann *et al.*, 2011).

5.1.6. Chapter aims

Initial findings reported in Hanczaryk (2014) suggested that crossing the strong mutant allele *ufo-1* with a T-DNA insertion line (located within the intergenic region between *NAN* and *GRA*, termed *nan-1 gra-1*) did not result in an enhancement of the *ufo* phenotype, but rather in a slight restoration of 2nd and 3rd whorl floral organ identity. The aims of this chapter were therefore centred around determining whether or not *NAN* and

GRA interacted genetically with *UFO* within the context of flower development. If they contributed to the established function(s) of *UFO*, such that they could be considered at least partially redundant, this could go some way to explaining the differences between the phenotypes of orthologous mutants observed in other species. To this end, *ufo* mutants were crossed with knockdowns of *NAN* and *GRA* to ascertain whether or not this led to an enhancement of the mutant phenotype and agreed with previous findings. Additionally, the expression profiles of *NAN* and *GRA* have been investigated to determine their potential areas of action, and if they overlap with that of *UFO*. Another question asked was whether or not *NAN* and *GRA* were functionally redundant between themselves. An analysis by Schumann *et al.* (2011) of FBKs suggests that those genes arranged in tandem repeats do not necessarily have the same spatial and temporal domains of expression, indicating that sub-functionalisation between homologous genes such as these can take place. To address this question, over-expression constructs of both genes have been generated and transformed into wild-type plants. Modifications to these constructs have also been generated that confer dominant activating or repressive activities to the proteins. These were created to begin to place the two genes in some regulatory pathway, so if transgenic plants showed defects or abnormalities that phenocopy established mutants or other transgenic lines, this could provide a helpful clue to understanding the positions of these genes in the floral regulatory network. Finally, the CRISPR-Cas9 genome editing technology was employed to generate plants with knockout mutations in both genes independently, making it possible to analyse each gene's function separate from the other in the event that they are not functionally redundant.

5.2. Results

5.2.1. Investigating the genetic relationship between *UFO*, *NAN*, and *GRA*

5.2.1.1. Phenotypic analysis of crosses between *ufo* and *nan/gra* mutants

To investigate the genetic interaction previously reported between *UFO* and *NAN/GRA*, where a slight restoration of petal and stamen numbers was observed, the established lines were regrown for examination. A *ufo-1 nan-1/+ gra-1/+* line was used to observe any modification of the *ufo-1* phenotype. The *nan-1 gra-1* line possesses a T-DNA insertion in the intergenic region between the coding sequences of *NAN* and *GRA* (**Fig. 5.1 C**), causing a marked reduction in the expression of *NAN*, while the expression level of *GRA* appears to be affected to only a small extent (**Fig. 5.1 B**). Flowers of *ufo-1* plants, a strong mutant that is the result of an early stop codon within the C-terminal region of the protein, typically exhibit a severely decreased level of B-class gene activity, such that the frequency of petal and stamen numbers are reduced to close to 0, and instances of chimeric sepaloid petals and filaments are prevalent in the majority of flowers. In addition, the number of fused carpels is frequently greater than 2. Initial analyses of the number of floral organs in *ufo-1 nan-1 gra-1* revealed a partial restoration of petal and stamen frequencies, with a concurrent reduction in sepaloid petal and filament numbers, indicating greater B-function activity within the 2nd and 3rd whorls (**Fig. 5.2**). RT-qPCR analysis of inflorescence tissue enriched for young flowers and meristematic tissue was performed to determine if the triple mutant indeed resulted in the differential expression of genes involved in 2nd and 3rd whorl development, such as *AP3*, *PI*, and *AG* (**Fig. 5.3**). Despite the observed phenotype, no differential expression of these genes could be detected in *nan-1 gra-1 ufo-1* samples, in comparison to *ufo-1*.

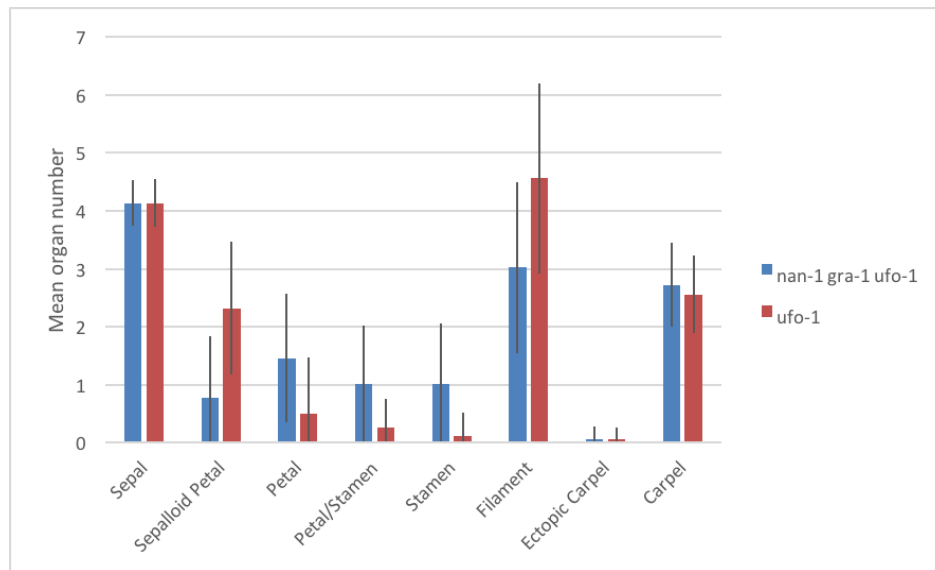


Figure 5.2 - Characterisation of *nan-1 gra-1 ufo-1* flowers. Counts of floral organ numbers in *nan-1 gra-1 ufo-1* (blue bars, n = 100) and *ufo-1* (red bars, n = 42) flowers. Values are the mean average. Error bars represent standard deviations.

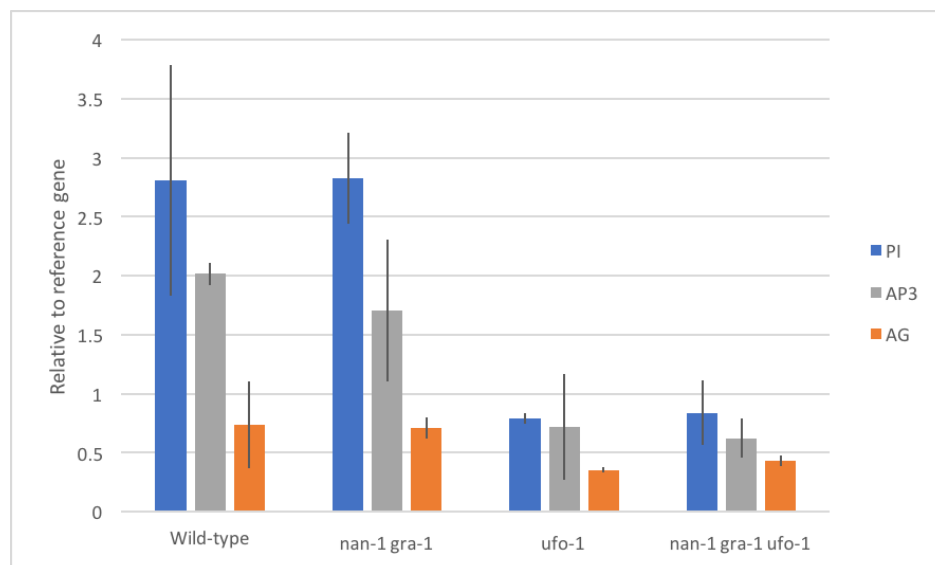


Figure 5.3 - Expression of B and C class homeotic genes. RT-qPCR results of *PI* (blue bars), *AP3* (grey bars), and *AG* (orange bars) mRNA levels in tissue enriched for the inflorescence meristem and young floral buds of wild-type, *nan-1 gra-1*, *ufo-1*, and *nan-1 gra-1 ufo-1* plants. Values are relative to the reference gene REF1. Values are the mean of three (or two for *ufo-1*) biological replicates for each genotype. Two

technical replicates were performed for each sample. Error bars represent standard deviations.

To determine whether the results obtained were due to the knocked down activity of *NAN* and/or *GRA*, further independent knockdown lines were crossed into the *ufo-1*, *ufo-2*, and *ufo-13* mutants. Two further T-DNA lines were also used, *gra-2* and *nan-2* (**Fig. 5.1 C**). Both exhibit reduced expression of the two genes to different extents. *gra-2*, possessing a T-DNA insertion immediately upstream of *GRA*'s coding sequence, retains approximately 40% of wild-type expression levels for *GRA*, and approximately 70% for *NAN* (**Fig. 5.1 B**). *nan-2* plants, containing a T-DNA insertion in *NAN*'s 5'UTR, express *NAN* and *GRA* at approximately 55% and 90% of wild-type levels, respectively (Hanczaryk, 2014). Below are summaries of the observations made for each of the crosses performed.

5.2.1.2. *nan-1 gra-1 x ufo-13*

nan-1 gra-1 plants were crossed into the *ufo-13* background. *ufo-13*, a weaker allele than *ufo-1* and *ufo-2*, possesses a SNP that results in early stop codon towards the C-terminus (W390*) (Durfee *et al.*, 2003). *ufo-13* flowers still have prevalent defects within the 2nd and 3rd whorls, although the increased frequency of correctly developed stamens and carpels (in comparison to stronger lesions) allows the plants to remain fertile. The F1 progeny were screened for the presence of the *nan-1 gra-1* T-DNA, and heterozygous individuals were allowed to self-fertilise. F2 plants that were homozygous for the wild-type or mutant *NAN/GRA* allele were selected in those individuals that displayed the *ufo-13* phenotype. While only a single individual was identified as being homozygous for the *nan-1 gra-1* allele, a reduction in sepalloid petal frequency was apparent when compared to *ufo-13* flowers (**Fig. 5.4**).

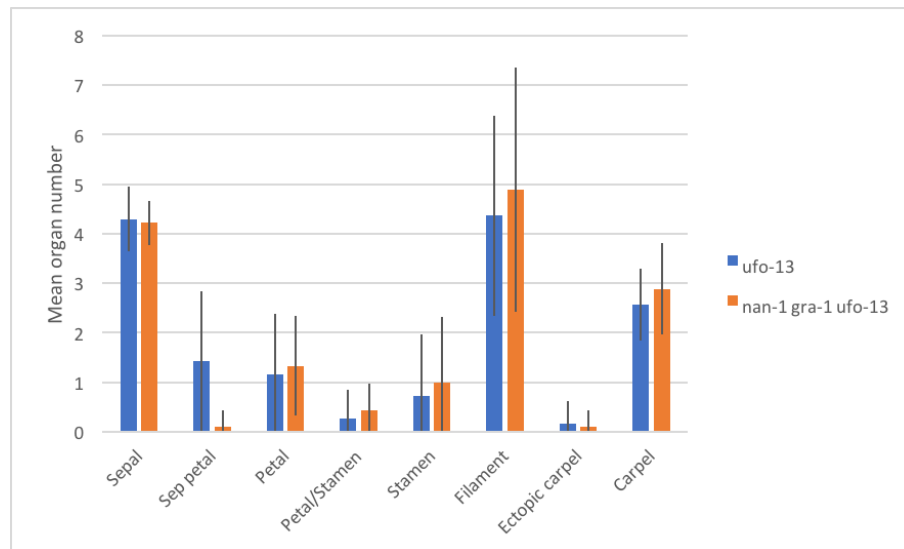


Figure 5.4 - Characterisation of *nan-1 gra-1 ufo-13* flowers. Counts of floral organ numbers in *ufo-13* (blue bars, n = 40) and *nan-1 gra-1 ufo-13* (orange bars, n = 9) flowers. Values are the mean average. Error bars represent standard deviations.

5.2.1.3. *gra-2 x ufo-1* and *gra-2 x ufo-2*

The *gra-2* homozygous T-DNA line, generated in the Col-0 background, was crossed into homozygous *ufo-1* and *ufo-2* mutants. The progeny was grown and individuals that were heterozygous for both alleles were allowed to self-fertilise. Heterozygous *ufo-1* and *ufo-2* mutants, both single nucleotide polymorphisms, were identified using the Tetra-primer Amplification Refractory Mutation System (T-ARMS), a PCR method which is based on the use of two sets of primers that will each allow the amplification of one allele but not the other (Medrano and De Oliveira, 2014) (detailed in Materials and Methods). To select for double mutants in the F2 population, plants that displayed the *ufo-1* or *ufo-2* phenotype were screened for the presence of the *gra-2* allele by PCR genotyping. Those plants that were homozygous for the T-DNA, or homozygous for the wild-type *GRA* locus, were used for the analysis. The phenotypes of the double mutants, for both crosses, did not appear to appreciably deviate from those of the single *ufo* mutants (**Fig. 5.5**).

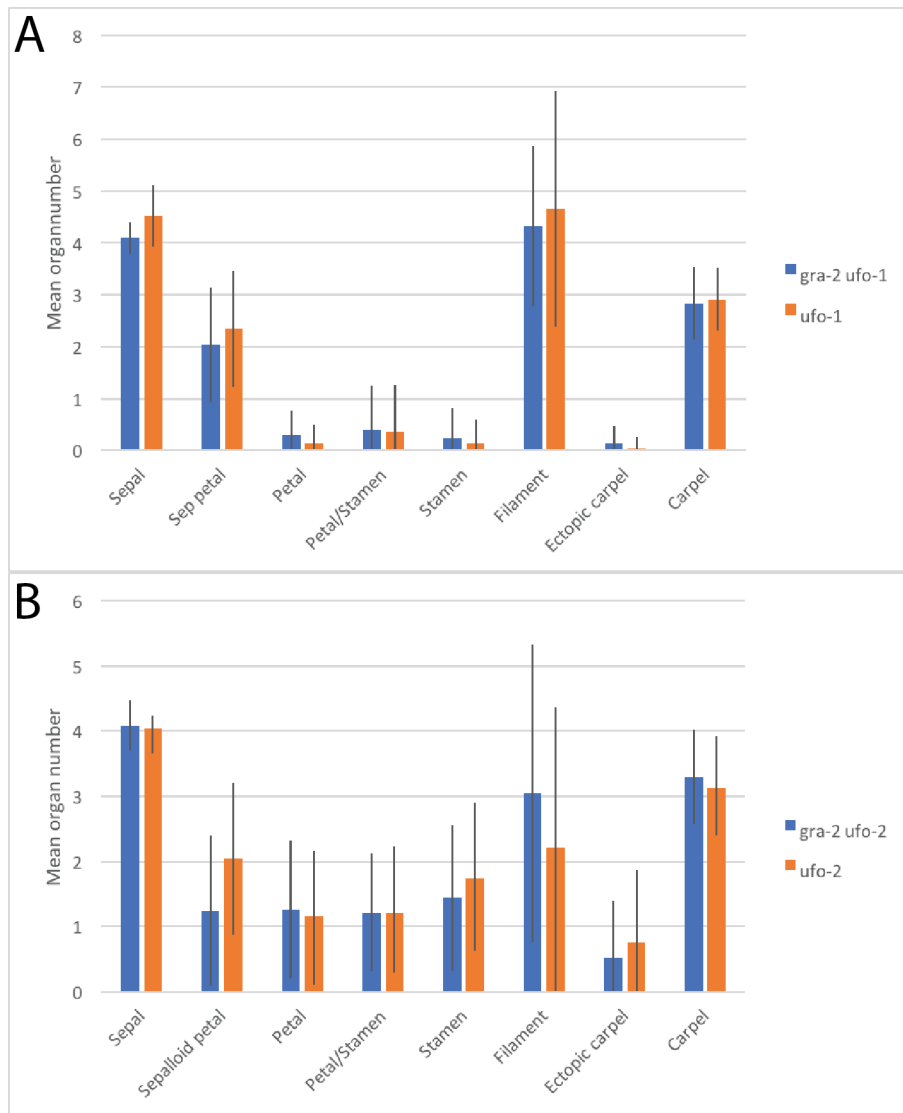


Figure 5.5 – Characterisation of *gra-2 ufo-1* and *gra-2 ufo-2* flowers. (A) Counts of floral organ numbers in *gra-2 ufo-1* (blue bars, n = 30) and *ufo-1* (orange bars, n = 23) flowers. Values are the mean average. (B) Counts of floral organ numbers in *gra-2 ufo-2* (blue bars, n = 41) and *ufo-2* (orange bars, n = 24) flowers. Values are the mean average. Error bars represent standard deviations.

5.2.1.4. *nan-2* x *ufo-2*

The *nan-2* homozygous T-DNA line, generated in an *L-er* background, was crossed into homozygous *ufo-2* mutants. The F1 progeny were screened for individuals heterozygous for the *ufo-2* allele, and these were allowed to self-fertilise. F2 plants that displayed the *ufo-2* phenotype were genotyped for the presence of the *NAN* wild-type locus that is perturbed in *nan-2* plants. Since a genotyping assay for the *nan-2* T-DNA could not be developed, individuals were selected for analysis if the genotyping assay for the wild-type locus did not produce a PCR product. Those individuals that produced a PCR product were used as a comparison, meaning that they could be heterozygous for the *nan-2* allele, or wild-type. *nan-2 ufo-2* flowers in this analysis did not show any significant deviation from the floral organ frequencies observed in *nan-2/+ ufo-2* flowers, except in the case of the average number of sepalloid petals, where a small difference was noted (**Fig. 5.6**).

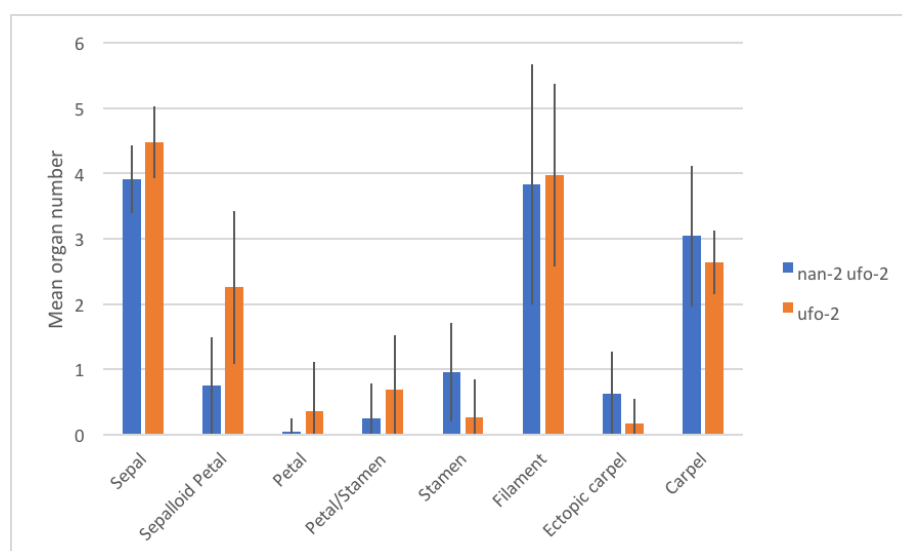


Figure 5.6 – Characterisation of *nan-2 ufo-2* flowers. Counts of floral organ numbers in *nan-2 ufo-2* (blue bars, n = 24) and *ufo-2* (orange bars, n = 42) flowers. Values are the mean average. Error bars represent standard deviations.

