



Trinity College Dublin

Coláiste na Tríonóide, Baile Átha Cliath

The University of Dublin

School of Genetics and Microbiology

**Investigating the roles of the floral-associated genes
TRIPTYCHON, *SHATTERPROOF* and *AGAMOUS* in
Arabidopsis thaliana and *Brassica rapa***

Caoilfhionn Breathnach

Supervisor: Prof. Frank Wellmer

March 2024

A thesis submitted to The University of Dublin
for the degree of Doctor of Philosophy

Declaration

I hereby 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.

Signed:  Date: 12/07/24

Summary

The genetic mechanisms responsible for flower development has been studied for over four decades now in *Arabidopsis thaliana*, a model organism for plants. However, more recently, plant research has become increasingly focused on additional species, and the translation of knowledge gained through studying *Arabidopsis* into other species used in agriculture. Through a combination of basic and translational research, in this thesis I sought to gain a deeper understanding of the development and regulation of the gynoecium in the family Brassicaceae.

The first results section of the thesis investigates the role of the trichome repressor *TRIPTYCHON* (*TRY*) in the establishment of the replum in the gynoecium of *Arabidopsis thaliana*. This work emerged from previous observations within our lab. Until now, loss-of-function mutant lines of *triptychnon* have been studied primarily in relation to the trichome clustering phenotype observed. The aim of this project was to independently assess the previous observation that *try* mutant plants also have a narrow replum, which was thought to be hypersensitive to exogenous cytokinin treatment. I developed a protocol for accurately measuring repla in large sample sizes to facilitate reproducing this observation. Novel *try* mutant lines were generated in this study using CRISPR/Cas9 genome editing, as well as complementation experiments to rescue the replum phenotype by reintroducing *TRY* activity. This was done to ascertain if the replum phenotype observed was a result of the loss of *TRY* function as opposed to any other artefact in the genetic background of the *try-29760* allele. Finally, through generating double mutants combining *try-29760* and other mutants carrying replum defects, as well as RT-qPCR analyses, I successfully identified co-factors of *TRY* in replum patterning. Through this work, a novel function of *TRY* in promoting replum formation was characterised, which seems to have evolved independently of the trichome formation machinery that it is normally associated with in the literature to date. Additionally, some interactors of *TRY* within the gynoecium patterning gene regulatory network were identified, namely *REPLUMLESS* and *ASYMMETRIC LEAVES1*.

Understanding the genetic regulation of developmental processes is highly important when seeking to increase the resistance of plants to common environmental stresses, such as drought and herbivory. This can be achieved by exploiting these genetic pathways to our advantage. A critical area of plant research is investigating methods of increasing and preserving yield. In *Brassica rapa* var. Yellow Sarson, the fruit is the most economically important organ of the plant, as the seeds are required to produce oil. Developing a method of protecting these crops from seed loss could potentially be a hugely beneficial addition to agriculture. The next section of the thesis sought to identify the homologs of the *Arabidopsis* *SHATTERPROOF1* & *2* in *Brassica rapa*, as they could be targeted to minimise seed loss. The aim of this work was to translate established work completed in *Arabidopsis thaliana* to the more agronomically valuable species *Brassica rapa*. Shatter resistance on the seed pod of *Brassica rapa* could protect them from opening due to weather conditions, thus preserving seed yield when

they are harvested. Four homologs were identified by sequence and synteny analyses. After identifying the homologs, they were functionally characterised by RT-qPCR experiments to illustrate the expression domains of these genes. This found that, while *Arabidopsis thaliana* SHP1/2 is exclusively expressed in the gynoecium, the *B. rapa* homologs are also expressed in the stamens. Complementation experiments were then carried out in *Arabidopsis thaliana*, which demonstrated that these identified genes were indeed true homologs of SHATTERPROOF1/2 as well as exploring the differences in expression domains across the two species. 3' RACE PCR allowed for the identification of the 3' UTR of each of the four genes in order to isolate unique segments of each of them. Additionally, TILLING mutant lines for each of these four homologs were acquired and higher-order mutants were subsequently generated. The pod shatter resistance of these mutant lines was quantified through the use of a random impact test. This revealed that even in a double mutant combination, the pod shatter resistance is increased when compared to the wild type.

Leading on from this work in *Brassica rapa*, the final section of the thesis examines the redundancy of the MADS box transcription factors AGAMOUS (AG) and SHATTERPROOF1 & 2 (SHP1/2) in the repression of trichome initiation on the gynoecium in the family Brassicaceae. It was previously shown in Ó Maoiléidigh et al. (2018) that the phenotype of *ag try-29760 cpc-1* triple mutants in *Arabidopsis* - with trichomes on their gynoecia - is exacerbated in a *shp1/2* mutant background and can be partially rescued by introducing SHP2 overexpression, suggesting that AG and SHP2 are at least partially redundant in repressing trichome initiation on the gynoecium. To combine the use of basic and translational research approaches, I endeavoured to further investigate this redundancy in *Arabidopsis thaliana*, before investigating the functional redundancy of AG and SHP genes identified in *Brassica rapa*. To do this, I generated higher order mutants in *Arabidopsis* and assessed their phenotypes, as well as examining the effect of exogenous cytokinin treatment, which is known to promote trichome initiation. Furthermore, I tested the function of the large intron in *BrSHP2* as a promoter, due to the large intron of *AtAG* being required for normal expression. This demonstrated that the large intron of *BrSHP2* does indeed function as a promoter. This suggests that *BrSHP2* may be more functionally similar to *AtAG* than *AtSHP2* is.

Acknowledgements

To begin, thank you to Prof. Frank Wellmer for taking me on as a Ph.D. student, providing so much valuable insight and providing encouragement when it was most needed. To Dr. Emmanuelle Graciet of Maynooth University, for sharing reagents, exchanging invaluable knowledge and being hugely supportive. Additionally, I would like to thank Prof. Cristina Ferrándiz of the University of Valencia and Dr. Stefan de Folter of Instituto Politécnico Nacional in Mexico City for kindly sharing reagents. To Prof. Lars Østergaard of Oxford University, for his assistance with TILLING screens, plant health advice and also sharing of seed stocks. And also, thank you to Nat Prunet of the University of North Carolina for the beautiful GFP images. Thanks to Science Foundation Ireland for making this Ph.D. possible.

The Smurfit Institute was a wonderful place to call home over the last few years, which is most definitely because of the people in it. To the technical staff of the department - Dr. David Noone, Phillip Campbell and Rachel Sullivan for keeping everything going for all labs. Particular thanks goes to Brenda Campbell for being arguably the most indispensable asset the department has, and also for always being there for a chat when it was needed. Many thanks to Oanh for providing the coffee that fuelled so much of my work and also her endless kindness. Thanks also to my friends of the Smurfit who always served as a respite from the day-to-day stress - Orla, Zoe, Dearbhaile and Claire being just a few amongst them.

To all members of the Wellmer lab, past and present, thank you for being a fantastic group to be a part of. To Andrea, Kevin and Christos for being extremely knowledgeable in the areas I struggled with and always making the time to assist me. To Joe for the dog pictures and camaraderie, especially during these final six months we floundered through together. To Bennett, the lab dad, for your absolutely bottomless pit of knowledge and your patience, and also for making some fond memories along the way. And always being up for a pint. Last but certainly not least are Kate and Christina. You two are the people who kept me going through the periods when I truly felt I wasn't able to see my research through, whether you were in the lab or had moved on. Without my work wives I wouldn't be so close to becoming a Doctor. Thank you.

My more personal thank-yous begin with my parents and also my late grandparents. They provided the foundation for my belief and enthusiasm in learning and education, as well as their chromosomes. My extended friend group have provided a fantastic escape from everything academic, but particular thanks goes to Lauren for being so close to me throughout it all - and not just physically. I think our friendship is officially timeless. Otis, Fiadh, Phillip and Kylie all made invaluable intellectual contributions. My final but biggest thank you goes to my person, Chris. My very own Consistency in a world gone mad. I owe so much of who I am to you.

For Daniel Darcy (1995-2011)
and all that you could have achieved.

Contents

1	Introduction	1
1.1	Induction of flower development	3
1.2	Floral patterning and floral organ specification	5
1.3	Control of floral organ development by floral organ identity factors	8
1.4	The Quartet model: MADS-domain protein tetramers	10
1.5	MADS-box genes and the evolution of flowers	13
1.6	Control of gynoecium and fruit development	14
1.7	Suppression of trichome formation on floral organs by MADS- domain proteins	18
1.8	Translational research for crop improvement	25
1.9	Aims of the thesis	27
2	Materials and Methods	31
2.1	Strains and growth conditions	31
2.1.1	Plants lines used in this work	31
2.1.2	Plant growth media	33
2.1.3	Plant growth conditions	33
2.1.4	Crossing of plant lines	34
2.1.5	Bacterial strains used in this work	34
2.1.6	Bacterial growth media	34

2.1.7	Preparation and transformation of competent <i>Escherichia coli</i> cells	34
2.1.8	Blue-white screening using XL1 Blue cells	35
2.1.9	Preparation and transformation of competent <i>Agrobacterium tumefaciens</i> cells	35
2.2	Treatment of <i>Arabidopsis</i> with 6-Benzylaminopurine (BAP)	36
2.3	Random impact test of <i>B.rapa</i>	36
2.4	Plant imaging	37
2.5	Prediction of binding sites	37
2.6	Genotyping and molecular work	38
2.6.1	"Edward's preparation" genomic DNA extraction	38
2.6.2	Cetyltrimethyl Ammonium Bromide (CTAB) genomic DNA extraction	38
2.6.3	Quantification of DNA and RNA	39
2.6.4	DNA sequencing	39
2.6.5	Quantitative real-time polymerase chain reaction (qRT-PCR)	40
2.6.6	Reverse-transcription polymerase chain reaction (RT-PCR)	41
2.6.7	Double-mismatch allele-specific (DMAS) quantitative PCR genotyping	41
2.6.8	3' RACE PCR	42
2.6.9	Isolation of plasmid DNA	42
2.6.10	Purification of PCR product	42
2.6.11	Oligonucleotide design	42
2.6.12	Agarose gel electrophoresis	43
2.6.13	Extraction of DNA from an agarose gel	43
2.6.14	Colony PCR to isolate plasmid of interest	43
2.6.15	GreenGate cloning	44
2.7	Generation of transgenic <i>Arabidopsis</i> lines	45