5.2.1.5. *ufo-2* x *35S::NAN/GRAamiR*

The results of the crosses performed above suggest, on that whole, that reducing *NAN/GRA* expression using T-DNA lines does not result in a genetic interaction with *ufo* mutants. To test whether further reducing the expression of *NAN* and *GRA* would modify the *ufo* mutant phenotype, flowers were counted from a previously generated *ufo-2* line harbouring a transgene that constitutively expressed an artificial microRNA simultaneously targeting *NAN* and *GRA* (*35S::NAN/GRAamiR*) (Hanczaryk, 2014). The amiRNA selected results in an almost full reduction in *NAN* mRNA levels, and approximately 80% of *GRA* mRNA levels (Hanczaryk, 2014). Flowers homozygous for *ufo-2*, and segregating for the amiRNA, were selected by PCR genotyping for the transgene. Flowers possessing the amiRNA did not significantly differ from *ufo-2* flowers (Fig. 5.7).

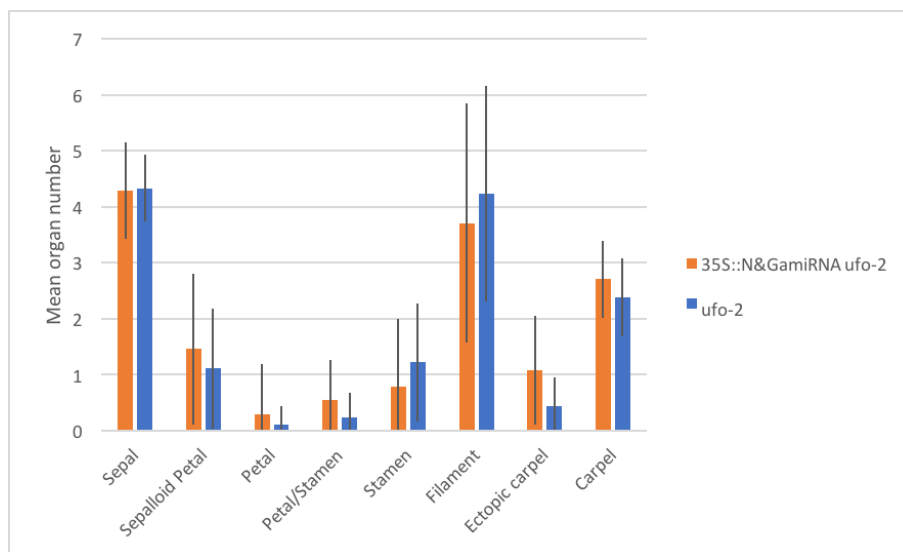


Figure 5.7 – Characterisation of *35S::NAN&GRAamiR ufo-2* flowers. Counts of floral organ numbers in *35S::NAN&GRAamiR ufo-2* (orange bars, n = 24) and *ufo-2* (blue bars, n = 18) flowers. Values are the mean average. Error bars represent standard deviations.

5.2.1.6. Investigating potential genetic interactions between *NAN*/*GRA* and B-class homeotic mutants

The results of the series of organ counts in mutant crosses outlined above strongly suggest that introducing knockdown mutants for *NAN* and *GRA* does not rescue the *ufo* mutant phenotype, and if these genes do have any genetic interaction it is likely too weak to be confidently detected using the methods above.

A previous report had suggested that the phenotypes of *ap3-3* and *pi-1* mutant lines were also modified in a *nan-1 gra-1* background, such that novel gynoecia defects were observed (Hanczaryk, 2014). To confirm one way or the other, the *pi-1* and *ap3-3* lines that had been previously crossed with the *nan-1 gra-1* T-DNA line were examined, and flowers of plants homozygous for both mutations were compared to the respective single mutants. Flowers of both *nan-1 gra-1 pi-1* and *nan-1 gra-1 ap3-3* were not phenotypically different from the respective single mutants (**Fig. 5.8**), and RT-qPCR analysis of inflorescence tissue enriched for young floral buds and meristematic tissue of the *nan-1 gra-1 ap3-3* and *ap3-3* lines did not reveal any change to the expression of *AP3* in the triple mutant. However, the expression of *NAN* was further reduced in the triple mutant by a factor of over 100 when compared to wild-type levels (**Fig. 5.9**), suggesting that *AP3* is involved in regulating *NAN*'s expression. Furthermore, ChIP-Seq analysis of PI-GFP in the floral induction system, carried out by Dr. Beibei Zheng in the Wellmer laboratory, has shown that binding peaks exist within the coding regions for both *NAN* and *GRA* (data not shown). As *AP3* and *PI* act as obligate heterodimers (Riechmann, Krizek and Meyerowitz, 1996), this suggests that *NAN* could be indirectly regulated by the B-class homeotic genes.

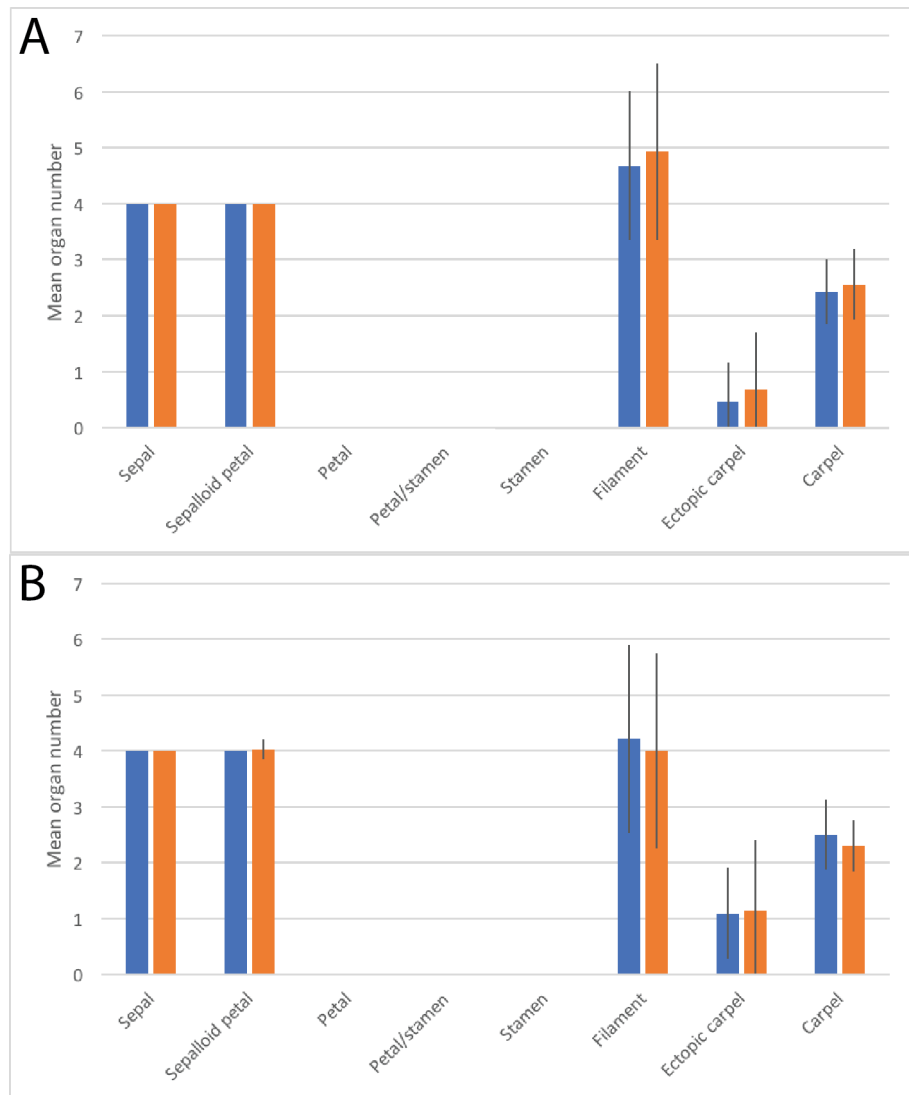


Figure 5.8 – Characterisation of *nan-1 gra-1 ap3-3* and *nan-1 gra-1 pi-1* flowers. (A) Counts of floral organ numbers in *nan-1 gra-1 ap3-3* (blue bars, n = 29) and *ap3-3* (orange bars, n = 28) flowers. (B) Counts of floral organ numbers in *nan-1 gra-1 pi-1* (blue bars, n = 32) and *pi-1* (orange bars, n = 34) flowers. Values are the mean average. Error bars represent standard deviations.

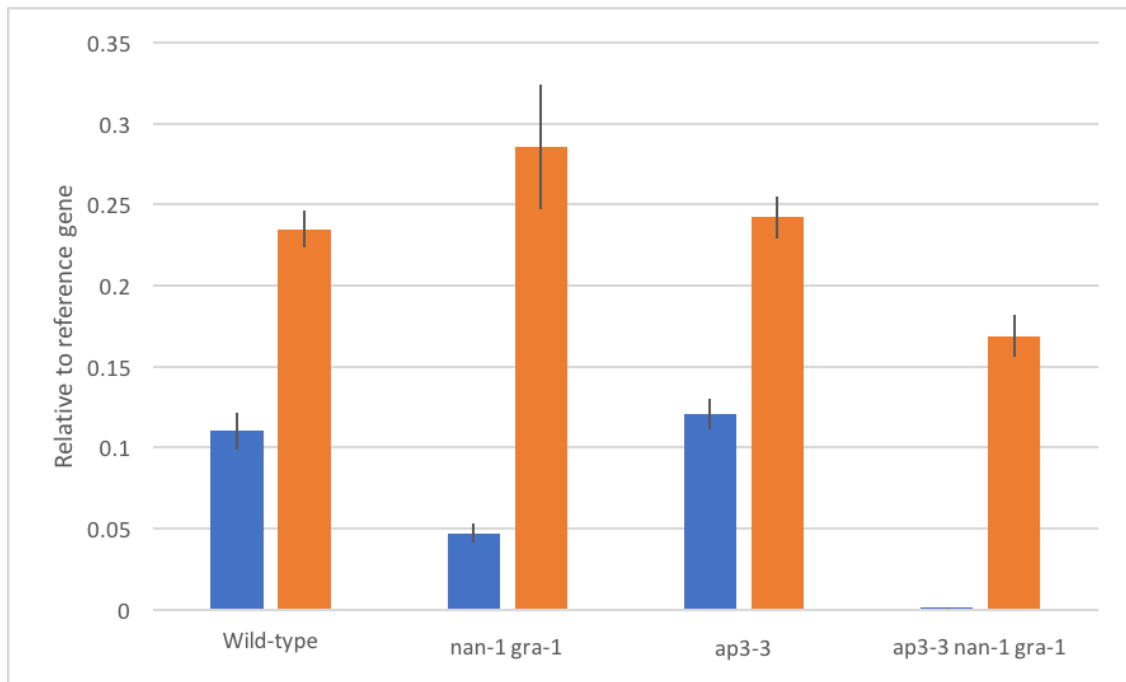


Figure 5.9 – Transcriptional response of *NAN* and *GRA* in *ap3-3* mutants. RT-qPCR results of *NAN* (blue bars) and *GRA* (orange bars) mRNA levels. Values are relative to the reference gene REF1. Values are the mean of three biological replicates for each genotype. Two technical replicates were performed for each sample. Error bars represent standard deviations.

5.2.1.7. Floral organ frequencies of *ufo* mutant flowers differ over time

As the phenotypic differences observed in the *nan-1 gra-1 ufo-1* line could not be explained by the differential expression of genes controlling 2nd and 3rd whorl development, and the phenotypes observed between *ufo* mutants and other crosses examined were largely similar, it was hypothesised that the manner in which *ufo* floral organ frequencies change over time (Wilkinson and Haughn, 1995) could be responsible for the partial rescue phenotypes previously noted in the *nan-1 gra-1 ufo-1* line. That is, comparisons could have been made between earlier or later arising flowers between the genotypes being examined. To test this, the *nan-1/+ gra-1/+ ufo-1* line was grown, and as many sequential flowers as possible were examined. In contrast to the former results,

when this factor is taken into account, the previously determined rescue phenotypes are no longer apparent (**Fig. 5.10**).

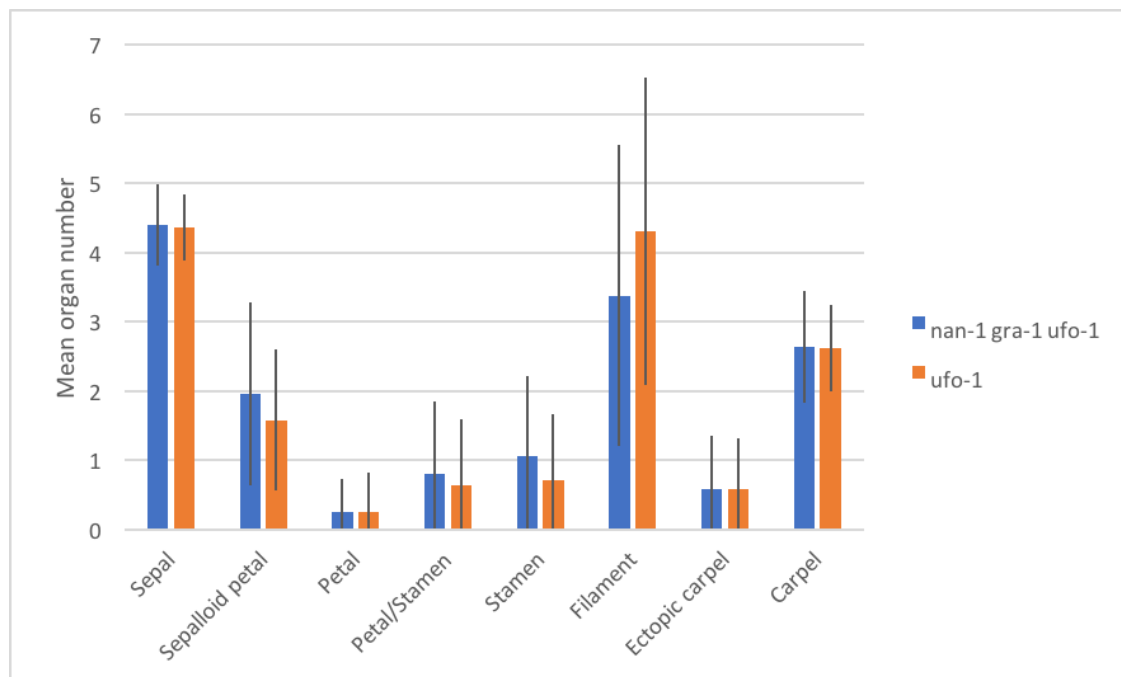


Figure 5.10 – Characterisation of the *nan-1 gra-1 ufo-1* floral phenotype with respect to flower number. Counts of *nan-1 gra-1 ufo-1* (orange bars, n = 120) and *ufo-1* (blue bars, n = 96) flowers. The first 24 sequential flowers were counted on 5 *nan-1 gra-1 ufo-1* plants and 4 *ufo-1* plants. Values are the mean average. Error bars represent standard deviations.

The core hypothesis of this project was that *NAN* and/or *GRA* had some function during flower development that partially relieved the *ufo* mutant phenotypes when they were knocked down. Analysis of floral organ frequencies in independent *nan/gra x ufo* mutant lines has indicated that the original rescue phenotypes observed in previous reports, and this report, were due to the method employed when examining the flowers. That is, examining flowers that have not arisen at the same stage of the plants' reproductive cycle introduces differences in floral organ frequency and composition that can lead to false positive results.

5.2.2. *Determining the expression profiles of GRA and NAN*

The analyses above of several mutant crosses for *GRA*, *NAN*, and *UFO*, indicated that knocking down *NAN/GRA* in a *ufo* background does not appreciably alter the floral organ identity defect phenotype. As these genes have not been studied before in any great detail, and the limited information so far available suggests that they may have some role in flower development, it was decided to further characterise their expression profiles and function. GUS reporter constructs were generated, and yeast 2-hybrid analysis was performed, to visualise the endogenous expression patterns of the two genes throughout the plant and determine if the proteins can physically interact with components of the SCF complex. Further, several over-expression constructs were generated to investigate whether *Arabidopsis* transformants would possess any phenotypes that could help place *GRA* and *NAN* in some established pathway.

5.2.2.1. *Determining the expression profiles of GRA and NAN with transcriptional reporters*

To determine the expression profiles of *GRA* and *NAN*, the respective upstream sequences of both genes, that were predicted to contain *cis*-regulatory elements (**Fig. 5.11**), were PCR amplified from Col-0 genomic DNA and fused upstream of the β -Glucuronidase (GUS) coding sequence (**Fig. 5.12 A**). The resulting constructs were transformed into wild-type (Col-0) plants. A 2kb region was cloned to assess the expression profile of *GRA* (*GRA::GUS*), and the ~680bp intergenic region between *GRA* and *NAN* was chosen for *NAN* (*NAN::GUS*). Each transgene's staining pattern was compared to the previously established transcriptional reporter line *UFO::GUS* (Lee *et al.*, 1997) in order to determine if they had any similarities.

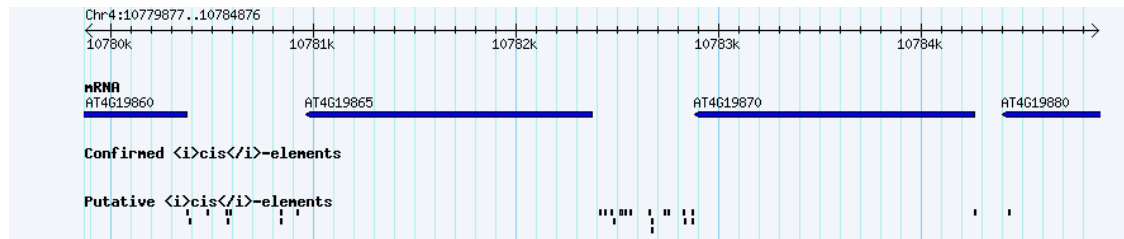


Figure 5.11 – Determination of likely promoter regions of *NAN* and *GRA*. Putative cis-regulatory elements of *NAN* (AT4G19865) and *GRA* (AT4G19870) are denoted by grey boxes upstream of the coding sequences (blue bars). Image obtained from AtCisDB (Yilmaz *et al.*, 2011).

Whole inflorescences were stained, cleared, and sectioned to visualise staining within the inflorescence meristems and young floral organs. Staining for the presence of GUS activity revealed similar yet distinct expression patterns in *NAN::GUS* and *GRA::GUS*. *UFO::GUS* exhibits heavy staining within IMs, FMs, and young floral buds. Neither *NAN::GUS* or *GRA::GUS* had any staining within these structures (**Fig. 5.12 B - G**). In contrast with *UFO::GUS*, both *GRA::GUS* and *NAN::GUS* exhibited staining within stamens, specifically the anthers (**Fig. 5.12 K-M**). Each construct appeared to be active at different stages of development, and within different tissues of the anthers. *NAN::GUS* was expressed in early-stage stamens up to around stage 9 within the vascular bundle, while *GRA::GUS* was expressed in later stages within the tapetum, a tissue which provides developing pollen with nutrients (Liu and Fan, 2013).

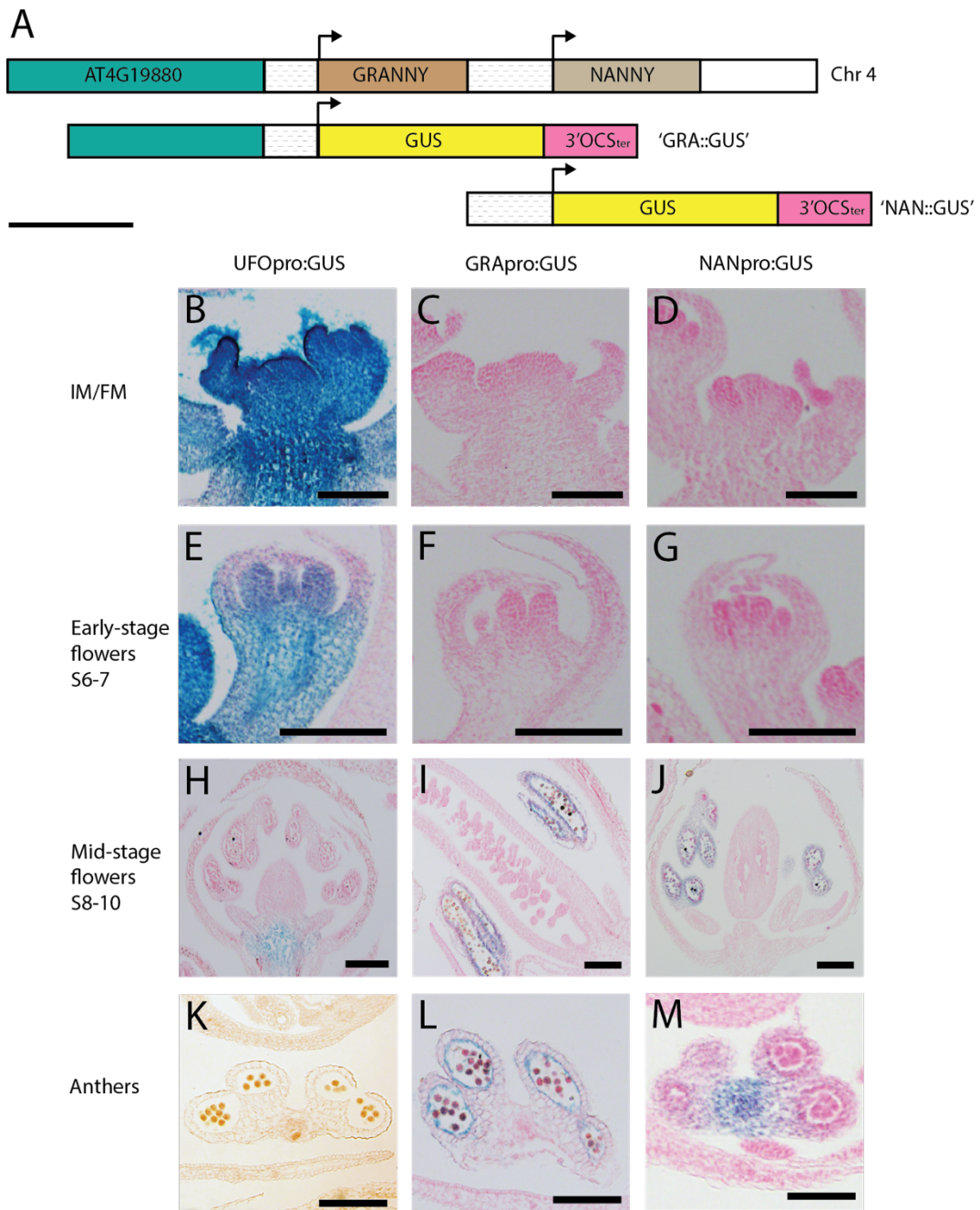


Figure 5.12 - GUS staining patterns of *UFO*, *NAN*, and *GRA* transcriptional reporters. (A) Schematic of GRA::GUS and NAN::GUS reporter cassettes, showing the regulatory regions used. Terminator sequences are derived from the *Octopine synthase* gene of *Agrobacterium*. Scale bar: 1kb. (B-M), sections of GUS-stained floral tissues. UFO::GUS exhibits staining within the IM and FM (B), and young floral buds (E, H), whereas this is not the case for GRA::GUS (C, F) or NAN::GUS (D, G). GRA::GUS and NAN::GUS exhibit staining within the anthers (I, L and J, M respectively) which does not occur in UFO::GUS (H, K). Scale bars for panels B-M: 100 μ M.

The differences observed in these staining patterns suggests that *GRA* and *NAN* do not have similar expression profiles to that of *UFO*. However, it could be the case that the sequences used in the *GRA::GUS* and *NAN::GUS* constructs do not fully represent the endogenous promoter regions. Several lines of evidence are in agreement with this. For instance, the microarray data curated by the *Arabidopsis* eFP browser states that at least *NAN* transcripts are detected within the shoot apex inflorescence region. Furthermore, *NAN* and *GRA* mRNA is detected in RT-qPCR analyses of inflorescence tissue enriched for young buds, and the T-DNA insertion lines *nan-1 gra-1* and *gra-2* demonstrate a clear reduction in expression when the same tissue is examined.

Another point to consider is that the staining patterns for *NAN::GUS* and *GRA::GUS*, while similar in that they occur in the same organ, appear to be specific to different tissues within the anther. Staining can be seen in the tapetum of *NAN::GUS* plants, while staining in *GRA::GUS* plants occurs in the vascular bundle. This could be evidence of sub-functionalisation between the genes, or it could be a limitation of the analysis if the full complement of endogenous regulatory elements is not present for the two genes.

5.2.2.2. Determining the endogenous expression profiles of GRA and NAN through in situ hybridisation

To further examine the endogenous expression profiles of *GRA* and *NAN*, *in situ* hybridisations were carried out on sectioned tissue inflorescence, using probes that were complementary to the 3'UTRs of either gene due to the similarity between their coding sequences. Preliminary analysis of the results suggests that both *GRA* and *NAN* are expressed in young floral tissues (**Fig. 5.13**). *GRA* expression was detected in the presumptive 4th whorl of stage 3 flowers, as well as in developing stamens and carpels of

stage 6 flowers. Furthermore, although only a weak signal was detected, expression can be seen within the anthers and ovules of flowers approaching stage 10. *NAN* expression was also detected in young floral buds and within ovules. The results obtained here suggest that the expression profiles determined using the transcriptional reporters do not fully capitulate the expression profiles of *NAN* and *GRA*. However, while the signals detected in this experiment agree with the findings of RT-qPCR experiments examining the levels of *NAN/GRA* mRNA in tissues enriched for young floral buds, it must be noted that these experiments were not optimised, and they require further validation before coming to any conclusions about the tissues in which these genes are expressed.

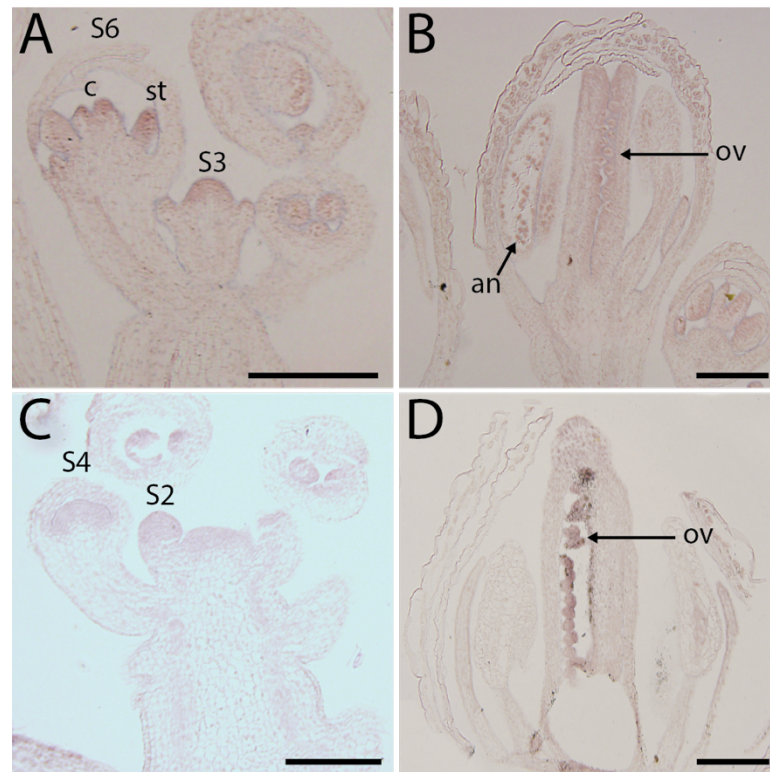


Figure 5.13 – Determining the endogenous expression profiles of *GRA* and *NAN*. Longitudinal sections of *in situ* hybridisations, using probes targeting *GRA* (A & B) and *NAN* (C & D) mRNA. *GRA* expression is apparent within the central dome of stage 3 flowers, and with the 3rd and 4th whorls of stage 6 flowers (A). Expression was also detected within anthers (an) and ovules (ov) of stage 10 flowers (B). *NAN* expression is apparent with stage 2 and stage 4 flowers (C), as well as within ovules (ov) in at stage 11 (D). Scale bars: 100 µm.

5.2.2.3. Physical interactions between GRA/NAN and ASK proteins

GRA and NAN are both described as putative F-box proteins, as their amino acid sequences are both predicted to contain N-terminal F-box domains (**Fig. 5.14**), as well as C-Terminal Kelch repeats acting as substrate interaction regions. A study by Koruda *et al.* (2012) showed that F-box proteins of the Kelch sub-family are likely to interact with *ARABIDOPSIS SKP1-LIKE 13 (ASK13)*, which is a member of the 21 gene *ASK* family (The *Arabidopsis* Genome Initiative, 2000). However, there is also evidence to suggest that a substantial amount of functional redundancy exists between different *ASK* members, such that an F-box protein could have more than one interacting partner (Gagne *et al.*, 2002; Kuroda *et al.*, 2012).

FBK84_ARATH	1	MNFQVEPPEKKTKNSSPHSPSSSSPSLSLLPEEIVVHCLARISRLYYPTLSLVSKSF	60
FBK85_ARATH	1	MKRQAKPPEKTKRTTNASSPTSSSSPSLSLLPDEIVENCLARISRSYYPTLSIVSKSF	60
		*: *:*****.:.:.:***** *:*** :***** *****:*****	
FBK84_ARATH	61	RSILSSTELYATRSHLGSTEQCVYLCLWDPSYQFPQWLRLLVNPNRTLANSIIKRRKKK	120
FBK85_ARATH	61	RSIISSSTELYVARSHLRNTEECVYVCLSDKSFEPKWFLLVWNPQ--ANSMVEKKRKKK	118
		:**.:****.***:***:* *:***:* *:****: ***:***:*****	
FBK84_ARATH	121	KTGQMLVPLTSSKFTSVSKATVVVGSEIYVLGGPVD---SAVRVLDCCSHTWRDAPSM	176
FBK85_ARATH	119	KTIGKLLVIPSSNLSPVSKSAIAGVSEIYVIGGKVDGALSSAVRILDCRSNTWRDAPSM	178
		** *:***: ***:***:*****:*** ** *****:*** ** *****:*** *	
FBK84_ARATH	177	NVSRMNAWACFHDGKIYVMGGCQGLK-DEPWAEVFNKTQTWEGLPEPGSEVRKCSIDRS	235
FBK85_ARATH	179	TVARKRPFICLYDGKIYVIGGYNKLSESEPWAIEVFDIKQTWECLSDPGTEIRNCTIYRI	238
		.:* . : *:*****:*** :*. *****: ***** *:***:***:*** *	
FBK84_ARATH	236	GVIEGKIEFGNVNEMCAYDTKLCKWEYCVNKSAAALRSECMENVSYGFWNMRLWYDNDI	295
FBK85_ARATH	239	AEIEGKIHFYTQKTYAYDPKQGEWECGEVAFPRSQCVMESVLYTFANNY-TWE-DDY	296
		. *****.*. :.: *** * :*** * .: * ***:***:*. * * * * *	
FBK84_ARATH	296	Q-----KDYWKRLGLESLDDKMYMRNGSSGNTTKLVACGGKLLLLWEGYMKHNPNNRK	349
FBK85_ARATH	297	GCKWSTDGYGEKVGLESLLEIHKRNGSSDNTTKLVACGGKLLLLWEGYMKHNPNNRK	356
		..* :.:***** : : *****.*****:*****:*****	
FBK84_ARATH	350	KIWCAVIAIEKCDGGGVGVGIVESVDVLYTVPISCQLLHCLVSV	393
FBK85_ARATH	357	KIWCAVIAIEKRDGGKVGIVGVVDVHIVPTSKLLHCLVSV	400
		*****:*** ** ***** ***: : ** ***:*****	

Figure 5.14 – Sequence alignment of NAN and GRA. Amino acid sequence alignment of NAN (FBK84_ARATH) and GRA (FBK85_ARATH). The predicted F-box domain is highlighted in yellow. Identical residues are denoted by asterisks (*). Strongly similar, and weakly similar, residues are denoted by colons (:) and periods (.), respectively. Alignment obtained using Clustal Omega (Söding, 2005).

To test whether NAN and GRA interact with members of the ASK family, ASK13 and ASK1 were used in a yeast 2-hybrid analysis (**Fig. 5.15**). ASK1 was chosen because of its high level of expression throughout the plant, and it has been shown to physically interact with other members of the SCF E3 ligase complex (discussed in Section 5.1). Physical interactions were detected for all combinations tested between NAN/GRA and ASK1/ASK13, using yeast 2-hybrid analysis, suggesting that NAN and GRA act as part of SCF complexes *in vivo*. Yeast that were auxotrophic for Tryptophan, Leucine, Adenine, and Histidine, were transformed with vectors containing bait proteins fused to either NAN or GRA (pGBKT7), and vectors containing prey proteins fused to either ASK1 or ASK13 (pGAD424). No growth was observed on selective media for cells transformed with 1 or 2 empty vectors. The interaction with both ASKs appears to be stronger for NAN, due to the ability of the transformed yeast to survive on dropout medium lacking both Adenine and Histidine. However, when the levels of the GRA and NAN proteins were checked in the yeast cells, NAN appeared to be present at higher levels. Hence, no conclusion can be made regarding the strength of the interactions.

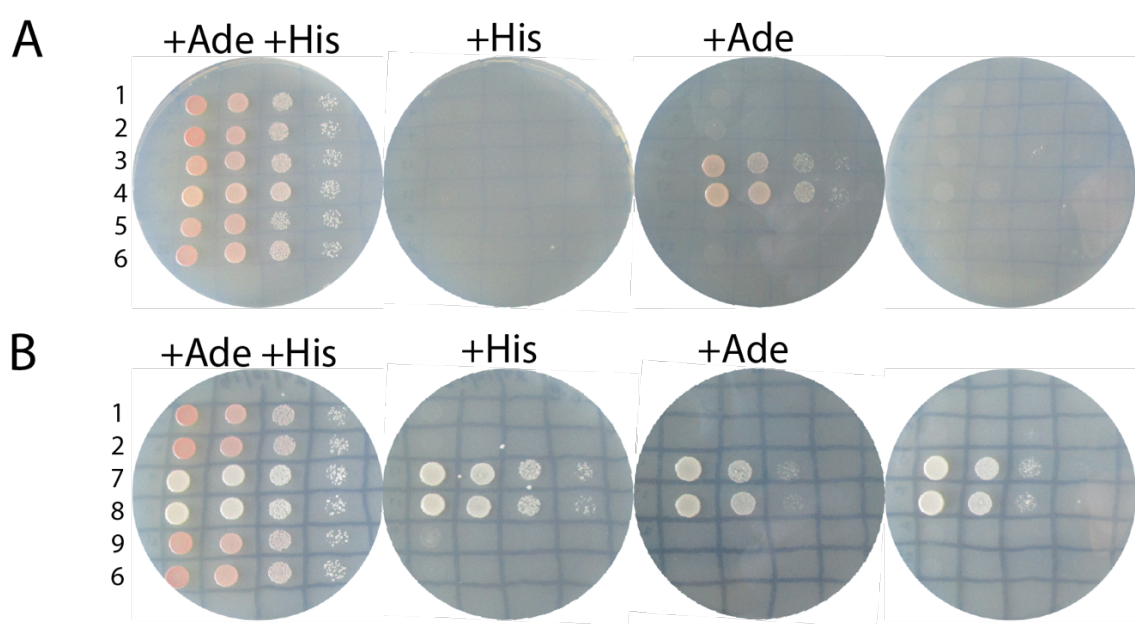


Figure 5.15 – Physical interactions between GRA/NAN and ASK1/ASK13. Yeast 2-hybrid analyses of GRA (A) and NAN (B) paired with ASK1 or ASK 13 by growth selection. Synthetic Complete medium was used (lacking Tryptophan and Leucine in all cases), with the addition of Adenine (+Ade) and/or Histidine (+His). **1** - pGBKT7 + pGAD424 ASK1; **2** - pGBKT7 + pGAD424; **3** - pGBKT7 GRA + pGAD424 ASK1; **4** - pGBKT7 GRA + pGAD424 ASK13; **5** - pGBKT7 GRA + pGAD424; **6** - pGBKT7 + pGAD424 ASK13; **7** - pGBKT7 NAN + pGAD424 ASK1; **8** - pGBKT7 NAN + pGAD424 ASK13; **9** - pGBKT7 NAN + pGAD424. The pGAD424 vector contains the *LEU2* nutritional marker, and the pGBKT7 vector contains the *TRP1* nutritional marker, allowing for the synthesis of Leucine and Tryptophan, respectively. This experiment was performed in collaboration with Dr. Emmanuelle Graciet, Maynooth University.

5.2.3. Generation of transgenic lines over-expressing GRA or NAN

To dissect the biological functions of both GRA and NAN within the context of flower development, several over-expression constructs were generated. All constructs are expressed using the Cauliflower Mosaic Virus 35S promoter (CaMV 35S / 35S::), which exhibits constitutive expression within *Arabidopsis* (**Figure 5.16**) (Ow *et al.*, 1987). First, the coding sequences of both genes were cloned into vectors harbouring just the CaMV 35S promoter (**Fig. 5.16 A & B**), and second, C-terminal fusions were generated with either a dominant repressive or activating domain (termed SRDX, and VP16, respectively) in order to gain further insight into the molecular functions of both proteins (**Fig. 5.16 C-F**).

The SRDX domain is a modified repressive amino acid sequence (LDLDLELR LGFA), derived from an Ethylene-responsive Element Binding Factor (ERF) associated Amphiphilic Repression (EAR) motif located within the zinc finger transcription factor SUPERMAN (SUP), an active repressor that is involved in flower development (Ohta *et al.*, 2001; Hiratsu *et al.*, 2002, 2003). Translational fusion constructs possessing this domain exhibit a dominant repressive activity, which are able to overcome the potential confounding effects of redundant genes, thereby providing a simple method of rapidly analysing the effects of repressing the activities of target genes.

The VP16 transcriptional activation domain is derived from the 78-amino acid carboxy-terminal sequence of the protein, encoded by Herpes Simplex Virus (Triezenberg, Kingsbury and Mcknight, 1988). It has been shown to interact with TATA-binding protein, TFIIB, and the SAGA histone acetylase complex *in vivo*. Translational fusion of this domain results in a dominant activation of the target protein. Multiple studies have

demonstrated the reliability of these activating and repressive domains, and although these techniques are more typically employed using fusions to transcription factors, it has been demonstrated that such fusions can also produce altered phenotypes when fused to F-box proteins, a recent example being the fusions of both domains to UFO (Chae *et al.*, 2008; Risseuw *et al.*, 2013; Hanczaryk, 2014). One possible reason for this is, at least in the case of UFO, that it is known to interact with the DNA-binding transcription factor LFY, and the steric proximity of the dominant domains when associated with this transcription factor could be sufficient to confer their respective activities upon the transcriptional machinery. Translational fusions between the SRDX and VP16 domains, and GRA and NAN, have therefore been generated to determine if these modifications could confer phenotypes reminiscent of mutants or over-expression lines that have already been established for other regulators of flower development, therefore providing clues to the placement of *GRA* and *NAN* in the gene regulatory network.