2.7.1	Transformation of <i>Arabidopsis</i> with construct-carrying <i>Agrobacterium tumefaciens</i> by floral dipping	45
2.7.2	pTRY::TRYg	45
2.7.3	p35S::TRYg	45
2.7.4	p35S::AtSHP2 CDS	46
2.7.5	p35S::BrSHP2 CDS	46
2.7.6	pAtSHP2::AtSHP2 CDS	46
2.7.7	pBrSHP2::BrSHP2 CDS	46
2.7.8	BrSHP2 intron 1::BrSHP2 CDS	47
2.7.9	pAtSHP2::BrSHP2 CDS	47
2.7.10	pBrSHP2::AtSHP2 CDS	47
2.7.11	Selection for transformed progeny using the BASTA TM herbicide	47
2.7.12	Generation of CRISPR/Cas9 lines targeting <i>TRIPTYCHON</i>	48
2.7.13	Selection of transformed plants carrying a CRISPR construct	49
2.8	Image manipulation and construction of figures	49
3	Investigating the role of <i>TRIPTYCHON</i> in replum development	51
3.1	Introduction	51
3.1.1	The role of <i>TRY</i> in trichome formation and flower development	51
3.1.2	Replum development	54
3.1.3	The role of cytokinin in gynoecium development and trichome formation	56
3.1.4	CRISPR/Cas9 genome editing	57
3.1.5	Aims of this chapter	60
3.2	Results	61
3.2.1	The localisation of <i>TRY</i> and <i>CPC</i> in the gynoecium	61
3.2.2	Establishment of experimental conditions for replum measurements	61

3.2.3	Reduction in replum width in the <i>try-29760</i> mutant	63
3.2.4	Complementation of the <i>try-29760</i> mutant	64
3.2.5	Generation of novel <i>try</i> mutant alleles through genome editing	69
3.2.6	Replum defects in the novel <i>try</i> mutant alleles	71
3.2.7	Effect of <i>CPC</i> function on replum development	71
3.2.8	Relationship between <i>TRY</i> and CK in replum development	73
3.2.9	Effect of cytokinin on other regulators of replum development and their genetic interactions with <i>TRY</i>	77
3.2.10	Response of regulators of CK signalling and replum formation to <i>TRY</i> perturbation	83
3.3	Discussion	87
3.3.1	Proposed model of the mechanism of <i>TRY</i> in the gynoecium	89
3.3.2	The localisation of <i>TRY</i> to the valve margin	94
3.4	Future Work	98
4	Identification and characterisation of the homologs of <i>SHATTERPR-OOF1/2</i> in <i>Brassica rapa</i>	101
4.1	Introduction	101
4.1.1	The function of the <i>SHP</i> genes in <i>Arabidopsis</i>	101
4.1.2	The dehiscence zone	103
4.1.3	Polyploidy within the <i>Brassica</i> genus	105
4.1.4	Potential of a shatter-resistant phenotype in <i>Brassic</i> as	108
4.1.5	Aims of the chapter	108
4.2	Results	110
4.2.1	Identification of homologs of <i>SHP1/2</i> in <i>B. rapa</i>	110
4.2.2	Synteny analysis	115
4.2.3	Characterisation of the identified <i>SHATTERPROOF</i> homologs	118

4.2.4	Complementation of SHATTERPROOF activity in <i>Arabidopsis</i> using both native and <i>B. rapa</i> coding sequences	121
4.2.5	The identification of the 3' UTRs of the <i>BrSHP</i> homologs .	124
4.2.6	Selection of mutant <i>brshp1/2</i> lines from TILLING screen by RevGen UK	127
4.2.7	Identification of mutant plants through Double-mismatch allele-specific (DMAS) PCR	132
4.2.8	Test via RT-PCR on a splice mutant line	133
4.2.9	Generating higher-order mutants of the <i>BrSHP</i> genes . . .	134
4.2.10	Phenotypic observations via Random Impact Test	136
4.3	Discussion	139
4.3.1	The identification and characterisation of <i>SHP</i> homologs in <i>B. rapa</i>	139
4.3.2	Isolating and examining <i>brshp</i> mutant lines	142
4.4	Future Work	145
5	Examining the roles of <i>AG</i> and <i>SHP1/2</i> in inhibiting trichome initiation on the gynoecium	147
5.1	Introduction	147
5.1.1	The role of <i>AG</i> in gynoecium development	147
5.1.2	The role of <i>AG</i> in floral trichome suppression	148
5.1.3	The role of <i>SHP1/2</i> in <i>Arabidopsis</i> and <i>B. rapa</i>	150
5.1.4	The role of <i>SHP</i> genes in other species	150
5.1.5	Aims of the chapter	151
5.2	Results	154
5.2.1	Characterisation of the <i>ag-10 shp1-1 shp2-1</i> triple mutant .	154
5.2.2	Interaction of <i>SHP1/SHP2</i> with <i>TRY</i> and cytokinin	154
5.2.3	Sequence comparison of <i>BrSHP2</i> and <i>AtAG</i>	156
5.2.4	Analysis of binding sites in <i>BrSHP2</i>	158

5.2.5	Complementation of <i>SHP</i> expression in <i>Arabidopsis</i> using the promoter from <i>B. rapa</i>	159
5.3	Discussion	163
5.4	Future Work	166
A1	Appendix	167
A1.1	Buffers used in molecular biology	167
A1.1.1	0.5 M ethylenediamine tetraacetic acid (EDTA), pH 8.0 . .	167
A1.1.2	DNA extraction buffer for "Edward's preparations"	167
A1.1.3	2% Cetyltrimethyl Ammonium Bromide (CTAB) DNA extraction buffer	167
A1.1.4	<i>Agrobacterium</i> pH8.0 cell lysis buffer	168
A1.1.5	Tris/EDTA (TE) pH8.0 buffer for resuspending DNA . . .	168
A1.1.6	Tris/acetate/EDTA (TAE) buffer for electrophoresis	168
A1.1.7	Agarose gel loading dye	168
A1.2	Solutions used for microbiology	169
A1.2.1	1M CaCl ₂ stock solution	169
A1.2.2	Super Optimal Broth (SOB)	169
A1.2.3	Transformation buffer (TB)	169
A1.3	Oligonucleotides used in this work	170
A1.4	Sequence alignments	173

List of Figures

1.1	The structure of the model plant <i>Arabidopsis thaliana</i>	4
1.2	The ABC(E) model of flower development	6
1.3	Structure of the MIKC domain of Type II MADS-box transcription factors	11
1.4	Model for the activity of MADS-domain protein tetramers	11
1.5	Structure and composition of an <i>Arabidopsis</i> fruit	15
1.6	The genes involved in valve and medial domain patterning	16
1.7	Model for the control of trichome formation in <i>Arabidopsis</i>	19
1.8	Suppression of trichome formation in <i>Arabidopsis</i> flowers	21
1.9	The effect of cytokinin on floral trichome formation	24
1.10	Model for the inhibition of trichome formation on floral organs of <i>Arabidopsis</i>	25
1.11	A flower of <i>Brassica rapa</i> var. Yellow Sarson (R-o-18)	26
1.12	Selected <i>Brassica</i> cultivars used as vegetables	28
3.1	Expression patterns of <i>TRY</i> and <i>CPC</i> in <i>Arabidopsis</i>	53
3.2	Phenotypes of trichome repressor mutants	54
3.3	Schematic overview of the fate of DNA cleavages induced by the CRISPR/Cas9 machinery	58
3.4	Confocal images of <i>TRY</i> and <i>CPC</i> localisation	62
3.5	Schematic representation of the method used for replum measurement	63

3.6	Stage-specific and spatial differences in replum width	64
3.7	Reduction of replum width in <i>try</i> -29760 mutant plants	65
3.8	Complementation of <i>TRY</i> function in a <i>try</i> -29760 mutant back- ground	66
3.9	Effects of <i>TRY</i> over-expression on trichome formation in a <i>try</i> - 29760 mutant background	67
3.10	Effects of the <i>TRY</i> complementation constructs on replum width .	68
3.11	Characterisation of the novel lines generated in this work	69
3.12	Generation of novel <i>try</i> null mutant lines through CRISPR/Cas9 .	70
3.13	Lesions in the novel <i>try</i> mutant alleles generated by CRISPR/Cas9	72
3.14	Trichome phenotypes of the two novel homozygous <i>try</i> lines gen- erated through CRISPR/Cas9	73
3.15	Replum width of two novel <i>try</i> mutant lines generated using CRISPR/Cas9 genome editing	74
3.16	Replum width in <i>cpc-1</i> single and <i>try</i> -29760 <i>cpc-1</i> double mutants	75
3.17	Effects of BAP treatment on replum width in <i>try</i> and <i>cpc</i> mutant plants	76
3.18	Effects of BAP treatment on replum width in <i>TRY</i> complementa- tion and overexpression lines	77
3.19	Effects of BAP treatment on replum width in novel <i>try</i> mutant lines	78
3.20	The effect of BAP treatment on the replum of the lines generated in this work, compared to the existing mutant lines	79
3.21	Effects of BAP treatment on replum width in different <i>Arabidopsis</i> accessions	80
3.22	Effects of BAP treatment on replum width in various mutants with known or potential defects in replum formation	81
3.23	The effect of BAP treatment on single and double mutants involv- ing known replum regulators	82