Arabidopsis transformants, possessing each of the transgenes described above, were obtained and were observed for any changes to the phenotypes of flowers. All transformants were screened for the presence of the transgenes by PCR genotyping, using primers within the CaMV 35S promoter and the coding regions of *NAN* or *GRA*. Organ counts for each of the lines suggest that over-expression of these genes does not produce any overt changes to the development of the flower, as the mean average floral organ frequency in each whorl matched that of wild-type flowers. In addition, no differences to organ morphology were observed. Details of the experiments can be found in Tables 5.1 and 5.2.

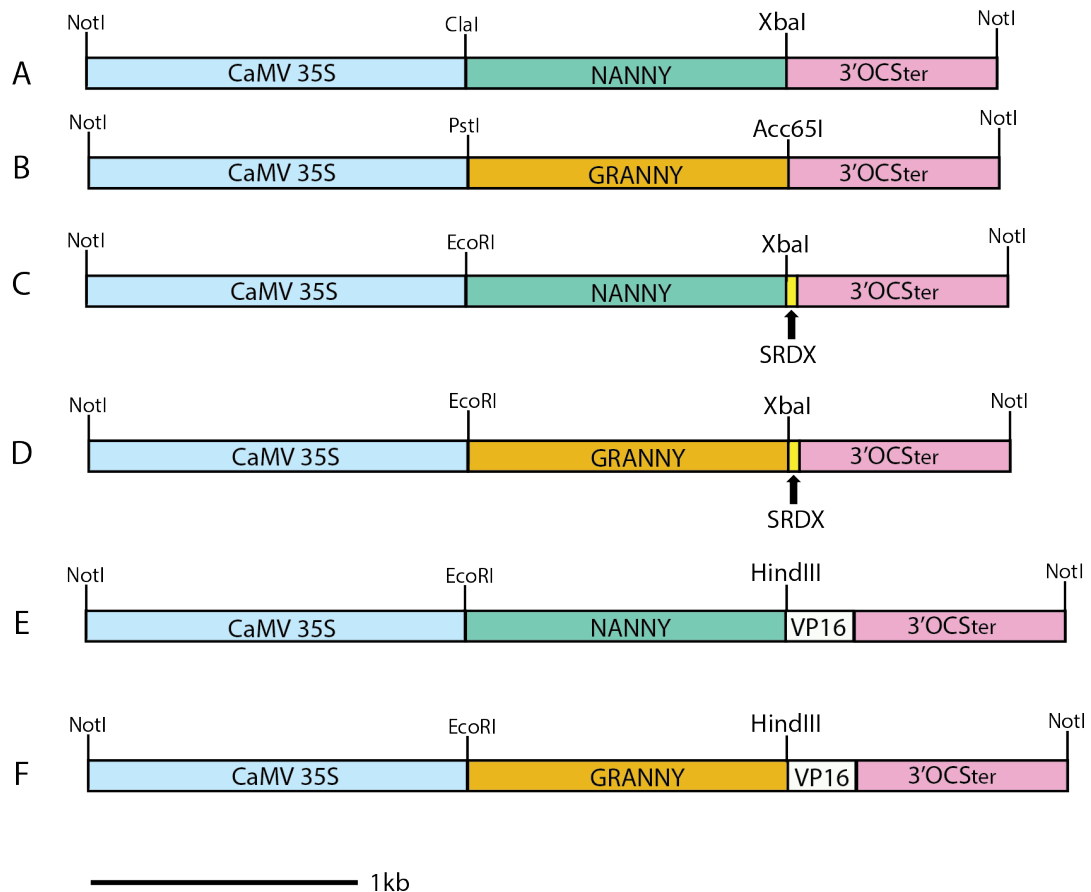


Figure 5.16 – Over-expression of *GRANNY* and *NANNY*. Simplified schematics of the over-expression constructs generated and transformed into wild-type Col-0 plants in this study: (A) 35S::*NAN* (B) 35S::*GRA* (C) 35S::*NAN*-SRDX (D) 35S::*GRA*-SRDX (E) 35S::*NAN*-VP16 (F) 35S::*GRA*-VP16. All of the coding sequences were expressed using the CaMV 35S promoter and followed by the *Agrobacterium*-derived *OCTOPINE SYNTHASE* (*OCS*) terminator sequence (3'OCS_{ter}). The coding sequences for *NANNY* and *GRANNY* in C-F were cloned without their respective stop codons to allow for the transcription of the SRDX or VP16 domains. Restriction sites used in the cloning procedures are labelled. All constructs were ligated into the pML-BART (Eshed *et al.*, 2001) binary vector for transformation into competent *Agrobacteria*. Scale bar: 1kb.

With the exception of the 35S::*GRA* construct, none of the other transgenic lines had been generated previously. In the case of 35S::*GRA*, it had previously been reported that its over-expression results in a progressive loss of petal numbers in affected plants (Hanczaryk, 2014). Petals in a few independent lines were reported as appearing

misshapen, and in one case petal development becomes fully ablated in later arising flowers (Hanczaryk, 2014). In this line (#6.4), petal numbers are progressively reduced in successive flowers until the 2nd whorl becomes absent in some T2 individuals by the time the 15th flower has arisen (**Fig. 5.17**). Beyond this point petal development in all successive flowers is largely absent.

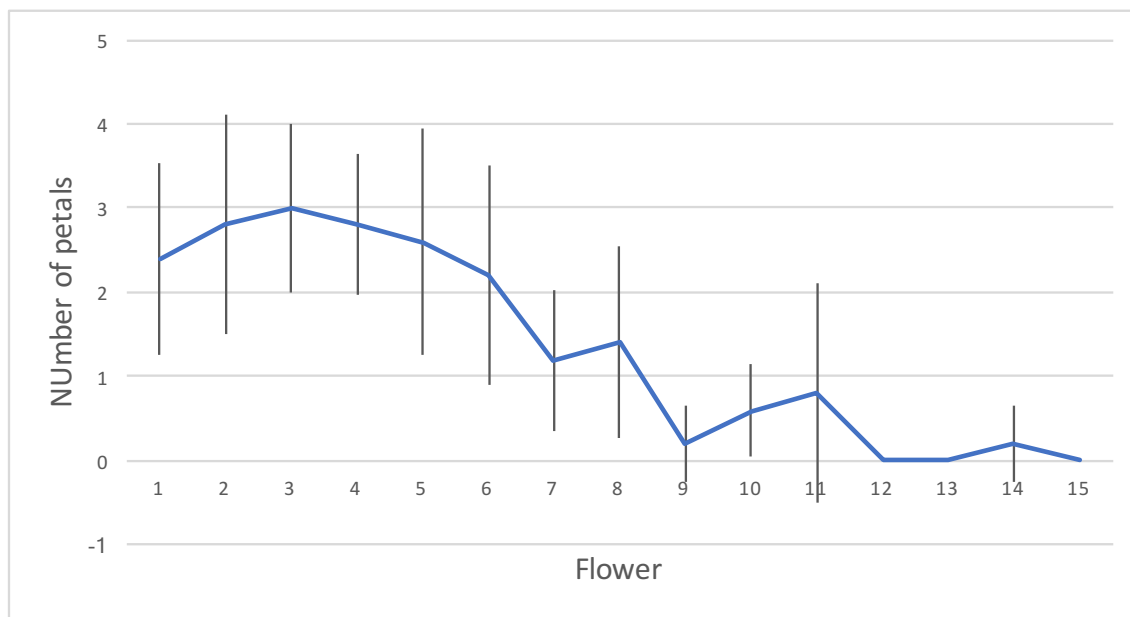


Figure 5.17 – Progressive reduction of petal numbers in 35S::GRA T2 #6.4 flowers. Counts of the number of petals per successive flower of the GRA over-expression line reported in Hanczaryk (2014). Flowers 1-15 were counted on 5 plants. Error bars represent the standard deviation.

As this phenotype was seen in a single line in the T2 generation, it is possible that the loss of petals is due to the insertion of the T-DNA within a region of the genome that is responsible for 2nd whorl development or maintenance. In addition, segregation analysis suggests that plants heterozygous for the transgene do not have the mutant phenotype, as approximately 25% of the plants in the T2 generation possessed the defect. This could indicate that either heterozygous plants do not express *GRA* to a sufficiently high level to

cause the phenotype, or it could be the case that the genomic region in which the transgene was introduced contained a gene required for petal development. To verify this, the locus in which the T-DNA is situated could be identified using thermal asymmetric interlaced (TAIL) PCR (Liu *et al.*, 1995). Considering that 47 35S::*GRA* transformants were obtained in this study, none of which displaying a phenotype similar to that observed above, it is likely that the petal development defect seen is a consequence of something other than over-expression of *GRA*.

One potential issue to be considered, in light of the fact that none of the transgenic lines that were generated resulted in any obvious phenotypes, is that the constructs used did not function properly when transformed into *Arabidopsis*. To confirm that these transgenic lines were ectopically expressing *NAN/GRA*, T2 seedlings were grown on 0.5X MS agar supplemented with 50 µM DL-phosphinothricin to select for those individuals carrying the transgenes. RNA was extracted from the seedlings of three randomly selected T2 lines of each genotype, and RT-qPCR was performed to determine the relative levels of *NAN* and *GRA* mRNA (**Fig. 5.18**). The values obtained were compared to those for a reference gene (REF1) and normalised against the wild-type expression levels of both genes found in seedlings. The results show that in the majority of cases, the lines examined were ectopically expressing *NAN* or *GRA* as intended. In the instances where the level of expression was similar to the wild-type samples, this could potentially be explained by the location of the transgene within the genome, leading to lesser expression at the developmental stage used. Furthermore, other lines of the same genotype showed high levels of ectopic expression, demonstrating that the transgenes used were transcriptionally functional.

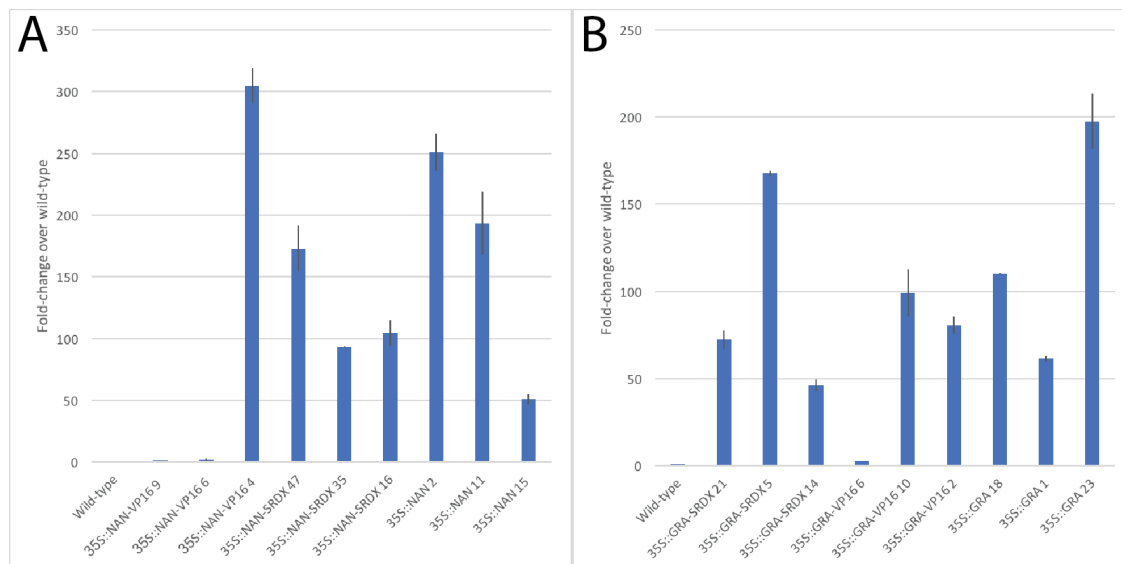


Figure 5.18 – *NAN* and *GRA* mRNA levels in seedlings of transgenic over-expression lines. Results of RT-qPCRs to determine the relative levels of *NAN* (A) or *GRA* (B) mRNA in seedlings of randomly-selected T2 over-expression lines. Two biological replicates were used for each line, and two technical replicates were performed for each sample. Error bars represent the standard deviation. Data are shown as fold-change over the wild-type results.

One aspect not fully considered during these experiments was that, following the results of the GUS reporter lines, the ectopic expression of *NAN/GRA* could have caused changes to the morphology within the anther, or some part of the pollen maturation process, as staining occurred in the anthers of *NAN::GUS* and *GRA::GUS* lines. While no obvious changes to any of the over-expression lines' fertility were observed, it remains a possibility that stamen development was affected in some manner not readily detectable through simple counts of the floral organs.

Table 5.1 – Organ counts of over-expression lines

Line	Sepal	Mosaic - Sepaloid petal	Petal	Mosaic - Stamen/Petal	Stamen	Filament	Ectopic Carpel (Whorl 3)	Carpel
35S::NAN	4	0	4.033 ± 0.02	0.03 ± 0.023	6 ± 0.05	0	0	2
35S::NAN-SRDX	3.97 ± 0.22	0.04 ± 0.28	3.95 ± 0.33	0.07 ± 0.29	5.74 ± 0.65	0	0.01 ± 0.1	2.01 ± 0.1
35S::NAN-VP16	4.11 ± 0.35	0	4.04 ± 0.31	0.04 ± 0.17	5.57 ± 0.82	0.02 ± 0.151	0	2
35S::GRA	4 ± 0.12	0	4 ± 0.13	0.04 ± 0.2	5.8 ± 0.5	0	0	2
35S::GRA-SRDX	4	0	4	0	5.91 ± 0.34	0 ± 0.09	0	2
35S::GRA-VP16	4	0	3.99 ± 0.18	0.04 ± 0.2	5.8 ± 0.49	0	0	2 ± 0.07
Col-0 (67)	4.044 ± 0.21	0	4	0.12 ± 0.33	5.57 ± 0.61	0.015 ± 0.12	0	2

Table 5.2 – Experimental details of NANNY/GRANNY over-expression lines

Line	Transformants obtained	Flowers counted
35S::NAN	22	317
35S::NAN-SRDX	30	100
35S::NAN-VP16	13	130
35S::GRA	47	431
35S::GRA-SRDX	24	120
35S::GRA-VP16	35	191

5.2.4. Generation of stable knockouts of *AT4G19870* and *AT4G19865*

CRISPR/Cas9 was used with the intention of disrupting the ORFs of the genes under study in order to obtain null mutants. As Cas9's nuclease activity is limited to sequences adjacent to the PAM sequence (Jinek *et al.*, 2012), in this case 5'NGG3', the scope for targeted mutations is restricted to discrete regions in the target gene. To obtain mutations with the greatest potential of producing a full knockout of *GRA* or *NAN*, such that the likelihood of a frameshifted ORF still possessing some residual activity was reduced, guide sequences were selected as close to the transcriptional start site (TSS) as possible while adhering to the parameters for successful mutagenesis. The online tool *Cas-Designer* (Park, Bae and Kim, 2015) was used to identify potential guide sequences. Sequences were selected that were close to the TSS, had zero predicted off-target effects when 1 or 2nt were mismatched, and had an out-of-frame score over 66%. The out-of-frame score is a metric which predicts the likelihood of induced mutations remaining in-frame due to microhomology-mediated end joining during the DNA repair process (Bae *et al.*, 2014). Appendix 6.3 lists the sequences used in this study. Potential off-target sites for regions of the genome that had up to 4nt mismatches within the guide sequence were identified using *Cas-OFFfinder* (Bae, Park and Kim, 2014).

Two CRISPR/Cas9 systems are used in this study, pGREEN U6-26::sgRNA-U6-26_{ter} ICU2::Cas9-NoS_{ter} and pHEE 401 U6-26::sgRNA-U6-26_{ter} EC1.2::zCas9-rbseE9_{ter} (herein referred to as pGREEN, and pHEE, respectively). The main functional difference between the vectors is the promoters used to drive Cas9 expression, and the codon optimisation of Cas9. The promoters used, *INCURVATA2* (*ICU2* – AT5G67100) and *EGG CELL 1.2* (*EC1.2* – AT2G21740), express Cas9 at different stages in the plant's life cycle. *ICU2* is primarily active in rapidly dividing cells, allowing for Cas9 to be expressed

in meristematic tissues (Hyun *et al.*, 2014), while the *EC1.2* promoter is active only in the plant egg cell and the embryo (Wang *et al.*, 2015). Thus, although the *ICU2*-driven Cas9 is active for a longer period of time throughout the plant's life cycle, any mutations generated in T1 plants are likely to be as part of a chimeric population within any given transformant, and will not be fully isolated until the T3 generation, as first an individual non-chimeric mutant must be isolated in the T2 population and left to go to seed. Conversely, the *EC1.2* driven Cas9 will only be active for a short period of time around embryogenesis, but as the number of cells available for mutagenesis is much lower, any acquired mutation is likely to be present at a higher frequency in an affected transformants. As such, heterozygotes and mutant homozygotes potentially can be identified in T1 plants by sequencing of PCR fragments, and it is possible to obtain a stable mutant population one generation earlier than with the former construct. Additionally, Cas9 driven by the *EC1.2* promoter, in the pHEE401 vector, has been codon optimised for *Zea mays* (referred to as zCas9), which might contribute to more efficient translation in comparison to the non-optimised Cas9 sequence used in the pGREEN vector. Both systems were used simultaneously (in separately transformed lines) in order to improve the chances of obtaining heritable mutations.

Identifying *Arabidopsis* transformants possessing mutations in the desired regions can be challenging if the targeted gene(s) do not have any obvious mutant phenotype, as additional screening is required to select those individuals that have undergone mutagenesis. Outlined below are the strategies used to isolate null mutants for GRA and NAN separately, and together as a double mutant. As the use of CRISPR/Cas9 systems has not been previously established in the Wellmer laboratory, some differing methods were used for the each of the isolated mutations.

5.2.4.1. Identification of transformants possessing mutations in the GRA coding sequence

Five sgRNAs were designed and incorporated in to the pGREEN Cas9 vector, targeting the *GRANNY* coding sequence, and of these GRAsg1 and GRAsg5 were selected for further use. Twelve *Arabidopsis* transformants were obtained for the pGREEN GRAsg5, and 24 transformants for the pGREEN GRAsg1 line. To determine if mutagenesis had occurred within these plants it was necessary to extract genomic DNA from individual transformants and PCR amplify the region surrounding the intended sites of cleavage. T7E1 endonuclease was used to detect heteroduplex formation as a result of DNA mismatching of the amplified fragments. In those individuals where mutagenesis had occurred, it was likely that the sample taken for genomic DNA extraction was chimeric for the wild-type and mutated sequences. Therefore, prior to the addition of T7E1 endonuclease the PCR product was denatured and cooled to room temperature slowly to promote the annealing of wild-type and mutated strands of DNA. As Cas9 has been shown to precisely cleave target regions at 3bp upstream of the PAM sequence (Jinek *et al.*, 2012), the size of digested PCR fragments could be easily predicted.