3.24	Characterisation of the response of replum regulators <i>ALC</i> , <i>FUL</i> , <i>RPL</i> and <i>SHP2</i> to the lines generated in this work	84
3.25	Characterisation of the response of CK effectors in the lines generated in this work	86
3.26	A proposed model of TRY interacting with gynoecium development genes	93
3.27	Comparing measurements taken of the replum and the medial domain	95
4.1	The expression domains of the <i>SHATTERPROOF1</i> & 2 mRNAs in <i>Arabidopsis</i>	104
4.2	The triangle of U	106
4.3	The possible fates of duplicated genes	107
4.4	Chromosomal loci of putative <i>BrSHP</i> homologs	111
4.5	Alignment of the CDS of putative <i>SHP1</i> homologs identified in <i>B. rapa</i>	112
4.6	Alignment of the CDS of putative <i>SHP2</i> homolog identified in <i>B. rapa</i>	113
4.7	Alignment of the amino acid sequences of the putative <i>SHP1</i> homologs	114
4.8	Alignment of the amino acid sequence of the putative <i>SHP2</i> homolog	115
4.9	Schematic representation of the exon/intron structures of the <i>Br-SHP</i> genes	116
4.10	Expression of <i>BrSHP</i> mRNAs relative to <i>EF1α</i>	119
4.11	Heat map of expression of <i>BrSHP</i> mRNAs relative to <i>EF1α</i>	120
4.12	Expression of <i>BrSHP</i> mRNAs relative to <i>TUB</i>	121
4.13	Heat map of expression of <i>BrSHP</i> mRNAs relative to TUBULIN	122
4.14	Scatter plot of the mean Ct values across various tissues of the reference genes tested, TUB and <i>EF1α</i>	123

4.15	Expression of <i>BrSHP</i> genes in stamens relative to <i>TUB</i>	124
4.16	An example of a 10 mm <i>B. rapa</i> flower	125
4.17	Overexpression of the <i>Arabidopsis</i> and <i>B. rapa</i> copies of <i>SHP2</i> . . .	126
4.18	The expression of the <i>Arabidopsis</i> and <i>B. rapa</i> copies of <i>SHP2</i> driven under their native promoters	127
4.19	The expression of the <i>Arabidopsis</i> and <i>B. rapa</i> copies of <i>SHP2</i> with the promoter of the converse	128
4.20	The rescue of the <i>shp1/2</i> phenotype with complementation con- structs	129
4.21	The phenotype of the overexpression of the <i>BrSHP2</i> CDS in a <i>shp1</i> <i>shp2</i> background	129
4.22	Alignment of the 3' UTRs of <i>AtSHP1</i> and the three copies of <i>BrSHP1</i>	130
4.23	Alignment of the 3' UTRs of <i>AtSHP2</i> and <i>BrSHP2</i> homolog	130
4.24	The position of the SNPs of the TILLING alleles selected	132
4.25	Splice donor site in <i>BrSHP2</i>	135
4.26	Crossing plan for <i>B. rapa</i> TILLING mutant lines	136
4.27	Schematic of the workflow of a Random Impact Test (RIT)	137
4.28	Logo sequence of the Type II MADS domain	143
5.1	The expression domain of <i>AG</i> in <i>Arabidopsis</i>	148
5.2	The phenotype of an <i>ag-10 try-29760</i> gynoecium	149
5.3	The expression of <i>SHP1/2</i> homologs in the floral organs of <i>S. alba</i>	152
5.4	The phenotype of the <i>ag-10 shp1-1 shp2-1</i> triple mutant	155
5.5	The effect of CK on trichome formation on siliques of <i>shp1-1 shp2-</i> <i>1 try-29760</i> triple mutant plants	156
5.6	CDS alignment of <i>AtAG</i> and <i>BrSHP2</i>	157
5.7	Amino acid sequence alignment of <i>AtAG</i> and <i>BrSHP2</i>	158
5.8	Expression of <i>BrSHP2</i> driven by its large intron	161
5.9	The phenotype of <i>brag1-1 brag2-1</i> double mutant flowers	164

A1.1 Alignment of the <i>AtSHP2</i> and <i>AtAG</i> CDS	174
A1.2 Alignment of the <i>AtSHP2</i> and <i>AtAG</i> protein sequences	175
A1.3 Alignment of the <i>AtSHP2</i> and <i>BrSHP2</i> genomic sequences	176
A1.4 Alignment of the <i>AtSHP2</i> and <i>BrSHP2</i> large introns	177
A1.5 Alignment of the <i>AtAG</i> and <i>BrSHP2</i> genomic sequences	178
A1.6 Alignment of the <i>AtAG</i> and <i>BrSHP2</i> large introns	179

List of Tables

2.1	<i>Arabidopsis</i> lines generated during the course of this study	32
4.1	Proposed nomenclature for the <i>B. rapa</i> homologs of <i>Arabidopsis</i> <i>SHP1/2</i>	110
4.2	Sequence conservation across the <i>SHP1</i> homologs	114
4.3	Sequence conservation in the <i>SHP2</i> homologs	115
4.4	Syntenly analysis for <i>BrSHP1</i> homologs	117
4.5	Syntenly analysis for the <i>BrSHP2</i> homolog	117
4.6	TILLING alleles selected for this work	131
4.7	Results of Random Impact Test of <i>brshp</i> mutants.	137
5.1	Predicted MADS-domain transcription factor binding sites	159

Abbreviations

Abbreviation	Term
aa	Amino acid
AG	AGAMOUS
ALC	ALCATRAZ
AlcRpro	Ethanol-inducible promoter
amiRNA	artificial micro RNA
AP1	APETALA1
AP2	APETALA2
AP3	APETALA3
ApE	A Plasmid Editor
ARR	Cytokinin response regulator
AS1	ASYMMETRIC LEAVES 1
BAP	6-Benzylaminopurine
BLAST	Basic local alignment search tool
bp	base pair
BP	BREVIPEDICILLUS
CAL	CAULIFLOWER
cDNA	Complementary DNA
CDS	Coding DNA sequence
CK	Cytokinin
CMM	Carpel margin meristem
CPC	CAPRICE
CRC	CRABS CLAW
DMAS	Double-mismatch allele-specific
DNA	Deoxyribonucleic acid

DZ	Dehiscence zone
EF1 α	Elongating factor1 alpha
EtOH	Ethanol
FIL	FILAMENTOUS FLOWER
FLC	Flowering Locus C
FM	Floral meristem
FUL	FRUITFULL
GA	Gibberellic acid
GFP	Green fluorescent protein
GIS2	Gibberellin insensitive2
GUS	β -glucuronidase
HDR	Homology-directed repair
IAA	Indole-3-acetic-acid
Indels	Insertions/deletions
IPTG	isopropyl beta-D-1-thiogalactopyranoside
JA	Jasmonic acid
JAG	JAGGED
KAN1	KANADI 1
kb	kilobases
mRNA	Messenger RNA
NHEJ	Non-homologous end joining
nt	Nucleotide
OD	Optical density
p35S::	Cauliflower Mosaic Virus 35S promoter
PAM	Protospacer-adjacent motif
PCR	Polymerase chain reaction
PI	PISTILLATA
PIN	PIN-FORMED

RNA	Ribonucleic acid
RPL	REPLUMLESS
RPM	Revolutions per minute
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAM	Shoot apical meristem
SEP	SEPALLATA
sgRNA	Single guide RNA
SHP	SHATTERPROOF
SNP	Single nucleotide polymorphism
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1
SPL9	SQUASOMA PROMOTER BINDING PROTEIN-LIKE 9
STK	SEEDSTICK
STM	SHOOTMERISTEMLESS
TF	Transcription factor
TFL	TERMINAL FLOWER
TILLING	Targeting induced local lesions in genomes
TRY	TRIPTYCHON
(v/v)	Volume per volume (mL per 100 mL)
(w/v)	Weight per volume (g per 100 mL)
WT	Wild-type
X-Gal	5-Bromo-4-Chloro-3-Indolyl-beta-D-Galactoside
X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid
ZFP8	Zinc-finger protein8

1 Introduction

Plants account for approximately 80% of the biomass on Earth and thus dominate life on the planet (Bar-On et al., 2018). The kingdom Plantae is comparable in size and complexity to the kingdom Animalia, which also contains predominantly multicellular eukaryotes, and comprises an estimated 390,000 extant species (Antonelli et al., 2023). A large majority (approximately 350,000) of these species belong to the seed plants (i.e. angiosperms or gymnosperms), but other vascular plants such as ferns, or non-vascular plants including mosses and liverworts, also contribute considerably to the large size of the plant kingdom (Antonelli et al., 2023). Plants have a multitude of essential functions in the planet's biosphere from contributing to the maintenance of the atmosphere through the production of molecular oxygen and the fixation of carbon dioxide to providing essential nutrition to other life forms through the transformation of light energy from the Sun to chemical energy primarily in the form of sugars (Raven, 2021).

In addition to their central role in biology, plants are vital for human societies (Raven, 2021). The domestication of plants (as well as animals) and the development of agriculture approximately 12,000 years ago have allowed humans to establish settled communities and gradually abandon their nomadic hunter-gatherer lifestyle (Purugganan, 2019). Throughout the millennia, plants have retained their central importance for human societies, with up to 80% of the human diet being comprised of plants (Ulijaszek, 1991). In fact, the large increase in the global population seen over the past centuries can be directly attributed to

increased food security resulting from improved agricultural production methods (Mulugeta Habtewold and Heshmati, 2023). An important example of this is the "Green Revolution" of the mid-20th century, where improved crop cultivars (such as the semi-dwarf IR8 rice variety) were developed and combined with advanced irrigation techniques as well as the application of synthetic fertilisers and pesticides to dramatically increase crop yields, especially in the developing world (Eliazer Nelson et al., 2019). Because of the continuing growth of the global population, there is renewed pressure on the agricultural sector to further increase food production. However, dwindling natural resources (land, water, as well as fertilisers) and the detrimental effects of current agricultural production techniques on biodiversity (e.g. through the use of pesticides) make this a difficult task. There is thus an urgent need for more sustainable food production. This challenge is further exacerbated by the effects of climate change, which are predicted to substantially reduce crop yields in many parts of the world (FAO, 2015). Meanwhile, a reduction in the consumption of animal products is being recommended by climate scientists due to their high water and land requirements, as well as greenhouse gas production (Hedenus et al., 2014).

Given the above, it is arguably more important now than ever to focus on plant research and to generate knowledge which can be used to breed improved crop cultivars with increased stress tolerance that can withstand the effects of climate change and that are less dependent on irrigation and the application of fertilisers and pesticides. In fact, it has been argued that there is an urgent need for a renewed "Green Revolution", which would heavily rely on the use of methods for the genetic modification of crop plant genomes, e.g. through the application of genome editing approaches (Custers and Dima, 2022).

The number of plant species that have been domesticated worldwide and adopted for agricultural production is estimated to be as high as 2,500 (Purugganan, 2019). A large majority of these plants belong to the angiosperms, or flowering plants, which are the largest group of land plants. While some an-

giosperms are cultivated exclusively for harvesting their vegetative organs including roots, shoots, leaves, tubers and axillary buds, their flowers and the fruits and seeds they produce are by far the most important food source for humans and their livestock. Historically, research on flowers and fruits has therefore attracted the attention of many scientists including those, who, in addition to being interested in basic biological phenomena, aim at applying new knowledge about the biology of plants for the improvement of agriculture. In the sections that follow, I will summarise what is currently known about these central developmental processes, starting with the transition from the vegetative to the reproductive phase of plant development which precedes the formation of flowers. My discussion will focus primarily on the small cruciferous plant *Arabidopsis thaliana* (*Arabidopsis*, hereafter), which has served as the main model for plant genetics over the past 40 years and has led to many fundamental insights into plant biology (Koornneef and Meinke, 2010; Meyerowitz, 2001; Provart et al., 2016) (**Figure 1.1**). Some of the many reasons for choosing *Arabidopsis* as a model organism include its short life span, high seed yield per plant, its ability to grow in a wide variety of conditions, its diploid and relatively small genome and its acclimation to many different regions across the world, with distinct genetic hallmarks allowing them to survive and tolerate the conditions they are subjected to in their environment.

1.1 Induction of flower development

The post-embryonic life cycle of *Arabidopsis* can be broadly divided into two phases, namely the vegetative and the reproductive phase. During the vegetative phase, the root system develops and leaves are formed by the shoot apical meristem, which contains a pool of pluripotent stem cells. The transformation of the shoot apical meristem into an inflorescence meristem marks the transition from the vegetative to the reproductive phase of development. This transition is

tightly regulated to ensure that flowering occurs under conditions that allow reproductive success and depends on both environmental and endogenous cues.

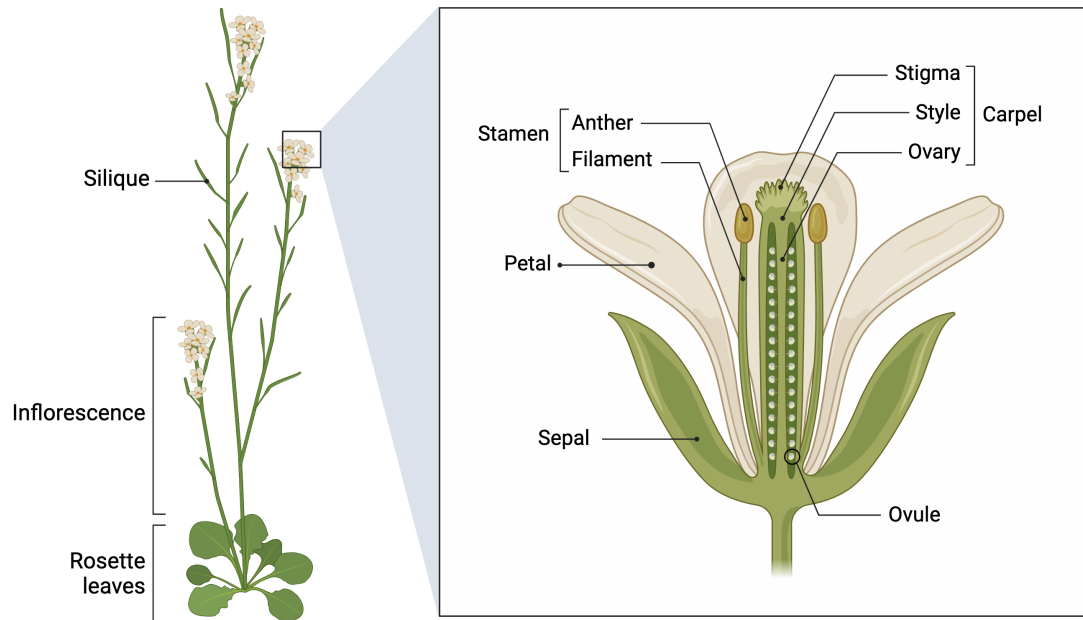


Figure 1.1: The structure of the model plant *Arabidopsis thaliana*.

On the left: schematic of an *Arabidopsis* plant after the transition to the reproductive phase of development. The positions of rosette leaves, the inflorescence and siliques are indicated. On the right: schematic of a flower. The different types of floral organs and their parts are indicated. Figure created with Biorender.com.

Of these cues, seasonal changes in day length are arguably most important with *Arabidopsis* being a facultative long-day plant, i.e. long days promote flowering but flowering eventually happens even when days are short (Simpson and Dean, 2002). Some *Arabidopsis* accessions, namely those that germinate in autumn, outlast winter and flower in spring, also require vernalisation, i.e. the exposure of the plant to a prolonged period of cold temperatures (Michaels and Amasino, 2008). Distinct genetic pathways exist that process the inputs from the different environmental and endogenous signals. These pathways converge on a small number of regulatory genes, such as *FLOWERING LOCUS C (FLC)* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)*, which are

known as floral pathway integrators (Parcy, 2005). These genes set in motion the genetic program needed for the formation of flowers. In the first step, they confer floral meristem identity to groups of cells that are initiated on the flanks of the inflorescence meristem and that develop into floral buds. This process depends on the activation of a small number of key regulators including LEAFY (LFY) and APETALA1 (AP1) which are crucial for the activation of genes that are needed for the formation of flowers, as well as for repressing genes that confer shoot identity, for example *TERMINAL FLOWER* (*TFL*) (Goslin et al., 2017; Kaufmann et al., 2010; Winter et al., 2011; Winter et al., 2015). Among the genes activated by LFY and AP1 are the floral organ identity genes (Parcy et al., 1998), which play key roles in the specification and development of floral organs. These genes will be discussed further in subsequent sections.

1.2 Floral patterning and floral organ specification

Arabidopsis flowers are composed of four different types of floral organs, which are arranged in four concentric circles or whorls, each containing one of the organ types (Smyth et al., 1990). As depicted in **Figure 1.1**, the first, outermost whorl contains four sepals, which are leaf-like organs that protect the developing floral bud. The second whorl contains four petals, which in *Arabidopsis* are small and inconspicuous as they do not serve, as in many other angiosperms, to attract pollinators (*Arabidopsis* reproduces primarily by self-pollination but outcrossing, e.g. through touch or wind, can also occur) (Abbott & Gomes, 1989). The third whorl consists of six stamens, which are the male reproductive organs and consist of a filament and an anther. The anther produces pollen grains which contain the male gametes. The innermost whorl consists of two carpels that fuse to form the gynoecium. The gynoecium is the female reproductive organ of the plant, which contains an ovary, a style and a stigma as well as a transmitting tract that facilitates the transport of male nuclei to the ovary to allow fertilisa-

tion to occur. The gynoecium in *Arabidopsis* develops a septum between the two fused carpels during later developmental stages before fertilisation, which will be discussed in greater detail in Section 1.6.

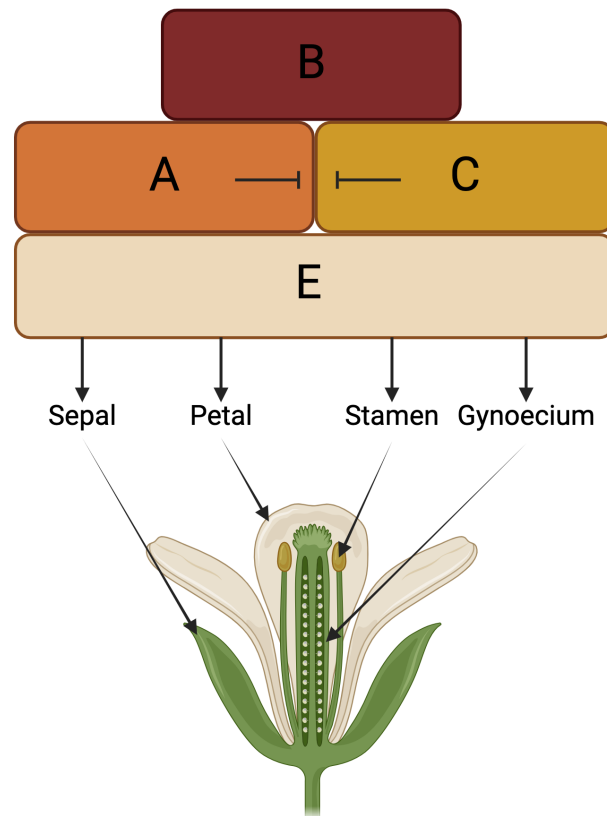


Figure 1.2: The ABC(E) model of flower development.

The combinations of the different gene activities and the floral organ types they specify are indicated. Figure created with Biorender.com.

The characterisation of a set of homeotic mutants that all exhibited floral organ identity defects in the 1980s and early 1990s led to the discovery of a small number of floral organ identity genes, which encode master regulators of floral organ specification and development. The activities of these genes were summarised in the ABC model of flower development (Coen and Meyerowitz, 1991), which has guided research in the flowering field since its publication. This model is based on the idea that three gene activities, termed A, B and C, act in a combinatorial manner to control the identities of the different floral organ types

(**Figure 1.2**). Specifically, the model posits that A-class gene activity alone results in the formation of sepals, while a combination of A and B-class activities confers petal identity. Furthermore, B-class combined with C-class gene activities lead to stamens, and C-class gene activity alone is sufficient for the formation of carpels. An important additional feature of the ABC model is that A- and C-class genes act antagonistically and repress each other's activities.

The detailed molecular characterisation of the floral organ identity genes showed that they are expressed primarily in the organs in which they are active (Ó Maoiléidigh et al., 2014). Thus, it appears that the patterning of the floral meristem into distinct whorls with different organ types is largely mediated by establishing spatially restricted expression domains for the floral organ identity genes. The control of the expression of these genes is complex and the exact regulatory mechanisms vary from gene to gene. However, they have in common that there are positive inputs, for example, through the above-mentioned LFY and AP1 regulators, as well as negative inputs that restrict the expression of the genes to certain domains within the floral meristem (Ó Maoiléidigh et al., 2014).