Using this method, mutations were detectable within transformants possessing the GRAsg5 guide RNA. Two individuals were selected for further analysis to determine the nature and frequency of the mutations, GRAsg5-1 and GRAsg5-9. To examine the mutations present from the inflorescence tissue samples used, a 1kb region of the genomic locus was amplified that contained the predicted cleavage site at ~300bp from the 3' end of the fragment. The purified fragment was sequenced to verify the result of the T7E1 assay from a qualitative standpoint (**Fig. 5.19**).

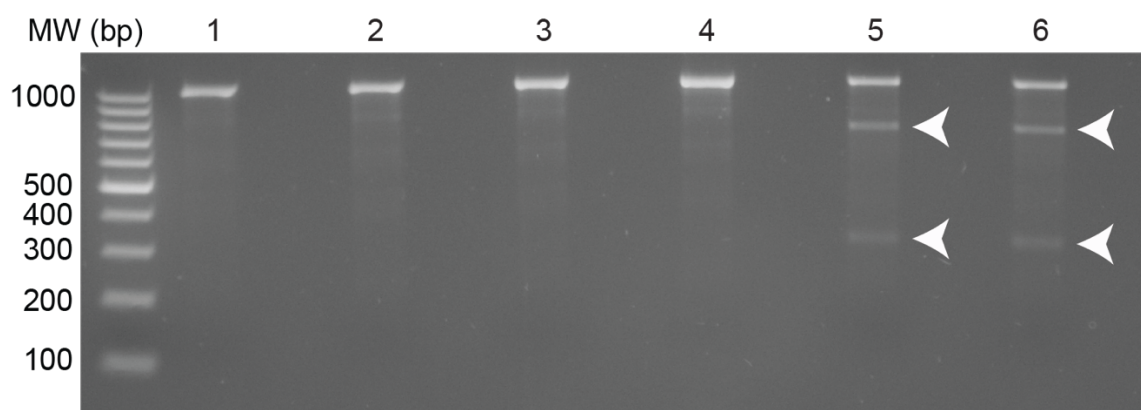


Figure 5.19 – Detection of Cas9-mediated mutations by analysis of heteroduplex formation and digestion. Agarose gel electrophoresis (2% (w/v)) of genomic DNA derived from inflorescences of independent T1 pGREEN GRAsg1 and GRAsg5 transformants. A ~1000bp region surrounding the target sites were amplified by PCR, and T7E1 endonuclease was used to detect heteroduplex formation. Lane 1 contains wild-type DNA, lanes 2-4 contain DNA derived from juvenile inflorescences of pGREEN sgGRA1 T1 transformants. Lanes 5 and 6 contain DNA derived from inflorescences of pGREEN sgGRA5 T1 transformants, and show bands suggestive of heteroduplex DNA digestion (arrowheads). The combined molecular weights of the bands agree with the predicted digestion pattern.

The presence of multiple nucleotide peaks downstream of the predicted cleavage site in the GRAsg5.1 and 5.9 samples indicated that the tissue collected contained more than one mutational event (**Fig. 5.20**). To analyse the different mutations the *Tracking of Indels by DEcomposition (TIDE)* software was used (Brinkman *et al.*, 2014), wherein sample Sanger chromatographs are compared against a wild-type chromatograph to assess the size and composition of indels. Both samples were predicted to be mosaics, consisting primarily of wild-type tissue, followed by several different low-frequency mutations. +1 Insertions were present among both samples to different degrees, though in 5.9 a 5bp deletion was also detected with greater frequency (**Fig. 5.21**).

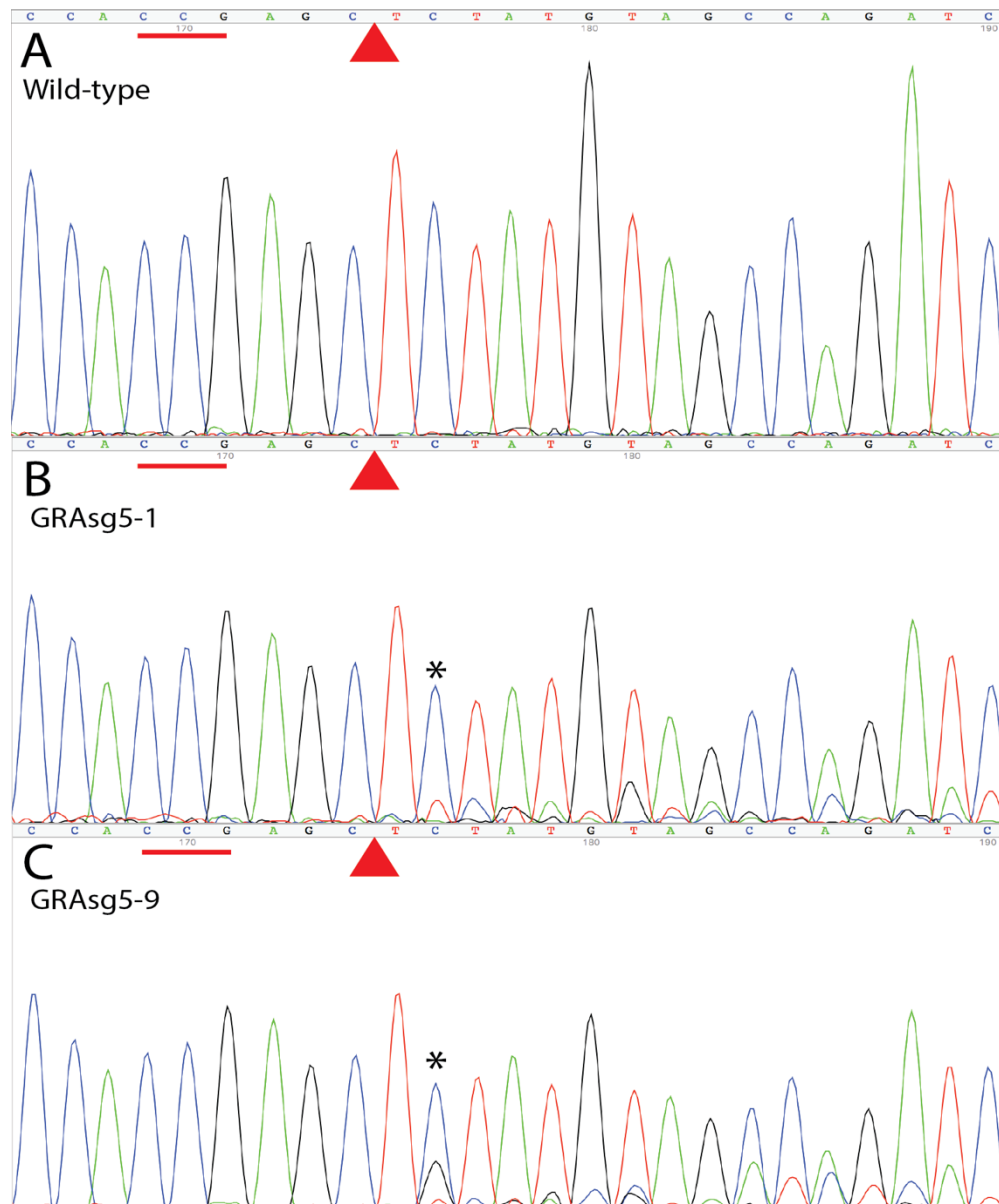


Figure 5.20 - Sanger sequencing of the predicted cleavage site of GRAsg5 transformants. Reverse strand Sanger chromatographs of PCR fragments amplified around the target site of the GRAsg5 guide RNA in wild-type (A), and two GRAsg5 transformants (B and C) reveal the presence of CRISPR/Cas9 induced mutations. PAM sequences are underlined in red and the predicted Cas9 cleavage sites are denoted by red triangles. Mutations within chimeric tissue samples used in (B) and (C) can be detected by the presence of multiple nucleotide peaks (*) occurring downstream of the cleavage site.

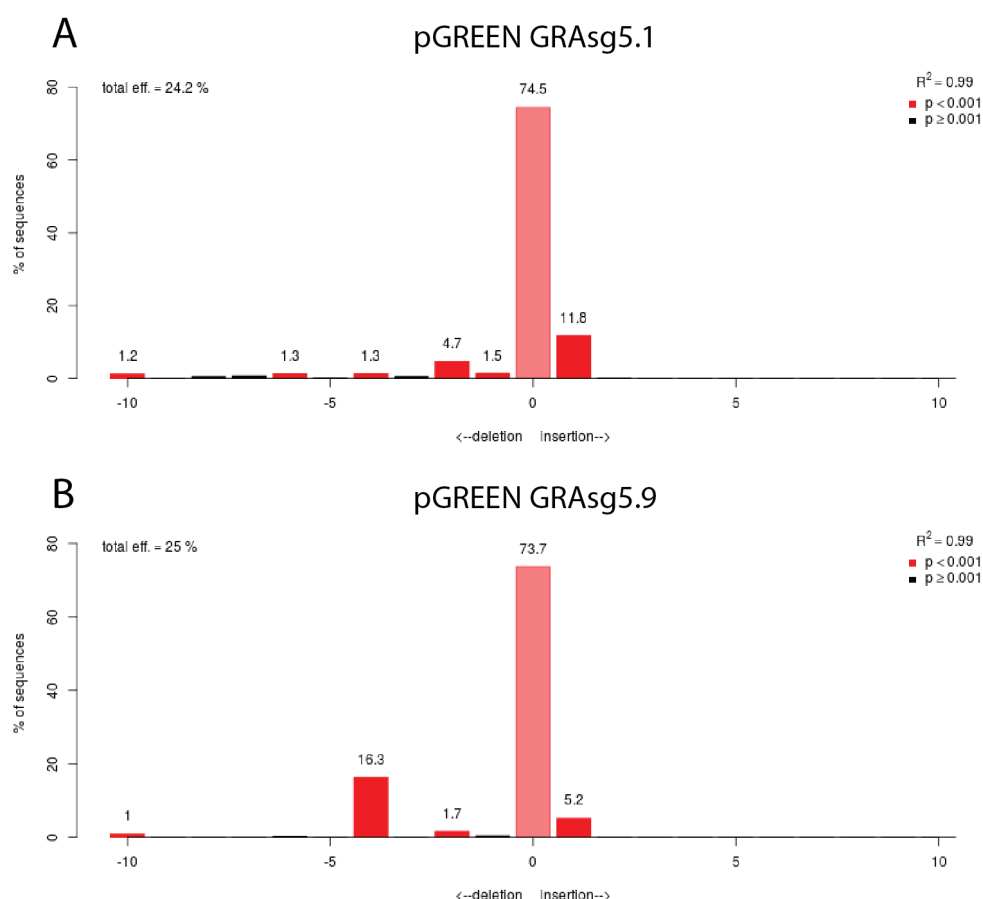


Figure 5.21 - Assessment of CRISPR-Cas9 genome editing in primary pGREEN GRAsg5 transformants. Distributions of predicted indel sizes and frequencies for pGREEN GRAsg5.1 (**A**) and pGREEN GRAsg5.9 (**B**). Red histogram bars signify a p -value of <0.001 . Black histogram bars have a p -value of ≥ 0.001 . Panels generated using *TIDE* (Brinkman *et al.*, 2014).

5.2.4.2. Establishment of a stable GRA mutant allele

Examination of the various mutations present within the GRAsg5.1/5.9 plants suggested that both individuals were chimeras, each possessing several different mutations at the intended target site at low frequencies. Both lines were allowed to self-fertilise in order to select individuals in the following generation that would potentially lack the transgene and be homozygous for some mutation, and the 5.1 line was used for further analysis.

As the T2 individuals did not display any overt phenotypes, and the range of predicted mutations was primarily single base insertions, simple PCR-based genotyping was not an adequate method of identification. Instead, mutants were identified by genomic DNA extraction using tissue from the first cauline leaf of each plant's primary stem, followed by sample pooling (4 samples in 6 pools) and nested PCR to amplify the genomic region around the predicted Cas9 cleavage site. The PCR products were then digested using Surveyor™ nuclease (similar to T7E1, discussed in Section 1.7.2.2) to detect the presence of heteroduplexes caused by the annealing of mismatching strands of DNA. Agarose gel electrophoresis analysis of the digested samples suggested that all samples pools contained DNA with mismatches around the predicted site, as the molecular weights of the products of digestion agreed with the predicted digestion pattern (**Fig. 5.22**). Further to this, the same region was amplified from 8 individual genomic DNA extractions (#9-16), and the PCR fragments were analysed using Sanger sequencing. The results of the sequencing showed that 6 (#10;11;12;14;15;16) of the samples were mosaics, 1 (#9) remained predominantly wild-type, and 1 (#13, hereafter referred to as GRAsg5.1-13) primarily contained a 17bp deletion at the cleavage site, which is predicted to cause a frameshift and result in an early stop codon 249bp downstream of *GRA*'s start site (**Fig. 5.23**). Indel spectrum analysis of the chromatograph for GRAsg5.1-13 indicates that, while the majority of the tissue sample used contained the deletion, a small percentage (5.4%) is predicted to remain wild-type. However, this result could be due to some error associated with the *TIDE* program's interpretation of the chromatograph.

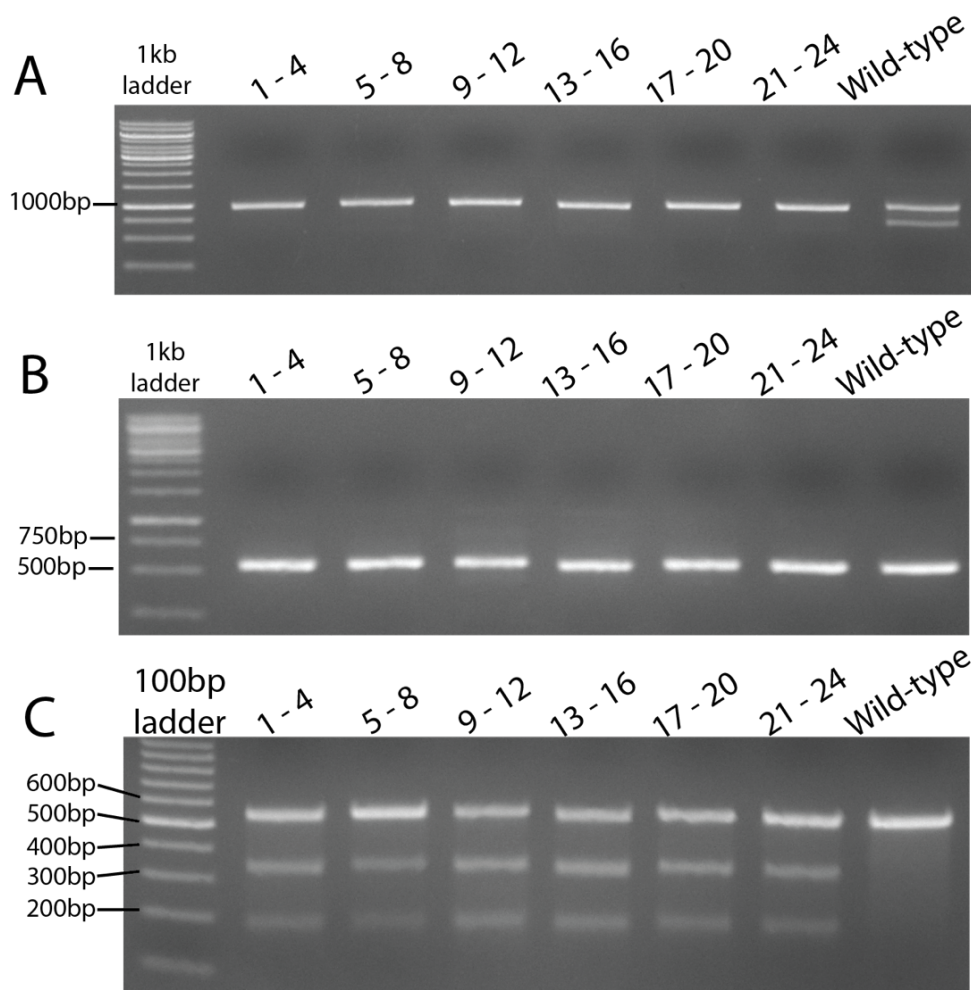


Figure 5.22 - Detection of DNA heteroduplex formation in pooled pGREEN GRAsg5.1 T2 plants through agarose gel electrophoresis. (A) PCR to amplify genomic region surrounding target site. Non-specific amplification can be seen in the wild-type lane, and faintly in the other lanes, which is eliminated through a subsequent round of PCR using nested primers **(B)**. **(B)** PCR using nested primers and a 1 μ l of a 1/15th dilution of the PCR products from **A**. **(C)** Surveyor™ nuclease digestion of 10 μ l of PCR products from **B** using a 2% (w/v) gel. The presence of bands at ~200bp and ~350bp corresponds to the predicted digestion pattern.