In *Arabidopsis*, *AP1* and *APETALA2* (*AP2*) are considered A-class genes. In the cells where these genes are being expressed, the development of carpels is being suppressed due to the above-mentioned antagonism of A- and C-class genes. Perturbation of the expression of A-class genes results in the transformation of sepals into carpel-like structures. In contrast, a perturbation of the C-class gene *AGAMOUS* (*AG*), which is responsible for the differentiation of the stamens and carpels, leads to the transformation of stamens and carpels into petals and sepals, respectively. Lastly, B-class genes in *Arabidopsis* are *PISTILLATA* (*PI*) and *APETALA3* (*AP3*). When B-class gene activity is perturbed, petals are converted to sepals and stamens to carpels.

Experiments in which different combinations of the floral homeotic genes were ectopically expressed led to changes in floral organ identities as predicted by the ABC model (Mizukami and Ma, 1992). However, in these experiments,

leaves could not be converted into floral organs as would be expected if the floral organ identity genes were indeed master regulators. This suggested that flower-specific co-factors might exist that are needed for the activities of the floral organ identity genes. These co-factors were later identified through reverse genetics and found to be encoded by four *SEPALLATA* (*SEP*) genes, which act in a largely redundant manner (Ditta et al., 2004; Pelaz et al., 2000). These genes were incorporated into the ABC model as E-class genes (Bowman et al., 2012; Causier et al., 2010), leading to a modified ABCE model (D-class genes, which were identified and named prior to the E-class genes, are involved in female reproductive development; see Section 1.6 below). It has been shown that the co-expression of combinations of the ABCE genes results in the conversion of leaves into floral organs (Goto et al., 2001; Honma and Goto, 2001; Pelaz et al., 2001), indicating that these genes are indeed master regulators for floral organ identity specification.

1.3 Control of floral organ development by floral organ identity factors

Expression of the floral organ identity genes commences during the early stages of flower development when organ specification takes place. However, the genes remain active throughout most of flower development, suggesting that they have important functions also at later stages (Ó Maoiléidigh et al., 2014). In agreement with this idea, gene perturbation experiments and other studies have shown that the floral organ identity genes control various processes during organ maturation (Wellmer et al., 2014). For example, in stamens, they control filament elongation, anther morphogenesis and microsporogenesis (Ito et al., 2004; Ito et al., 2007). From molecular analyses, it appears that in some cases, the transcription factors encoded by the floral organ identity genes act directly on genes involved in the differentiation of specific cell types, while in others, they activate

additional regulatory genes that then control developmental processes in parallel with or without further involvement of the floral organ identity genes. An example of the latter mechanism is the activation of *NOZZLE/SPOROCTELESS* (*NZZ/SPL*) by the C-class regulator AG, which is essential for microsporogenesis (Ito et al., 2004). Thus, it appears that the floral organ identity genes have a multitude of functions during floral organ development and that they employ different regulatory mechanisms to control the gene regulatory networks underlying flower formation.

Using genomic approaches, gene expression during flower development has been characterised in numerous studies (Alves-Ferreira et al., 2007; Jiao and Meyerowitz, 2010; Ryan et al., 2015; Wellmer et al., 2006; Wellmer et al., 2004). This work has led to detailed insights into the floral transcriptome with both spatial and temporal resolution. At the same time, target genes of the floral organ identity factors were identified on a genome-wide scale through a combination of genome-wide localisation and gene perturbation studies (Ó Maoiléidigh et al., 2013). It was found that in each case they either directly or indirectly regulate the expression of several hundred genes. Many of these target genes encode other regulatory proteins or microRNAs, suggesting that the floral organ identity factors provide various inputs into the gene regulatory network underlying flower formation. However, the architecture of this network is at present not well understood. Similarly, the precise mechanism through which the floral organ identity factors act on their target genes remains to be elucidated. In the following section, I will review what is known about how these transcription factors control the expression of their target genes.

1.4 The Quartet model: MADS-domain protein tetramers

As described above, the floral identity genes encode transcription factors. With the exception of AP2, all belong to the family of MADS-domain proteins. MADS-domain proteins are encoded by MADS-box genes, which are not plant-specific and can be found in animals and fungi as well. However, the size of the MADS-box gene family is much enlarged in plants compared to other eukaryotes. For example, in *Arabidopsis*, well over 100 genes encode MADS-domain proteins of the 20,000 total genes in the organism. This is much larger in comparison to animals, which tend to possess only up to 5 MADS-box genes (Gramzow and Theissen, 2010). Members of the MADS-domain protein family can be divided into two main classes, denoted as Type I and Type II (Gramzow and Theissen, 2010). Type II MADS-domain transcription factors are of particular importance for the control of flower development as they include most of the floral organ identity factors as well as many other important floral regulators, such as SEED-STICK (STK), FRUITFULL (FUL) and CAULIFLOWER (CAL) (Gramzow and Theissen, 2010). These proteins are commonly referred to as MIKC-type proteins because of their domain structure (**Figure 1.3**): the N-terminal, eponymous MADS domain (M) is followed by an Intervening (I) domain; both domains are crucial to enable the transcription factors to bind to recognition motifs in DNA and for dimerisation. These domains are followed by a keratin-like (K) domain, and a carboxy-terminal C domain, which facilitate higher-order protein-protein interactions and in the case of the C domain can mediate transcriptional activation of target genes (Gramzow and Theissen, 2010).

MADS-domain proteins bind as hetero- and/or homodimers to so-called CArG-box motifs (consensus sequence: 5'-CC(A/T)₆GG-3'). However, they can also be part of higher-order protein complexes. Notably, the above-mentioned

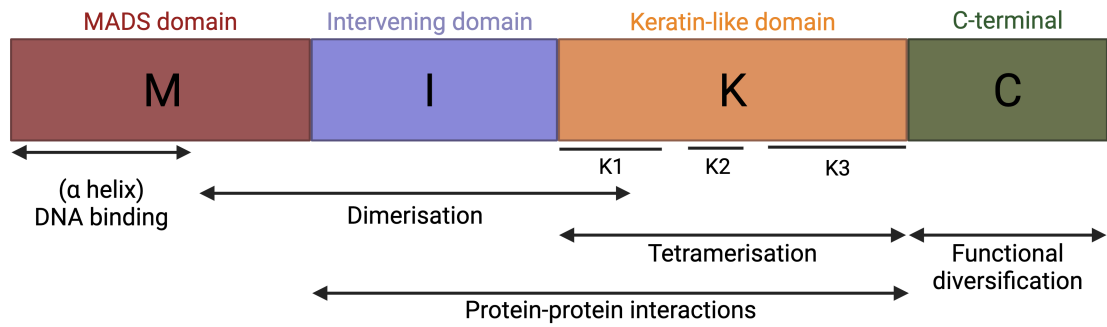


Figure 1.3: Structure of the MIKC domain of Type II MADS-box transcription factors.

The four domains of MIKC-type MADS-domain proteins and their functions are shown. K1-3: amphipathic α -helices. Figure created with Biorender.com.

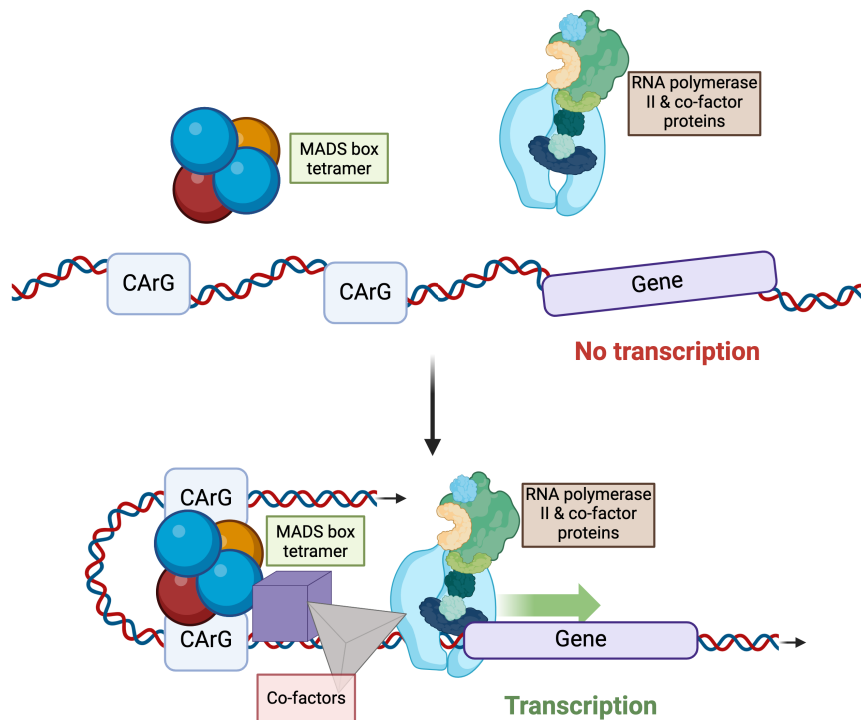


Figure 1.4: Model for the activity of MADS-domain protein tetramers.

At the top: target gene of MADS-domain proteins with two CArG-boxes in its promoter in an inactive state. Binding of a MADS-domain protein tetramer to the CArG-boxes (at the bottom) leads to looping of the intervening DNA, the possible recruitment of additional transcriptional regulators and the onset of transcription. Figure created with Biorender.com.

SEP co-factors have been shown to facilitate the formation of MADS-domain protein tetramers, in combination with other floral organ identity factors to specify a particular organ type (Melzer and Theissen, 2009). For example, in the second whorl where both A- and B-class genes act in concert to initiate petal formation, a tetramer may be formed by the A-class factor AP1, the B-class proteins AP3 and PI (which act as an obligate heterodimer) and a SEP protein. This tetramer would then bind to two adjacent CArG boxes possibly resulting in the looping of the intervening DNA. This model, which is known as the Quartet model (**Figure 1.4**), thus predicts that the four different types of floral organs are specified by four different MADS-domain protein tetramers with the composition of the tetramers being predicted by the ABCE model (Theissen et al., 2016; Theissen and Saedler, 2001; Espinosa-Soto et al., 2014; Hugouvieux et al., 2018).