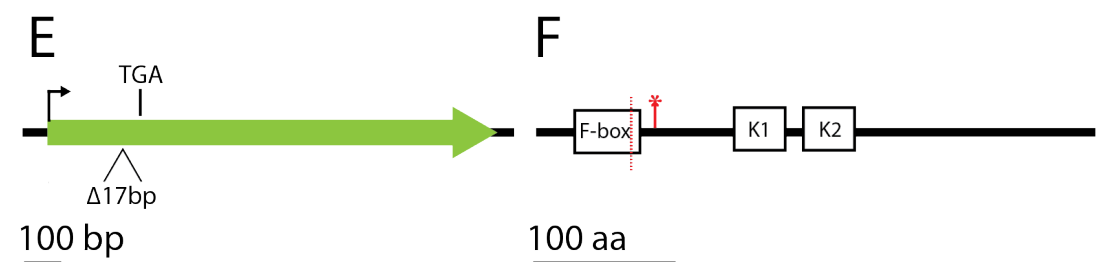
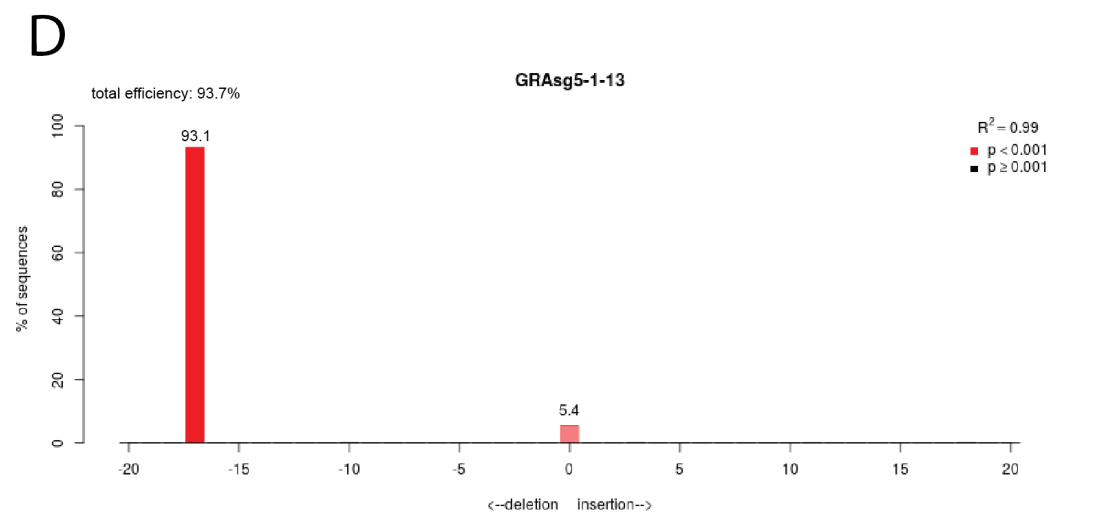
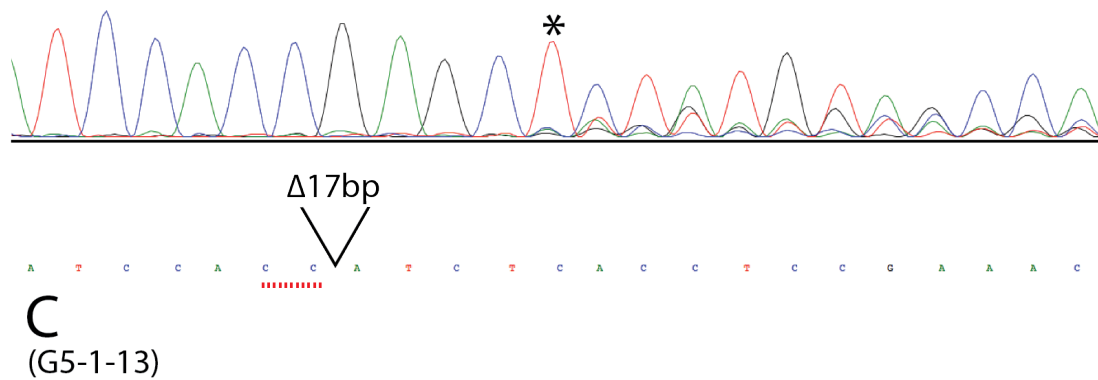
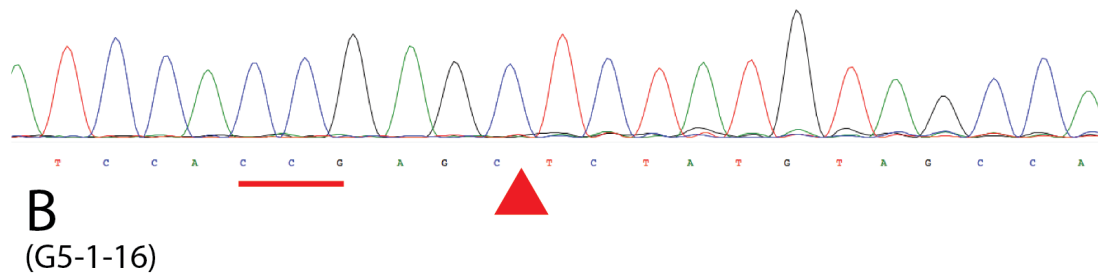
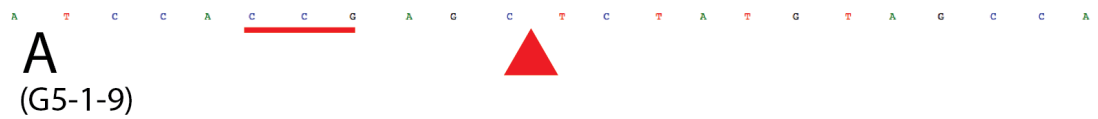


Figure 5.23 – Detection of mutations in pGREEN GRAsg5.1 T2 plants. Sanger chromatographs of PCR fragments amplified around the target site of the GRAsg5 guide RNA in representative T2 individuals. PAM sequences are underlined in red and the predicted Cas9 cleavage sites are denoted by red triangles. **(A)** A T2 plant that has not undergone any significant mutation events. **(B)** A mosaic individual possessing multiple indel alleles, denoted by the presence of more than 2 peaks for the majority of nucleotides downstream of the Cas9 cleavage site (*). **(C)** An individual primarily possessing a 17bp deletion within the *GRA* coding sequence. The dashed red line denotes the remaining nucleotides of the PAM sequence. **(D)** Predicted indel frequency for GRAsg5.1-13 in **(C)**. The deletion is estimated to comprise 93.1% of the tissue sample used for PCR and sequencing. Red histogram bars indicate a *p*-value <0.001. **(E & F)** Schematic representations of the predicted effects of a 17bp deletion in the *GRA* coding sequence from **C**. **(E)** The deletion (marked by the arrowhead) results in an early stop codon at 249bp after the start codon. **(F)** The frameshift results in premature termination (red *) of translation 16 amino acids after the cleavage event (red dashed line). F-box: F-box domain; K1 and K2: Predicted Kelch repeat domains.

5.2.4.3. Analysis of Cas9 off-target effects in the pGREEN GRAsg5-1.13 T2 individual

When generating targeted mutations using the CRISPR/Cas9 system it is important to ensure that Cas9's nuclease activity is limited to the intended genomic site. In *Arabidopsis*, and other plant species, the present consensus is that off-target effects are a rare occurrence (Peterson *et al.*, 2016, discussed in Section 1.7.2.3). However, to confirm that the results of any downstream analyses of newly generated mutants are caused because of the intended disruption to the gene(s) under investigation, and not unwanted disruptions to other regions in the genome, it is prudent to analyse sequences that have a high level of identity to the guide RNA used.

To investigate the sequence integrity of the potential GRAsg5 off-target sequences, which were flagged by the online program *Cas-OFFinder* (Bae, Park and Kim, 2014) (**Table 5.3**), primers were designed to PCR amplify 200-400bp regions either side of all genomic regions that contained the guide RNA sequence, with up to 4 mismatches, from the GRAsg5-1-13 T2 individual. Sanger sequencing of the five identified loci suggests that, in this instance, there have been no detectable off-target events as the sequences fully match their respective entries in the TAIR10 annotated genome, and the chromatographs for each sequencing reaction show that all base calls are associated with single nucleotide peaks (**Fig. 5.24**).

Table 5.3 – Predicted off-target sites for the GRAsg5 guide RNA.

Target (5' – 3') *	Chromosome	Position (AGI)	Mismatches
AGATCTGGtTcCATAGAAcG	3	2747506 (AT3G09000)	4
AGATCTGcCTtCATAAtCT	1	9893611 (AT1G28290) [†]	4
AGATCTGtgTACcTAGAACT	1	17700931 (AT1G47990) [†]	4
AGActTGGCTACATAGAGgT	1	23772138 (AT1G64060)	3
AGgTCTGGCgACATAAtCT	5	3629539 (AT5G11370)	4

* Mismatching nucleotides are denoted in lower case.

[†] Closest TAIR10 annotated gene

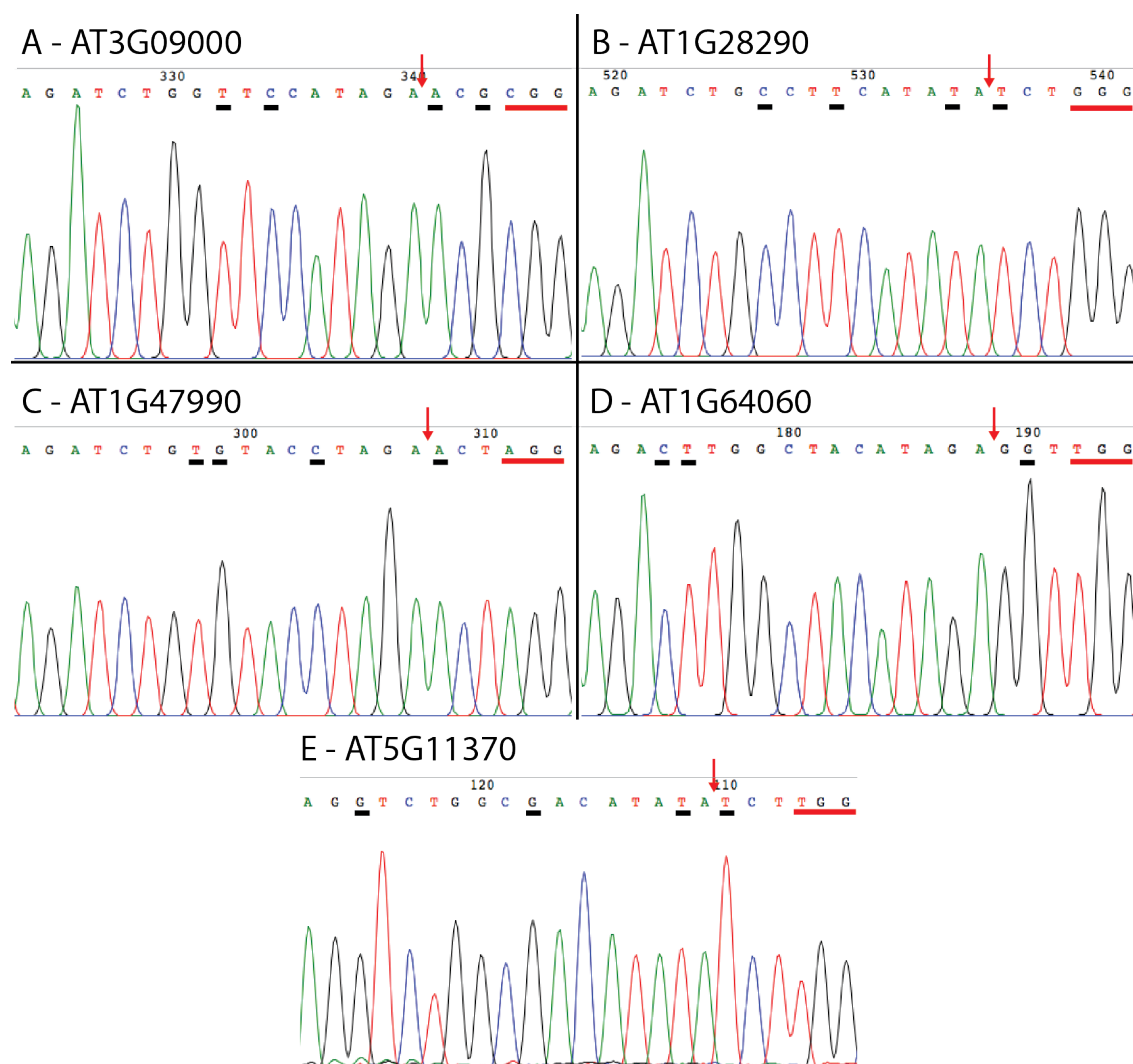


Figure 5.24 – Analysis of potential off-target effects of Cas9 in the GRAsg5-1-13 T2 plant. Sanger chromatographs of five loci predicted by the *Cas-OFFinder* program to be potentially subject to Cas9 nuclease activity, mediated by imperfect base-pairing between the genomic sequences and the GRAsg5 guide RNA. Black bars underline bases that are mismatched to the GRAsg5 target sequence. Red bars underline PAM sequences, and red arrows show the predicted Cas9 cleavage sites. AGI codes denote the TAIR10 annotated gene in which the off-target site is located, or the closest gene to the site in the cases of (B) and (C). No mutational events are detectable in all panels.

5.2.4.4. Detection of homozygous *GRAsg5.1-13* mutants

Progeny of the self-fertilised *GRAsg5.1-13* T2 plant were grown to observe any phenotypic differences, between the mutants and wild-type Col-0, that were a result of the disruption of the *GRA* ORF. To confirm the inheritance of the mutation, primers were designed to amplify a ~200bp region either side of the deletion. A third primer was designed that would anneal to the sequence that had been deleted, thereby allowing for the amplification of a ~200bp fragment should the wild-type allele be present. PCR genotyping of 16 T3 plants suggested that all were either heterozygotes or wild-type, as agarose gel analysis revealed products of amplification that corresponded to the presence of the deleted sequence in all samples. Sanger sequencing of the PCR products showed that the majority of the plants genotyped appeared to be heterozygous for the mutation, suggesting that the silique from which the seeds were obtained might have been accidentally fertilised by a wild-type plant. Due to time constraints, further batches of seeds from this plant have not been grown and analysed, although it is expected that homozygous *gra* mutants will be present.

5.2.5. Generation of a mutant *NANNY* allele by CRISPR/Cas9

Several vectors containing guide RNAs targeting the *NANNY* coding sequence were generated in order to obtain a transgenic line with a frameshift mutation. 40 *Arabidopsis* transformants harbouring the pHEE 401 U6-26::NANsg10-U6-26_{ter} EC1.2::zCas9 (Hereafter referred to as NANsg10) transgene were selected for analysis. As the frequency of mutation is predicted to be higher in the pHEE vector system when compared to the pGREEN system, primary transformants that were identified using hygromycin selection were screened for mutations by directly sequencing PCR products of the amplified target region instead of preliminary detection using DNA mismatch-sensitive nucleases as described above.

PCR fragments were generated using genomic DNA extracted from each of the transformants, and Sanger sequencing was performed on 8 individuals around the predicted target site for the NANsg10 guide RNA. One individual was identified that was heterozygous, NANsg10.18, possessing a 10bp deletion at the predicted zCas9 cleavage site, and wild-type for the other allele (**Fig. 5.25**). No mutations were detected in the remainder of the sequenced samples. The deletion is located 116bp after the start codon of *NAN* and the subsequent frameshift is predicted to result in an early stop codon at 97 amino acids after the initiation of translation. As the mutation is located within the F-box domain of NAN, any product of translation should not closely resemble the wild-type sequence, therefore producing a knockout allele of *NAN*.

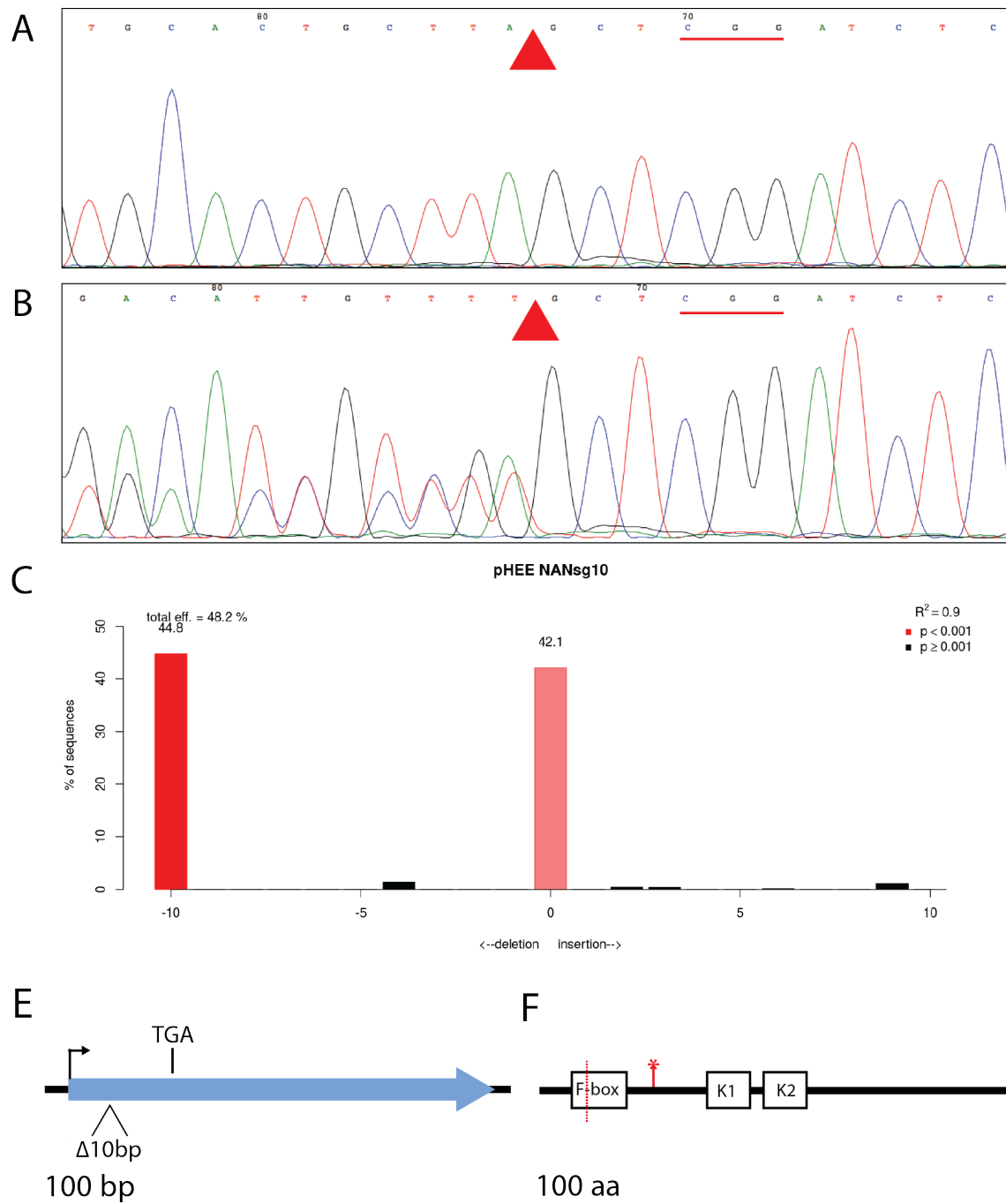


Figure 5.25 – Detection of a mutation in the *NAN* coding sequence in pHEE NANsg10 primary transformants. (A & B) Sanger chromatographs of the forward strands of PCR fragments amplified from a wild-type (A) and a mutated transformant – NANsg10.18. (B). PAM sequences are underlined in red, and red triangles denote the predicted zCas9 cleavage site. (C) Predicted indel frequency analysis of the mutant sequences from (B). The deletion is estimated to compromise 44.8% of the tissue sample used for PCR and sequencing. Red histogram bars indicate a p-value <0.001. The tissue used for PCR predominantly contains either a wild-type allele or a 10bp

deletion. (E & F) Schematic representations of the predicted effects of a 10bp deletion in the *NAN* coding sequence from (C). The arrowhead in (E) and dashed red line (F) denote the point of deletion. The deletion is predicted to result in a premature stop codon and termination of translation after 97 amino acids (red *). F-box: F-box domain; K1 and K2: Predicted Kelch repeat domains.

As the NANsg10.18 plant is predicted to be heterozygous for its mutation, seeds were collected and the following generation was grown to select for those individuals homozygous for either the mutant or wild-type alleles. A genotyping strategy to easily discriminate between the segregating plants was devised. PCR was performed using two primers flanking either side of the deletion, and a third primer was designed that annealed to the 10bp immediately upstream of the deleted sequence and then to the deleted sequence (**Fig. 5.26**). As DNA polymerases require the 3'-end of the primer to be annealed to the template (Kwok, 2001), PCR using tissue from plants that possessed the deletion would not be permissive for amplification around the site of the deletion. Hence, amplification using homozygous mutants produced a single fragment (~330bp), while the same PCR using heterozygotes produced two fragments (~300bp and ~230bp). PCR using wild-type plants results in a single smaller fragment. An explanation for this could be that even though both fragments are initially amplified, the amplification of the smaller fragment is thermodynamically preferable and therefore becomes subject to exponential amplification.

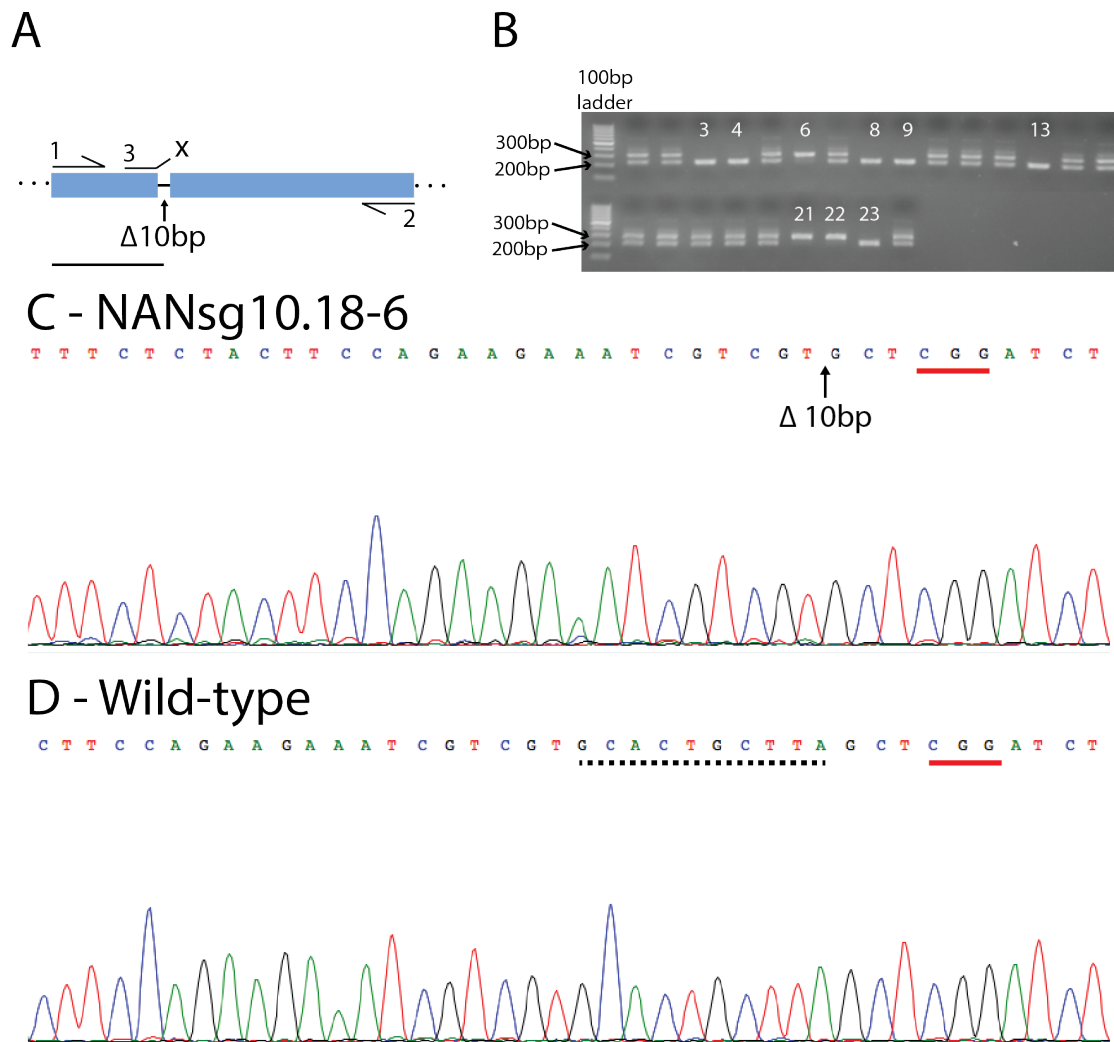


Figure 5.26 – Identification of a homozygous mutation in the *NAN* coding sequence. (A) Schematic depicting the genotyping strategy used to identify plants with a 10bp deletion within the *NAN* coding sequence (blue). Primers 1 and 3 are forward primers, while primer 2 is the reverse. The deletion prevents the 3'-end of the primer 3 from annealing to the template, preventing extension by DNA polymerases. This results in amplification of a single fragment spanning the width of the coding region between primers 1 and 2. Scale bar: 100bp. (B) Agarose gel (2% (w/v)) electrophoresis analysis of PCR reactions performed on T2 individuals. Lanes 6, 21, and 22, predicted to be homozygous mutants, possess a single band (~330bp). Lanes 3, 4, 8, 9, 13, and 23, possess a single band (~220p), and are predicted to be wild-type. All other lanes are predicted to be heterozygotes. (C & D) Sanger chromatographs of a homozygous mutant, NANsg10.18-6 (C) and a wild-type individual (D). Red lines indicate the PAM sequences and the dashed black line in panel D indicates the deleted sequence.

Agarose gel electrophoresis analysis of 24 T2 individuals resulted in 3 that were predicted to be homozygous for the deletion, 6 wild-type, and 15 heterozygotes (**Fig. 5.26 B**), roughly adhering to standard segregation ratios when two alleles are present in the preceding generation. Sanger sequencing of the PCR fragments of those that were suspected of being homozygous mutants confirmed the results of the genotyping assay, as all of the samples had a 10bp deletion in the intended region.

5.2.5.1. Off-target analysis of *NANsg10* homozygous mutants

The *Cas-OFFinder* program located two loci in the *Arabidopsis* genome that had high homology to the guide sequence used (**Table 5.4**). In one instance, the potential off-target is within the coding sequence of *GRA*. The selection of this particular guide sequence was a deliberate action to determine if the low number of mismatches still prohibited the nuclease activity of zCas9 at the target site within *GRA*. The two potential off-target loci were amplified, and examined using Sanger sequencing, to verify that no unintended mutational events had occurred.

Table 5.4 – Off-targets of the NANsg10 guide sequence

Target (5' – 3')*	Chromosome	Position (AGI)	Mismatches
TCGTCGacCA ^t TGCaTAGCTTGG	4	6087264 (AT4G09630)	4
TCGTCGaGaACTGCTTAGCTCGG	4	10784124 (AT4G19870)	2

*Mismatching nucleotides are denoted in lower case

5.2.5.2. Analysis of off-target effects of zCas9

To investigate the sequence integrity of the potential NANsg10 off-target sequences, which were flagged by *Cas-OFFinder*, the genomic regions were amplified (as with the off-target detection outline for the GRAsg5 line). Sanger sequencing of the PCR fragments for all homozygous mutants showed no changes to the wild-type sequences, and the chromatographs for each sequencing reaction showed that all base calls are associated with single nucleotide peaks (**Fig. 5.27**). Hence, no off-target effects were detected.

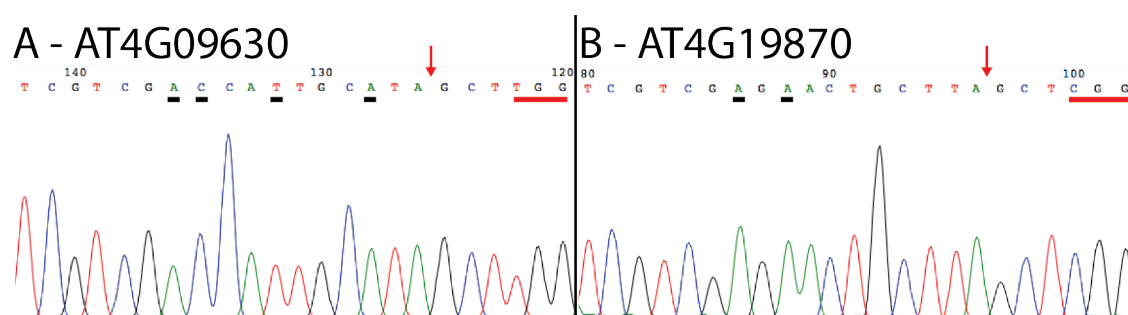


Figure 5.27 – Analysis of potential off-target effects of zCas9 in the NANsg10.18 T2 plants. Sanger chromatographs of two loci predicted by the *Cas-OFFinder* program to be potentially subject to zCas9 nuclease activity, mediated by imperfect base-pairing between the genomic sequences and the NANsg10 guide RNA. Black bars underline bases that are mismatched to the NANsg10 target sequence. Red bars underline PAM sequences, and red arrows show the predicted zCas9 cleavage sites. AGI codes denote the TAIR10 annotated gene in which the off-target site is located, or the closest gene to the site in the cases of. No mutational events are detectable in both panels.

5.2.5.3. Phenotypic analysis of *NANsg10* mutants

NANsg10 plants that were determined to be homozygous mutants were grown and compared to the wild-type samples. No obvious deviation from the wild-type state was observed for vegetative development, and a preliminary examination of the flowers suggests that they also do not differ from those of wild-type plants. The first three flowers of one of the homozygous mutants did have slight defects relating to the 2nd and 3rd whorls, such that the number of petals and stamens was greater in the first three flowers, and these defects did not occur in successive flowers. All flowers examined produced pollen, though due to time constraints no further examination of the anther morphology has been performed.

5.2.6. Generation of a *GRANNY-NANNY* double mutant line

GRA and *NAN* are located extremely closely to one another on chromosome 4, with 688bp separating the stop codon of *GRA* from the start codon of *NAN*. Isolating a line that is mutant for both genes is therefore not tenable by crossing *gra* and *nan* single mutants. To circumvent this issue the pHEE 401 vector was used to generate binary plasmids that contained two guide RNAs, targeting both the *GRA* and *NAN* coding sequences, in order to disrupt the ORFs of both genes and isolate a stable double mutant line. Overlap-extension PCR was used to create a *BsaI*-U6-26::*GRAsg*-U6-26_{ter}-U26-29::*NANsg*-U6-26_{ter}-*BsaI* amplicon that was subsequently ligated into the pHEE 401 vector using Golden Gateway cloning techniques. After hygromycin selection, transformants were transferred to soil and allowed to grow under constant illumination. For the following experiments, as a proof of concept, previously generated guide sequences were used that individually target the coding sequences of *NAN* or *GRA* (termed *NANsg7*, and *GRAsg9*, respectively). Ideally, guide sequences would be designed encompassing the entire locus

in order to wholly remove both genes' coding sequences and proximal regulatory elements.

As the likelihood of a DSB occurring at one target site is probably not affected by zCas9 activity at any other site, the potential cleavage activity of zCas9 at two independent sites on a given allele could result in four types of mutants: Those that are 1) singly mutant for *GRA*, 2) singly mutant for *NAN*, 3) doubly mutant for *GRA* and *NAN* by the presence of small indels at both target regions, or 4) doubly mutant for *GRA* and *NAN* by the generation of a chromosomal deletion encompassing the genomic region contained between the two target sites, estimated to be approximately 1900bp.

Forty transformants were isolated and primers flanking both target sites were used to amplify the locus from genomic DNA to first determine if any of the plants had undergone a mutation event resulting in a deletion of the region between the two target sites. Agarose gel electrophoresis analysis of the PCR products suggested that this was indeed the case, as 11 lanes of 36 successful PCRs contained two bands, one at ~3kb and another at ~1kb, agreeing with the predicted amplicon sizes had the deletion occurred (**Fig. 5.28 B**). Furthermore, two independent wild-type samples used for the sample PCR did not possess the smaller band, an indication that the smaller size bands were not a result of non-specific amplification.

To investigate the sequence composition of the smaller DNA species, a second round of nested PCR was performed using the prior PCR products as templates and an extension time unfavourable to the amplification of the longer product. Sequence analysis of six of the resulting amplicons revealed that all of the samples had undergone a large deletion

event, as the Sanger chromatographs reported bases aligning with the *GRA* genomic sequence up until the predicted cleavage site of GRAsg9, at which point the base calls begin to align to the *NAN* genomic sequence after the NANsg7 predicted cleavage site (**Fig. 5.28 C**). This demonstrates that, although no single individual was homozygous for the large deletion, its frequency within the sampled population is at least 16%, and likely up to 31% if the rest of the samples positive for the ~1kb band are also due to the simultaneous action of zCas9 at two independent target sites. Moreover, 5 of the 6 samples sequenced possessed multiple nucleotide peaks at most positions downstream of the cleavage sites, signifying that more than one deletion event had occurred within those transformants while zCas9 was active (**Table 5.5**). Seeds from these lines were isolated, but due to time constraints further analysis has not been performed. However, this experiment demonstrates the ease with which chromosomal deletions can be obtained and represents a conduit for advancing our knowledge about the biological relevance of *NAN* and *GRA*.

**Table 5.5 – Independent deletion events detected in
pHEE G9N7 transformants**

Individual #	Deletion events
2	3
3	1
4	2
9	3
19	3
22	2

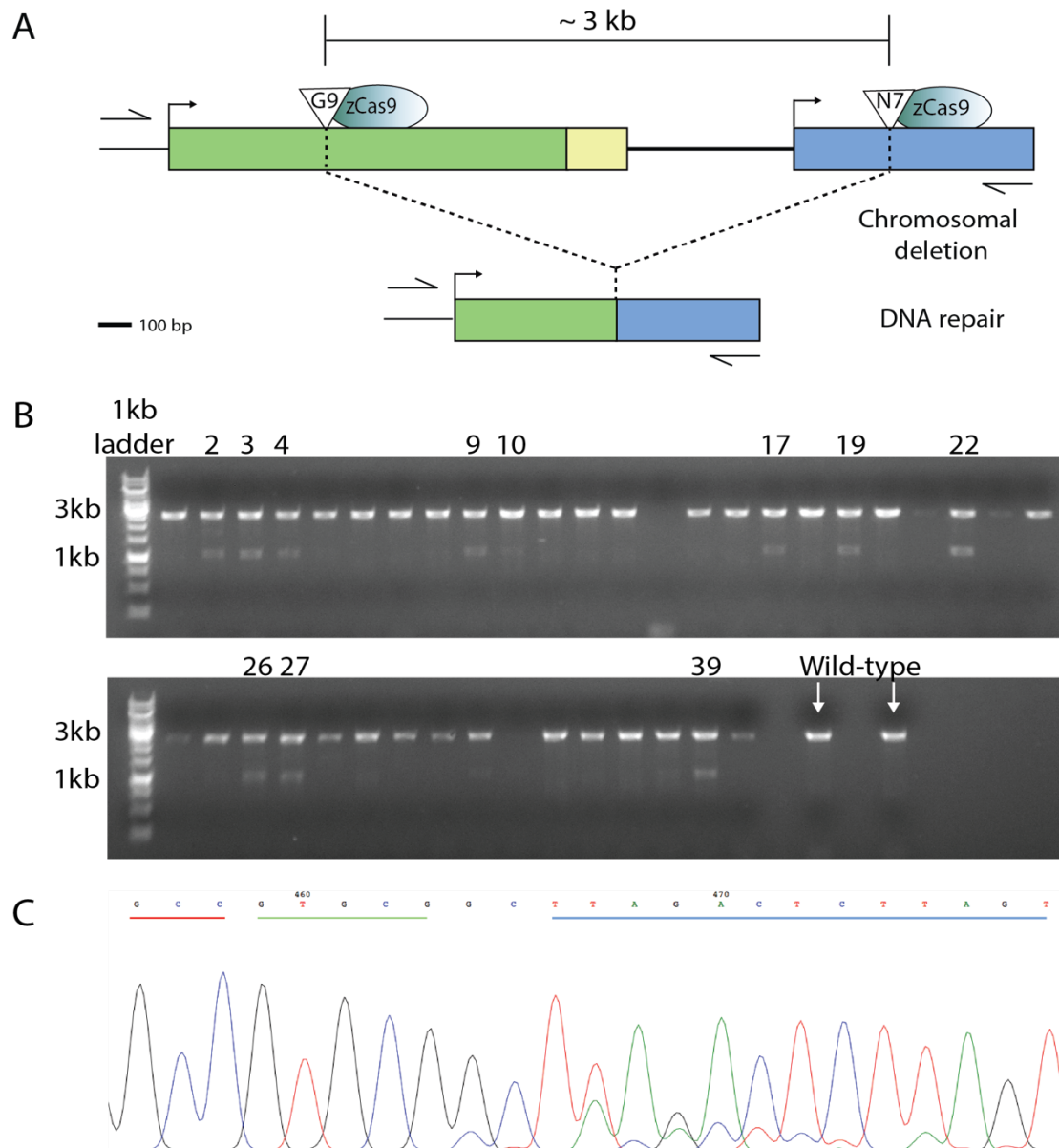


Figure 5.28 – Detection of CRISPR/Cas9-mediated chromosomal deletion events in pHEE GRAsg9-NANsg7 transformants. (A) Schematic representation of a chromosomal deletion resulting from the DNA repair of simultaneous double strand breaks caused by zCas9's nuclease activity at the GRAsg9 (G9) and NANsg7 (N7) target sites (dashed lines). The repaired locus consists of approximately the first 490bp of the *GRANNY* (green) coding sequence, and the latter 696bp of the *NAN* (blue) coding sequence. Half arrows indicate the primers used in a genotyping assay to identify transformants possessing a chromosomal deletion. Yellow box: *GRA* 3'UTR. (B) Agarose gel analysis of transformants. Individuals with a detectable deletion event, at approximately 1100bp, are numbered. The wild-type amplification product is visible at approximately 3kb. (C) Sanger chromatograph of a sequenced representative

individual (#3 in panel B) possessing a chromosomal deletion between *GRA* and *NAN*. The junction at which the DNA repair has taken place is flanked by the GRAsg9 and NANsg7 predicted cleavage sites. Sequences corresponding to the GRAsg9 adjacent PAM sequence, GRAsg9 target site, and NANsg7 target site are underlined in red, green, and blue respectively.

One potential issue to be cautious of when using this strategy to obtain a double mutant is that the fusion of the remnants of the *GRA* and *NAN* coding sequences could produce an open reading frame that is not frameshifted, and therefore translation of a GRA-NAN hybrid protein could take place and potentially fulfil the role of at least one of the wild-type proteins. In the best-case scenario, a frameshift occurs at the DNA repair site, allowing only for the translation of the first 163 amino acids of GRA, which contains the F-box domain but excludes its C-terminal substrate interaction regions. To select for a plant containing a frameshifted *NAN* ORF, seeds collected from the self-fertilised transformants would be PCR genotyped to identify those possessing the mutation, and then sequenced to verify that a frameshift had occurred.

5.3. Discussion

This chapter was based on the original hypothesis that the putative F-box genes, *NAN* and *GRA*, acted in an antagonistic manner to the floral homeotic F-box gene, *UFO*, such that introducing mutations to the two former genes in a *ufo* mutant background resulted in a partial restoration of floral organ identity within the 2nd and 3rd whorls. Past reports concluded that *NAN* and *GRA* therefore had some influence over the regulation of the B-function genes that govern floral organ formation. To investigate this idea, several independent crosses were made between different mutant lines for *NAN/GRA* and *UFO* in order to determine if the genetic interaction previously observed was repeatable. Initial findings in this report concluded similarly that knocking down *NAN* and *GRA* in a *ufo* background did indeed rescue floral organ identity defects to a small degree. However, further analysis of other crosses produced variable results, and eventually it was determined that the likely cause of the initial observations was due to the tendency of the defects associated with *ufo* flowers to change over the course of the plant's reproductive cycle. No genetic interactions were observed when the *nan-1 gra-1* line was crossed with the B-function mutant lines, *ap3-3* and *pi-1*, also indicating that *NAN/GRA* do not regulate floral organ specification. Moreover, examination of the levels of expression of some of the homeotic genes in the *nan-1 gra-1 ufo-1* line showed that a reduction in *NAN/GRA* mRNA within the *ufo-1* background did not result in the differential expression of these master regulators, providing further evidence that the homeotics are not subject to regulation by these F-box genes. Preliminary RT-qPCR data suggests, however, that the B-function regulator AP3 might indirectly affect the expression of *NAN*, as its expression was reduced to a much greater degree in the *ap3-3 nan-1 gra-1* when compared to *nan-1 gra-1*. This could be due to some combination of factors, such as the downregulation of a direct target of AP3, and the expression of *NAN* is further reduced

due to the compounding effects of having a disrupted promoter in combination with the reduced expression of a direct regulator. Further investigation is required to determine whether this is the case.