Genome-wide localisation studies for the different floral organ identity factors (Kaufmann et al., 2009; Kaufmann et al., 2010; Ó Maoiléidigh et al., 2013; Pajoro et al., 2014; Wuest et al., 2012) showed that they indeed bind preferentially to CArG boxes. What's more, their binding patterns exhibit large overlaps despite their in some cases (in particular, the A-class regulator AP1 and the C-class regulator AG) clearly distinct functions. Gene perturbation experiments then showed that binding of the floral organ identity factors to the promoters of target genes does not always lead to changes in expression. In fact, many binding events appear to be transcriptionally silent. An unanswered question in the field is therefore how the floral organ identity factors achieve regulatory specificity. It has been proposed that this may be in part due to differences in the sub-types of CArG-box sequences to which they bind with high affinity. Alternatively, other regulatory proteins may interact with the floral organ identity factors on the promoters of some, but not all, target genes, leading to changes in gene expression. In agreement with this idea, it has been shown that the floral organ identity factors are part of larger protein complexes, and some candidates for interacting proteins have been identified. However, the function of these

interactors is currently not well understood.

1.5 MADS-box genes and the evolution of flowers

In 1790, long before any knowledge of the genetics of plants, the writer and polymath Johann Wolfgang von Goethe published his seminal essay “The Metamorphosis of Plants” (Goethe, 1790). In it, Goethe introduced his idea that all plant parts are derived from a single basic organ, which he referred to as the “Urblatt”, or primordial leaf. According to Goethe, the apparent diversity of plant organs (e.g., leaves, sepals, petals, etc.) is a result of modifications of this primordial leaf. Thus, he sought to emphasise the unity of plant morphology, rather than viewing different plant parts as entirely separate entities. He believed that specific developmental changes, such as folding, elongation, or fusion of parts, led to the diversity of plant structures. Goethe’s hypothesis is consistent with the concept of homology, which suggests that different plant parts share a common evolutionary origin. In his view, the similarity among various organs is a result of their shared ancestry from the primordial leaf.

Goethe’s ideas were confirmed, at least in part, 200 years after the publication of “The Metamorphosis of Plants” through the groundbreaking work on the ABCE model. Specifically, it was shown that the simultaneous loss of A-, B- and C-class gene activities results in flowers which consist of four whorls of leaf-like organs (Alvarez-Buylla et al., 2010). A similar phenotype was observed in plant mutants for all four *SEP* genes (Ditta et al., 2004). Thus, the floral organ identity factors appear to transform leaf-like structures into floral organs. This hypothesis is further supported by the finding that known regulators of leaf development were found to act downstream of the floral organ identity factors. Based on these observations, it was concluded that these regulators modulate and partially suppress the genetic program for leaf development. While these results show that the floral organ identity genes play an important role in the evolution

of flowers, it is important to note that they are not specific to angiosperms but are also found in gymnosperms where they are involved in determining male and female reproductive organs (Winter et al., 2002). Thus, they must have evolved before the groups of gymnosperms and angiosperms diverged.

1.6 Control of gynoecium and fruit development

One of the final processes undertaken by an annual plant, such as *Arabidopsis*, in its life cycle is the initiation of fruit development post-fertilisation and the production of seeds contained within the fruit. Fruits form from the gynoecium in the centre of the flower. As described above (Section 1.2), the gynoecium of *Arabidopsis* is composed of two congenitally fused carpels. Along the apical-basal axis of the gynoecium (i.e., from top to bottom), four distinct regions can be defined: style, stigma, ovary and gynophore (**Figure 1.5 (A)**). The ovary, which makes up most of the *Arabidopsis* gynoecium, is composed of the valves (or carpel walls), the replum, and the septum (**Figure 1.5 (C)**). In a mature fruit, the valves open along a tissue termed valve margins, which form between the replum and the valves (**Figure 1.5 (B)**).

During pollination, the first point of contact for a pollen grain is with the stigma at the top of the gynoecium. The desiccated pollen grain relies on the stigmatic papillae on the surface of the stigma to provide water for hydration, and this occurs when the pollen is recognised as compatible. In the case of *Arabidopsis*, this includes a flower's own pollen as it is a self-compatible species. Following hydration, the pollen grain forms a pollen tube that enters the stigma and grows down the transmitting tract to an ovule in the ovary where sperm cells are released to allow fertilisation of an egg cell.

Both the formation of the gynoecium and of fruits have been studied in great detail over the past 40 years, leading to the discovery of many key regulators and pathways that control different aspects of the development of these or-

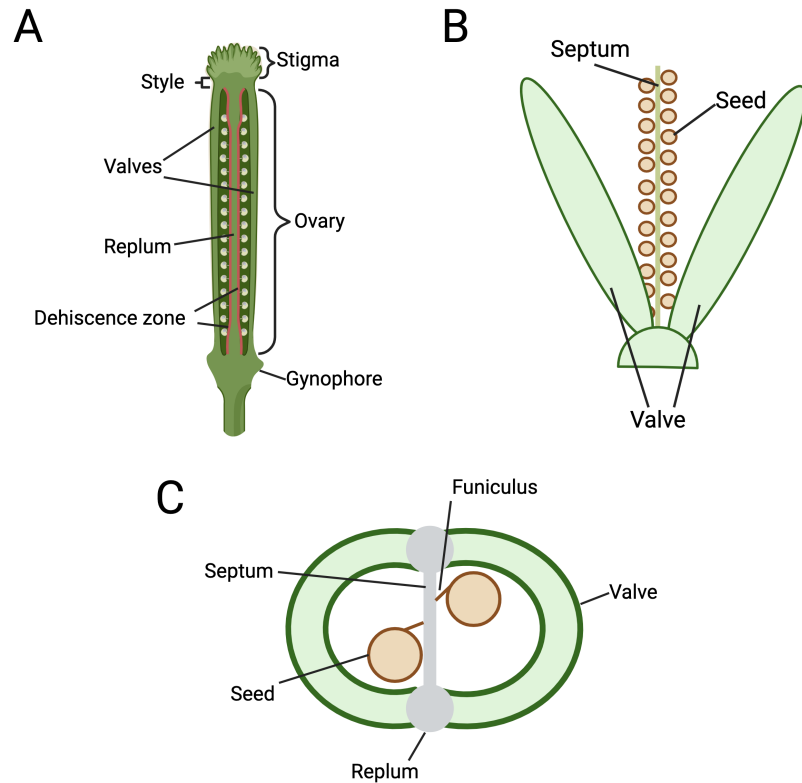


Figure 1.5: Structure and composition of an *Arabidopsis* fruit.

(A) Schematic of a longitudinal section of a fruit. The different parts of the fruit are indicated. (B) Schematic of a fruit after opening. Valves separate exposing the seeds. (C) Schematic of a longitudinal section of a fruit. Different tissues are indicated. Figure created with Biorender.com.

gans. As described in Section 1.2, the C-class regulator AG is crucial for the specification of carpels and thus initiates gynoecium development. Among the many genes that have been identified to act downstream of AG are *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF2* (*SHP2*), which both encode MADS-domain transcription factors that are closely related to AG. Their expression commences in the gynoecium during early floral stages and becomes restricted to the valve margins and ovules around the time of fertilisation. Double mutants affected in both *SHP* genes form fruits that do not open upon maturation, indicating that they are required for the dispersal of seeds. Morphological analyses showed that in the absence of *SHP* gene activity, the valve margin cells fail to develop properly, and the dehiscence zone does not differentiate correctly.

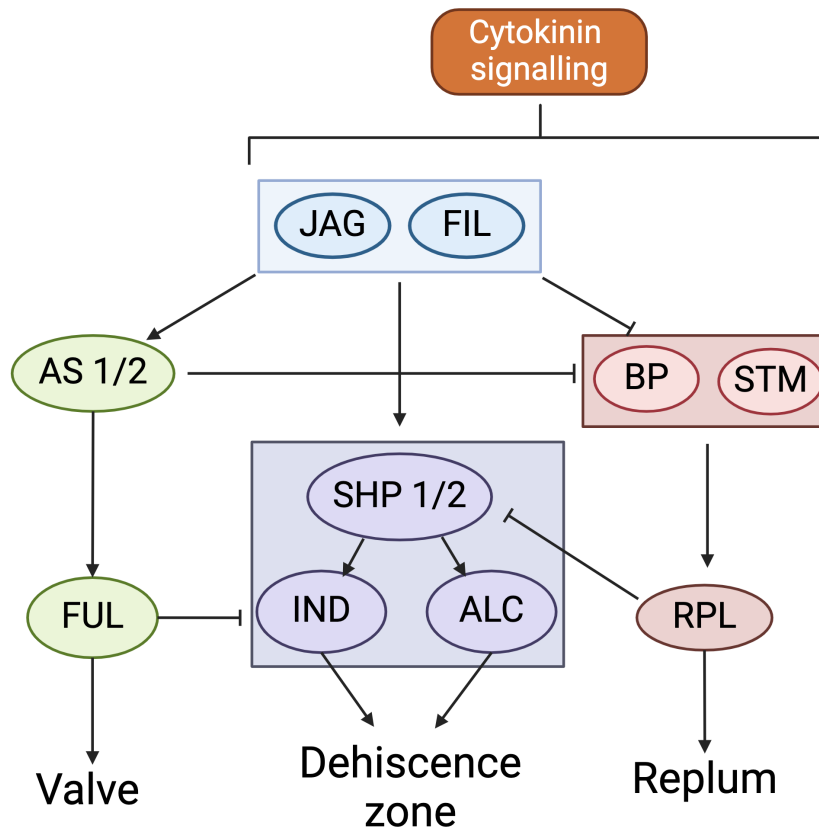


Figure 1.6: The genes involved in valve and medial domain patterning.

A model illustrating the interaction of genes regulating the medio-lateral patterning of the gynoecium. In blue at the top, upstream regulators JAGGED and FILAMENTOUS FLOWER. In green, the valve tissue regulators ASYMMETRIC LEAVES1/2 and FRUITFULL. In purple, the dehiscence zone regulators SHATTERPROOF1/2, ALCATRAZ and INDEHISCENT. In red, the replum tissue regulators BREVIPEDICELLUS, SHOOTMERISTEMLESS and REPLUMLESS. Figure created with Biorender.com

Several other genes have been identified to be involved in the specification of the valve margins. These include *FRUITFULL* (*FUL*), *REPLUMLESS* (*RPL*), *ALCATRAZ* (*ALC*), and *INDEHISCENT* (*IND*). *ALC* and *IND* act downstream of the *SHP* transcription factors and directly contribute to conferring valve margin identity (**Figure 1.6**). *FUL* encodes a MADS-domain transcription factor that is involved in multiple aspects of fruit development. It is primarily expressed in valves where it has been shown to negatively regulate the *SHP* genes, *ALC* and *IND* to restrict their expression to the valve margins. On the other side of the

valve margin, in the replum, the gene *RPL* appears to play a role that is similar to that of *FUL* in valves – it represses the valve margin identity genes, restricting their expression to a narrow strip of cells in between the valves and the replum (**Figure 1.6**).

In addition to their essential role in controlling seed dispersal, the *SHP* genes are also key regulators of ovule identity where they act together with the MADS-domain transcription factors SEEDSTICK (STK) and AG. The *SHP* genes and STK together are also referred to as ‘D class genes’ that when added to the above-mentioned ABCE model (Section 1.2) lead to an ABCDE model of flower development, which can explain the formation of most structures and organs of the flower. However, the D-class genes are no longer considered to fit into the model of floral development regulators of the four whorls of *Arabidopsis* flowers, as these genes act specifically to regulate ovule and seed coat development, which is a subset of the fourth whorl.

As well as to a multitude of regulatory genes of which many encode transcription factors, gynoecium and fruit development depend on the activities of different phytohormones, with auxin and cytokinin being arguably the most important. One example where these hormones play an essential role is the formation of the lateral and the medial domain in the gynoecium around stage 7 of flower development (Smyth et al., 1990). The lateral domain corresponds to the carpel valves and the medial domain includes the replum and septum, the placenta and ovules, as well as the stigma and style. The tissues of the medial domain are generated by a specialised meristem, the carpel margin meristem (CMM). In order to distinguish between the lateral and medial domains, antagonism between auxin and cytokinin is exploited. Cytokinin signalling appears to be restricted to the medial domain where it induces auxin biosynthesis and transport of auxin to the cells that will become in the lateral domain. In the cells of the lateral domain, the auxin that is being brought in from the medial domain acts to suppress cytokinin signalling. Thus, the establishment of differences in

cytokinin signalling appears to be the main distinguishing factor for patterning of the gynoecium into a medial and a lateral domain.

Auxin and cytokinin also play important roles in other processes during gynoecium and fruit development. For example, cytokinin appears to be the main determinant for the development of the replum. Low levels of cytokinin or cytokinin signalling lead to a narrow replum, while high levels of cytokinin or cytokinin signalling result in an enlarged replum. In fact, it has been shown that an exogenous treatment of wild-type flowers with a cytokinin solution leads to over-proliferation of the replum (Zúñiga-Mayo et al., 2014; Marsch-Martínez et al., 2014).

1.7 Suppression of trichome formation on floral organs by MADS-domain proteins

An early example of floral organ identity genes suppressing the genetic network for leaf development is the control of trichome formation on floral organs. Trichomes are epidermal outgrowths that are commonly found on leaves and stems of many plant species. Trichomes are usually not, with the exception of the abaxial side of sepals, found on floral organs in *Arabidopsis*. They have several different functions, which include the reduction of transpiration and the reflection of UV light (Dalin et al., 2008). Furthermore, they have been shown to act as repellents of insect pests by limiting their movement on the surface of a plant.

Genetic and molecular analyses have led to detailed insights into the mechanism underlying the control of trichome initiation in *Arabidopsis*. Trichome development is promoted by a transcriptional activator complex composed of an R2R3 MYB-type transcription factor, GLABRA1 (GL1) (Oppenheimer et al., 1991), a WD40-repeat protein, TRANSPARENT TESTA GLABRA1 (TTG1) (Galway et al., 1994; Walker et al., 1999), and a basic Helix-Loop-Helix transcription

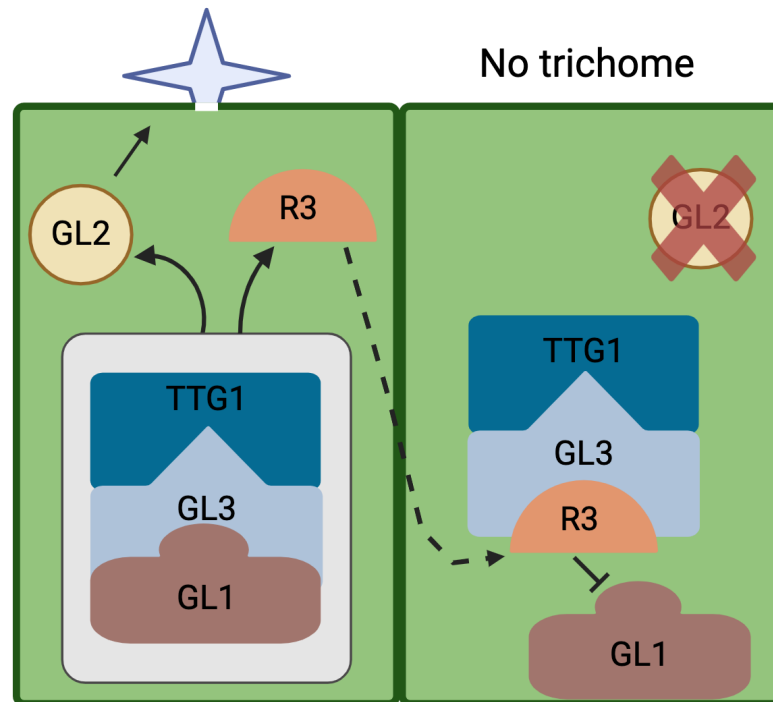


Figure 1.7: Model for the control of trichome formation in *Arabidopsis*.

In a trichome-forming cell (on the left), the GL1-GL3-TTG1 activator complex initiates the expression of GL2, which controls trichome morphogenesis. The activator complex also triggers the expression of small R3 MYB proteins (R3), such as TRIPTYCHON (TRY) and CAPRICE (CPC), which can move to neighbouring cells (on the right), where they compete with GL1 for binding to the activator complex rendering it inactive and also inhibiting GL1 expression. This results in the suppression of trichome formation. Figure created using Biorender.com

factor, either GLABRA3 (GL3) or the closely related ENHANCER OF GLABRA3 (EGL3) (Bernhardt et al., 2003; Payne et al., 2000) (**Figure 1.7**). This complex triggers the expression of the homeodomain-containing protein, GLABRA2 (GL2) (Masucci et al., 1996; Rerie et al., 1994), which is required for the initiation of trichomes. At the same time, the activator complex also induces the expression of a number of genes that encode single-repeat R3 MYB transcription factors (Pattanaik et al., 2014). These proteins can move from trichome-forming cells to neighbouring cells where they compete with GL1 for binding to the complex and render it inactive, resulting in the inhibition of trichome initiation in those

cells (Hülkamp et al., 1994; Pesch and Hülkamp, 2004; Pesch and Hülkamp, 2009; Schellmann et al., 2002). Furthermore, it has been shown that at least some of these R3 MYB proteins can directly suppress *GL1* expression, adding to the negative effect on trichome initiation they confer (Wang and Chen, 2014). Thus, trichome patterning in the leaf epidermis appears to be largely mediated by an activator–inhibitor feedback loop although it has been shown that the removal of TTG1 from non-trichome cells is an important contributing factor as well (Bouyer et al., 2008).

In *Arabidopsis*, seven small single-repeat R3 MYB transcription factors have been identified as repressors of trichome formation (Wang and Chen, 2014). Of these, TRIPTYCHON (TRY) and CAPRICE (CPC) are arguably most important. Over-expression of these transcription factors leads to a strong reduction of trichome number in agreement with their proposed function as trichome repressors. However, the analysis of single mutants in the corresponding genes revealed that they have different effects on trichome formation (Wang and Chen, 2014). For example, mutations in *TRY* lead to increased trichome clustering, while mutations in *CPC* result in a higher trichome density on leaves. Genetic analyses revealed that these genes, despite their distinct single-mutant phenotypes, act in a largely redundant manner.

The small R3 MYB proteins, as well as several components of the transcriptional activator complex involved in trichome formation, are also part of the mechanism that initiates the formation of root hairs (Wang and Chen, 2014). However, in the root, activation of *GL2* leads to non-hair cells, while an absence of *GL2* activity results in root hair development. In fact, the role of the regulatory components is inverted between trichomes and root hairs so that the small R3 MYB proteins are activators of root hair development and repressors of trichome formation.

Through a combination of genome-wide localisation studies and gene perturbation experiments using artificial microRNAs (amiRNAs) (Schwab et al.,

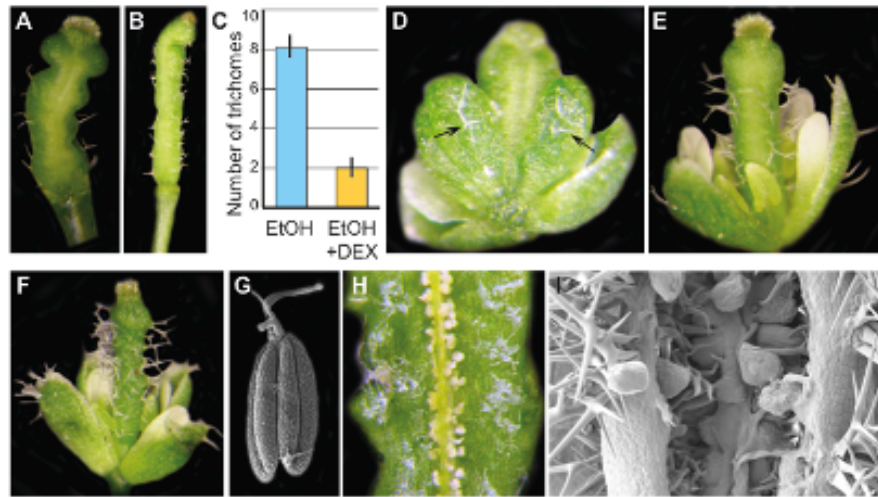


Figure 1.8: Suppression of trichome formation in *Arabidopsis* flowers.