Further work to delineate the roles of NAN and GRA included investigations into their expression profiles through the employment of transcriptional reporters and *in situ* hybridisations, and a yeast 2-hybrid study to show that the genes encoded proteins that physically interacted with members of the ASK family, thereby indicating that they function as F-box proteins within the SCF complex. Results from the expression profile experiments tentatively indicate that these genes are expressed within developing floral tissues, although comparisons between the results suggests that the putative promoter regions used in the transcriptional reporter assays are not fully representative of the endogenous regulatory elements that contribute to the genes' expression. Further optimisation of these experiments is required to be able to make any confident conclusions regarding the endogenous expression profiles.

One of the limitations of the mutant lines used in this study is that they are wholly composed of T-DNA insertion lines within the proximal regulatory regions of *GRA* and *NAN*, and these lines only lead to partial reductions in either of the genes' expression. Therefore, it is likely that a significant portion of the respective proteins are still present, potentially precluding proper analysis of their functions through mutant studies. In order to obtain mutants in which the genes have been fully knocked out, CRISPR/Cas9 was successfully implemented for the first time in the Wellmer laboratory in order to induce small indels within the coding sequences of each gene separately, and another vector was designed in order to obtain a chromosomal deletion encompassing a ~1.9kb region

between the two genes, ideally resulting in a double knockout. Although it was not possible in this study to perform any genome-wide analyses to investigate potential off-target effects associated with aberrant Cas9 activity, Sanger sequencing of the off-target sites identified indicates that the action of Cas9 was specific to the intended loci, therefore providing some confidence that any novel phenotype arising from the establishment of these line are likely to be reflective of null mutants for *NAN* and/or *GRA*. Using methods such as these will allow us to study the effects of mutations in both genes separately and together, therefore likely providing us with insights into their functions and whether they share any functions. While we are still unable to come to any conclusions as to biological relevance of these genes, we are now in a position to advance our knowledge concerning any functions they have during flower development.

6. Appendix

6.1. Buffers and solutions

CTAB buffer

100 mM Tris-HCl (pH8.0)
20 mM EDTA
1.4 M NaCl
2% (w/v) CTAB
1% (w/v) PVP 40,000

50X TAE buffer

242 g Tris-base
57.1 ml glacial acetic acid
37.2 g Na₂EDTA.2H₂O
H₂O to 1 liter

Edward's Prep extraction buffer

200 mM Tris-HCl (pH 7.5-8.0)
250 mM NaCl
25 mM EDTA
0.5% (w/v) SDS

TE buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

***Agrobacterium* colony PCR lysis buffer**

10 mM Tris-HCl (pH 8)
0.1% (v/v) Triton X-100

GUS staining solution

0.2% (v/v) Triton X-100
2 mM K₄Fe(CN)₆
2 mM K₃Fe(CN)₆
50 mM NaPO₄
2 mM X-Gluc (excluded for first round of washing tissue)

FAA

50% Ethanol
3.7% Formaldehyde
5% acetic acid

6.2. GO-terms associated with DEGs, during *AG* or *AP3*

knockdowns, at different stages in flower development

Table 6.1 – Enriched GO terms associated with upregulated genes during knockdown of AG at 3 days after floral initiation	
Term	Enrichment
plastid translation	16.14228799
chloroplast rRNA processing	16.14228799
chloroplast ribulose biphosphate carboxylase complex biogenesis	16.14228799
NADH dehydrogenase complex (plastoquinone) assembly	16.14228799
cytochrome b6f complex assembly	14.12450199
protein import into chloroplast thylakoid membrane	13.83624685
photosynthesis, dark reaction	13.83624685
reductive pentose-phosphate cycle	13.72094479
photosynthetic electron transport in photosystem I	13.11560899
transcription from plastid promoter	12.55511288
carbon fixation	12.10671599
glycine catabolic process	12.10671599
PSII associated light-harvesting complex II catabolic process	12.10671599
photosystem II assembly	11.73984581
serine family amino acid catabolic process	10.76152533
photosynthesis, light harvesting in photosystem I	10.52757912
photosystem I assembly	10.27236509
chloroplast RNA processing	10.19512926
protein nitrosylation	10.08892999
peptidyl-cysteine S-nitrosylation	10.08892999
photosystem II stabilization	10.08892999
photosystem II repair	9.933715687
photosynthetic electron transport chain	9.800674852
thylakoid membrane organization	9.770332205
peptidyl-cysteine modification	9.685372795
nonphotochemical quenching	9.685372795
energy quenching	9.685372795
plastid membrane organization	9.519810866
negative regulation of photosynthesis	9.495463524

FDR-adjusted *p*-values < 0.05

Table 6.2 – Enriched GO terms associated with upregulated genes during knockdown of AG at 5 days after floral initiation	
Term	Enrichment
plant-type cell wall modification involved in multidimensional cell growth	11.72952854
anthocyanin-containing compound biosynthetic process	11.43629032
cell wall modification involved in multidimensional cell growth	10.89170507
flavonoid biosynthetic process	10.4935137
response to karrikin	10.48326613
multidimensional cell growth	10.1655914
flavonoid metabolic process	8.953181415
plant-type cell wall loosening	8.471326165
plant-type cell wall modification	8.055751674
anthocyanin-containing compound metabolic process	7.624193548
nitrate metabolic process	7.438237608
nitrate assimilation	7.438237608
nitrogen cycle metabolic process	6.62973352
reactive nitrogen species metabolic process	6.62973352
phenylpropanoid biosynthetic process	6.55302586
phenylpropanoid metabolic process	5.950590087
cellular response to brassinosteroid stimulus	5.848696421
anatomical structure arrangement	5.544868035
steroid hormone mediated signaling pathway	5.154384371
brassinosteroid mediated signaling pathway	5.154384371
regulation of cell proliferation	5.154384371
response to steroid hormone	5.154384371
cellular response to steroid hormone stimulus	5.154384371
response to brassinosteroid	5.136298812
response to toxic substance	4.783807717
lateral root development	4.773408135
hormone transport	4.737362982
plant-type cell wall organization	4.65599606
lignin metabolic process	4.49426146
symbiosis, encompassing mutualism through parasitism	4.443238624

FDR-adjusted *p*-values < 0.05

Table 6.3 – Enriched GO terms associated with upregulated genes during knockdown of AG at 8 days after floral initiation

Term	Enrichment
plant-type cell wall modification involved in multidimensional cell growth	25.84943493
cell wall modification involved in multidimensional cell growth	24.00304672
anthocyanin-containing compound biosynthetic process	21.00266588
plant-type cell wall loosening	18.66903633
multidimensional cell growth	14.00177725
anthocyanin-containing compound metabolic process	14.00177725
plant-type cell wall modification	12.68085487
glycolipid metabolic process	12.00152336
liposaccharide metabolic process	11.39127641
cellular response to phosphate starvation	11.08594323
cellular lipid catabolic process	9.206648056
anatomical structure arrangement	8.728380624
membrane lipid biosynthetic process	8.616478308
membrane lipid metabolic process	7.906885977
flavonoid biosynthetic process	7.226723743
cellular response to starvation	7.028343091
cell wall modification	6.893182647
response to starvation	6.637879586
cellular response to nutrient levels	6.325508782
response to karrikin	6.300799763
flavonoid metabolic process	6.165920257
lateral root development	5.84422007
response to salicylic acid	5.788294519
anatomical structure formation involved in morphogenesis	5.719874962
response to nutrient levels	5.659665752
auxin-activated signaling pathway	5.659665752
cellular response to extracellular stimulus	5.271257318
post-embryonic root development	5.250666469
cellular response to auxin stimulus	5.220080063
cellular response to external stimulus	5.169886985

FDR-adjusted *p*-values < 0.05

Table 6.4 – Enriched GO terms associated with downregulated genes during knockdown of AG at 3 days after floral initiation	
Term	Enrichment
assembly of actomyosin apparatus involved in mitotic cytokinesis	23.80161128
assembly of actomyosin apparatus involved in cytokinesis	23.80161128
phragmoplast assembly	23.80161128
DNA unwinding involved in DNA replication	22.21483719
cell cycle phase	15.86774085
biological phase	15.86774085
mitotic spindle organization	11.42477341
DNA strand elongation involved in DNA replication	10.98535905
DNA strand elongation	10.98535905
specification of floral organ identity	10.20069055
response to cyclopentenone	10.08068242
regulation of G2/M transition of mitotic cell cycle	10.08068242
DNA duplex unwinding	9.996676737
regulation of cell cycle G2/M phase transition	9.520644512
DNA geometric change	9.520644512
G2/M transition of mitotic cell cycle	9.019557958
DNA replication initiation	8.56858006
cell cycle G2/M phase transition	8.56858006
floral organ formation	8.462795121
mitotic cell cycle checkpoint	8.400568687
regulation of mitotic cell cycle phase transition	8.292174252
cytokinesis by cell plate formation	8.160552438
microtubule-based movement	8.160552438
adaxial/abaxial axis specification	7.99734139
response to mechanical stimulus	7.933870426
polarity specification of adaxial/abaxial axis	7.789618237
specification of axis polarity	7.789618237
mitotic cytokinetic process	7.719441496
cytokinetic process	7.719441496
membrane invagination	7.516298299

FDR-adjusted *p*-values < 0.05

Table 6.5 – Enriched GO terms associated with downregulated genes during knockdown of AG at 5 days after floral initiation

Term	Enrichment
regulation of response to alcohol	8.263986014
regulation of abscisic acid-activated signaling pathway	8.263986014
post-embryonic organ development	5.893402597
floral organ development	4.484110672
cellular component biogenesis	2.472403487
peptide metabolic process	2.343966942
translation	2.320527273
peptide biosynthetic process	2.299069293
cellular amide metabolic process	2.291878788
amide biosynthetic process	2.266139915
organonitrogen compound biosynthetic process	2.049277457
organonitrogen compound metabolic process	1.906925227
cellular nitrogen compound biosynthetic process	1.743708501
macromolecule biosynthetic process	1.743296185
cellular macromolecule biosynthetic process	1.736271809
gene expression	1.62946933
organic substance biosynthetic process	1.547977492
cellular biosynthetic process	1.537354809
cellular nitrogen compound metabolic process	1.532360817
nitrogen compound metabolic process	1.466622482
biosynthetic process	1.463339874
cellular macromolecule metabolic process	1.444022819
macromolecule metabolic process	1.430481613
primary metabolic process	1.381731777
organic substance metabolic process	1.377124506
cellular metabolic process	1.357170858
metabolic process	1.28543264

FDR-adjusted p -values < 0.05

Table 6.6 – Enriched GO terms associated with downregulated genes during knockdown of AG at 8 days after floral initiation	
Term	Enrichment
cold acclimation	15.64886339
androecium development	7.657954423
stamen development	7.657954423
floral organ development	5.633590819
response to alcohol	4.445028776
response to abscisic acid	4.234398328
response to water deprivation	3.73408037
response to water	3.64993208
response to lipid	3.170313774
response to osmotic stress	2.942773681
plant organ development	2.846432175
cell differentiation	2.81190514
response to acid chemical	2.8038099
carboxylic acid metabolic process	2.666102651
cellular developmental process	2.588862914
response to hormone	2.383309421
response to endogenous stimulus	2.327452199
response to oxygen-containing compound	2.311649697
response to organic substance	2.27732347
response to chemical	1.968037919
multicellular organism development	1.792577022
single-multicellular organism process	1.752672699
single-organism developmental process	1.7473148
anatomical structure development	1.738983674
developmental process	1.736718894
multicellular organismal process	1.709400908
single-organism process	1.356190658

FDR-adjusted *p*-values < 0.05

Table 6.7 – Enriched GO terms associated with upregulated genes during knockdown of AP3 at 3 days after floral initiation

Term	Enrichment
indole-containing compound biosynthetic process	21.00577692
glucosinolate biosynthetic process	20.5283729
glycosinolate biosynthetic process	20.5283729
sulphur compound biosynthetic process	14.38802773
indole-containing compound metabolic process	11.7304988
glucosinolate metabolic process	10.94846555
glycosinolate metabolic process	10.94846555
response to karrikin	9.879279459
alpha-amino acid biosynthetic process	8.21134916
post-embryonic organ development	7.225987261
cell wall modification	6.948064674
sulphur compound metabolic process	6.948064674
response to gibberellin	5.942423734
organic acid biosynthetic process	5.443304905
cellular response to extracellular stimulus	5.313225927
meristem development	5.247086185
regulation of growth	5.236222653
alpha-amino acid metabolic process	5.225404011
cellular response to external stimulus	5.211048506
monocarboxylic acid biosynthetic process	5.038038927
response to extracellular stimulus	4.817324841
secondary metabolite biosynthetic process	4.646937145
root development	4.497502445
root system development	4.478917724
small molecule biosynthetic process	4.426332166
animal organ development	4.28079814
response to water deprivation	4.164834156
carboxylic acid biosynthetic process	4.124421953
response to water	4.070978739
secondary metabolic process	4.038915644

FDR-adjusted p -values < 0.05

Table 6.8 – Enriched GO terms associated with upregulated genes during knockdown of AP3 at 5 days after floral initiation	
Term	Enrichment
response to gibberellin	6.524199485
lipid catabolic process	5.312562438
single-organism catabolic process	3.390353237
hormone-mediated signaling pathway	3.382094154
cellular response to chemical stimulus	3.20440274
cellular response to hormone stimulus	3.158929807
cellular response to endogenous stimulus	3.119886855
cellular response to organic substance	3.102024076
response to oxygen-containing compound	2.929813924
growth	2.925304784
response to acid chemical	2.854958758
positive regulation of biological process	2.701507346
regulation of transcription, DNA-templated	2.679114582
regulation of RNA biosynthetic process	2.676923077
regulation of nucleic acid-templated transcription	2.676923077
regulation of RNA metabolic process	2.645544431
regulation of nucleobase-containing compound metabolic process	2.588009855
response to inorganic substance	2.570224592
transcription, DNA-templated	2.557669763
nucleic acid-templated transcription	2.555672364
RNA biosynthetic process	2.550692488
organic substance catabolic process	2.540729087
regulation of cellular macromolecule biosynthetic process	2.537979897
regulation of gene expression	2.537343254
regulation of macromolecule biosynthetic process	2.529412073
response to endogenous stimulus	2.519275182
catabolic process	2.496532838
regulation of cellular biosynthetic process	2.466500581
regulation of nitrogen compound metabolic process	2.461998024
regulation of biosynthetic process	2.450367946

FDR-adjusted p -values < 0.05

Table 6.9 – Enriched GO terms associated with upregulated genes during knockdown of AP3 at 8 days after floral initiation	
Term	Enrichment
response to acid chemical	3.146824725
anatomical structure morphogenesis	2.979760609
response to oxygen-containing compound	2.512524085
response to chemical	2.39957939
cellular response to stimulus	2.054621849
response to stress	1.896862521
response to stimulus	1.689984
FDR-adjusted <i>p</i> -values < 0.05	

Table 6.10 – Enriched GO terms associated with downregulated genes during knockdown of AP3 at 3 days after floral initiation	
Term	Enrichment
anatomical structure morphogenesis	6.385201306
plant organ development	5.088449532
regulation of cellular biosynthetic process	3.577176298
regulation of biosynthetic process	3.55377907
system development	3.548989114
regulation of cellular macromolecule biosynthetic process	3.31275875
regulation of macromolecule biosynthetic process	3.301575394
regulation of cellular metabolic process	3.281879195
regulation of nitrogen compound metabolic process	3.213581599
regulation of transcription, DNA-templated	3.20261973
regulation of nucleic acid-templated transcription	3.2
regulation of RNA biosynthetic process	3.2
regulation of RNA metabolic process	3.162489895
regulation of gene expression	3.12793177
regulation of nucleobase-containing compound metabolic process	3.09371293
regulation of metabolic process	3.074504873
transcription, DNA-templated	3.057444314
nucleic acid-templated transcription	3.055056619
RNA biosynthetic process	3.049103663
regulation of primary metabolic process	3.046728972
regulation of macromolecule metabolic process	2.935957305
aromatic compound biosynthetic process	2.878351864
organic cyclic compound biosynthetic process	2.7731569
biological regulation	2.258660508
response to stimulus	2.19072

FDR-adjusted p -values < 0.05

Table 6.11 – Enriched GO terms associated with downregulated genes during knockdown of AP3 at 5 days after floral initiation

Term	Enrichment
leaf senescence	19.13765182
organ senescence	19.13765182
defence response, incompatible interaction	12.83348416
aging	12.53846154
animal organ development	9.305796573
leaf development	8.57968885
innate immune response	8.105358419
immune response	7.861954262
immune system process	7.094934334
phyllome development	6.871471835
response to other organism	5.409154482
response to external biotic stimulus	5.400228484
response to organic cyclic compound	5.386894587
response to biotic stimulus	5.223525078
defence response to other organism	5.12790927
response to external stimulus	4.688845668
response to fungus	4.568989126
plant organ development	4.540462659
shoot system development	4.382864011
defence response	4.179487179
multi-organism process	4.0129227
system development	2.94059098
signal transduction	2.886717557
single organism signaling	2.843308271
signaling	2.840460691
cell communication	2.747970518
multicellular organism development	2.483605615
single-multicellular organism process	2.428318395
response to stress	2.364640835
multicellular organismal process	2.302577633

FDR-adjusted p -values < 0.05

Table 6.12 – Enriched GO terms associated with downregulated genes during knockdown of AP3 at 8 days after floral initiation	
Term	Enrichment
cell fate specification	28.22651274
cell fate commitment	17.37016169
flavonoid biosynthetic process	15.53975755
flavonoid metabolic process	13.25869222
response to karrikin	9.879279459
androecium development	9.609025613
stamen development	9.609025613
photoperiodism	8.685080843
pigment biosynthetic process	7.323635738
response to wounding	6.659896093
response to organonitrogen compound	6.529506561
floral organ development	6.283467184
pigment metabolic process	5.988387233
carboxylic acid biosynthetic process	5.774190734
floral whorl development	5.765415368
monocarboxylic acid biosynthetic process	5.757758774
fatty acid biosynthetic process	5.716762074
positive regulation of response to stimulus	5.530092292
phenylpropanoid metabolic process	5.507612242
negative regulation of response to stimulus	5.344665134
response to red or far red light	4.882423825
ethylene-activated signaling pathway	4.882423825
response to jasmonic acid	4.817324841
organic acid biosynthetic process	4.762891792
regulation of reproductive process	4.753938988
response to ethylene	4.707483558
regulation of post-embryonic development	4.392697423
flower development	4.168838805
small molecule biosynthetic process	4.149686406
response to nitrogen compound	4.10567458

FDR-adjusted *p*-values < 0.05

6.3. Cas9 guide sequences used in this study

Table 6.13 – CRISPR sgRNA guide sequences used (excluding PAM sequences)	
Guide	Sequence (5' - 3')
GRAsg5	AGATCTGGCTACATAGAGCT
GRAsg9	GCGACAATCGAGGATTCGCA
NANsg7	TAACTAAGAGTCTAAGCCAT
NANsg10	TCGTCGTGCACTGCTTAGCT

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