(A) Gynoecium of a wild-type plant after an amiRNA-mediated knockdown of AG. (B) Gynoecium of a *shp1 shp2* double-mutant plant after an AG knockdown. (C) Number of trichomes forming on gynoecia of ethanol (EtOH)-treated 35SPro:AlcR/AlcAPro:AG-amiRNA 35SPro:GR-LhG4/6xop:SHP2-GFP *ag-1* plants with or without dexamethasone (DEX) treatment. (D) Flower of a *try* mutant plant after a simultaneous knockdown of AG and AP3. Trichomes on stamens are marked by arrows. (E) Flower of a *cpc* mutant plant after an AG knockdown. (F-I) Phenotypes of *try cpc* double-mutant plant after an AG knockdown. (F) A Flower. (G) Trichome on the tips of an anther. (H-I) Trichomes inside of a gynoecium. Images taken from (Ó Maoiléidigh et al., 2018).

2006) driven under the control of an ethanol-inducible promoter system, it was shown that the C-class regulator AG represses the expression of activators of trichome initiation and activates the repressors (Ó Maoiléidigh et al., 2013). Based on these results it was hypothesised that AG might be involved in the suppression of trichome formation on reproductive floral organs. In agreement with this idea, it was found that a specific knockdown of AG activity led to the formation of trichomes on carpel valves (Figure 1.8 (A)). However, this phenotype was relatively weak with only a few trichomes being formed per gynoecium. Also, trichomes on stamens were never observed despite the fact that AG is active in this organ type. It was therefore hypothesised that other pathways may exist that suppress trichome formation in the absence of AG activity. To test this, several likely candidates for redundant genes were investigated. For example, it had been shown previously that the genes *SHP1* and *SHP2*, which are

closely related to *AG* and together control gynoecium and fruit development (see Section 1.6 above), can functionally replace *AG* (Pinyopich et al., 2003). To test whether *SHP1*/*SHP2* may act in the suppression of trichome development, *AG* activity was knocked down in a *shp1 shp2* double-mutant background. This led to a strong increase in trichome number on carpel valves (**Figure 1.8 (B)**), in agreement with the idea that *AG* and *SHP1/2* act redundantly in the suppression of trichome initiation. Also, when *AG* activity was knocked down and simultaneously *SHP2* was over-expressed, a significant reduction in the number of trichomes on carpels was found when compared to an *AG* knockdown alone (**Figure 1.8 (C)**), suggesting that the *SHP* genes can partially compensate for a loss of *AG* activity.

Candidates for genes acting redundantly with *AG* in male reproductive organs were the B-class regulators *AP3* and *PI*, which control stamen identity together with *AG* (see Section 1.2 above). When the activities of both B- and C-class genes were knocked down simultaneously, the formation of trichomes on the anthers of stamens was observed (Ó Maoiléidigh et al., 2013), suggesting that these regulators act redundantly in the suppression of trichome formation. However, the penetrance of this phenotype was low, implying that additional redundant genes or pathways may exist.

By mining a dataset on temporal gene expression during early flower development (Wellmer et al., 2006), it was found that expression of the repressor of trichome initiation *TRY* is strongly up-regulated during early flower development (Ó Maoiléidigh et al., 2013). This increase in expression also occurred when either *AG* or *AP3* were knocked down, suggesting that up-regulation of *TRY* is independent of B and C function (Ó Maoiléidigh et al., 2013). In fact, it has been shown that SQUAMOSA Promoter Binding Protein-Like (*SPL*) transcription factors, which mediate the age-dependent control of trichome distribution on leaves (Yu et al., 2010), promote the expression of *TRY* (and other trichome repressor genes) in flowers. To test whether *TRY* plays a role in the suppres-

sion of trichome formation in flowers, the functions of AG and/or AP3 were perturbed in flowers of *try* mutant plants. This led to a considerable increase in the number of trichomes formed on the reproductive floral organs (including stamens when AP3 function was perturbed; **Figure 1.8 (D)**) when compared to the aforementioned knockdown experiments in a wild-type background (**Figure 1.8 (A)**). Thus, it appears that *TRY* and the floral organ identity genes act in independent pathways in the suppression of trichome formation. Similar results were obtained when the role of another key repressor of trichome formation, *CAPRICE CPC*, was tested (**Figure 1.8 (C)**). These results showed that an impairment of C-class gene activity, when combined with a knockdown of a trichome repressor gene, leads to the formation of trichomes on gynoecia.

To further investigate the interactions between *TRY*, *CPC* and floral organ identity genes, AG activity was knocked down in a *try cpc* double-mutant background. Flowers of *try cpc* plants exhibited an increased number of trichomes on sepals. This included the formation of trichomes on the adaxial sides of sepals (**Figure 1.8 (F)**), which are normally glabrous. A specific inactivation of AG in *try cpc* mutant flowers resulted in the formation of trichomes on the tips of anthers (**Figure 1.8 (G)**) and in the appearance of a large number of trichomes on gynoecia (**Figure 1.8 (F)**). These trichomes were often arranged in clusters, in agreement with the phenotype of *try cpc* double mutants in leaves (Schellmann et al., 2002). Strikingly, the formation of trichomes was observed not only on the outside of carpel valves but also on their inner surfaces (**Figure 1.8 (H-I)**). Thus, it appears that when both *TRY* and *CPC* are mutated, not only is the suppression of trichome formation abolished, but also organ polarity in the gynoecium is affected.

Additional factors in the suppression of trichome formation in flowers were discovered when the role of several different phytohormones, which had been shown previously to promote trichome initiation in leaves were tested (Pattanaik et al., 2014). It was shown that all hormones applied caused an in-

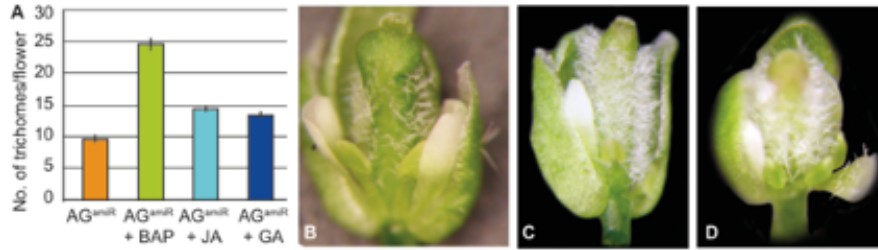


Figure 1.9: The effect of cytokinin on floral trichome formation.

(A) Effect of different hormones on trichome numbers on gynoecia after AG perturbation. AG knockdown denoted by "AG^{amiR}" (AGAMOUS artificial microRNA). BAP: 6-benzylaminopurine (a cytokinin); GA: gibberellin; JA: jasmonic acid. (B) Flower of *try* mutant plants after an AG knockdown and treatment with 100 μM BAP. (C-D) Flowers of *try cpc* double mutants after an AG knockdown and treatment with 100 μM BAP. Data from (Ó Maoiléidigh et al., 2018).

crease of trichome numbers on carpel valves when AG activity had been perturbed, with the strongest effect being observed for the synthetic cytokinin 6-Benzylaminopurine (BAP) (Figure 1.9 (A)). This result was in agreement an earlier finding showing that the expression of several genes known to be involved in the cytokinin-dependent control of trichome formation in leaves are regulated by AG (Ó Maoiléidigh et al., 2013). One of these genes encodes the cytokinin oxidase CKX6, which inactivates cytokinin and is up-regulated by AG (Werner et al., 2003). Thus, it appears possible that AG reduces cytokinin levels in carpels by promoting the expression of CKX6, and that the resulting low cytokinin concentration in the epidermis contributes to the repression of trichome formation.

To better understand the role of cytokinin in the suppression of trichome formation in floral organs, different mutant combinations were treated with BAP. The most dramatic phenotypes were observed for *try* single mutants (Figure 1.9 (B)) and *try cpc* double-mutant plants, respectively, in which AG activity had been knocked down. Flowers of the latter plants formed a very large number of trichomes on all floral organs so that the flowers were fully pubescent (Figure 1.9 (C-D)). Thus, when the trichome repressor genes and AG are inactivated simultaneously, flowers appear to be hypersensitive to BAP treatment.

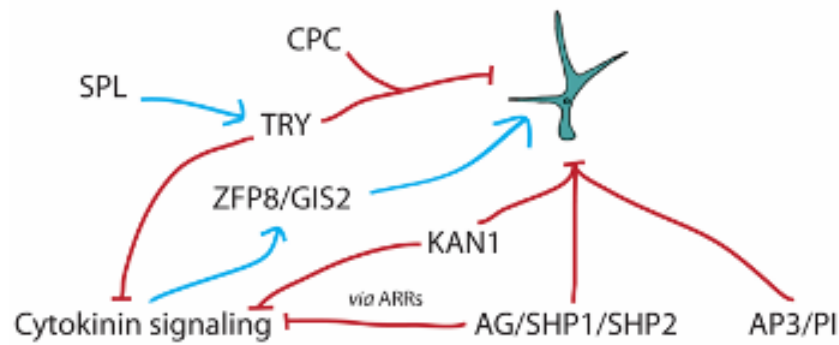


Figure 1.10: Model for the inhibition of trichome formation on floral organs of *Arabidopsis*.

Genes that suppress trichome formation are indicated by red blunted lines, positive inputs by blue arrows. ARR: cytokinin response regulator; KAN1: KANADI1; ZFP8/GIS2: ZINC-FINGER PROTEIN8/GIBBERELLIN INSENSITIVE2. Figure adapted from (Ó Maoiléidigh et al., 2018).

Taken together, the results of the experiments discussed above provided detailed insights into how the suppression of trichome formation on floral organs is controlled and revealed a complex interplay of several distinct pathways (Figure 1.10).

1.8 Translational research for crop improvement

As described at the beginning of this chapter, modern agriculture faces many stark challenges. One of the main threats to agriculture are pathogen infections, insect pests and weed contamination, which result in an estimated loss of around 40% of the global crop production every year (Oerke et al., 1994). This reduction in yield is a main cause for food shortages and famines in the developing world, a problem that is exacerbated by strong population growth in many of the affected countries. In industrialised societies, where population size is comparatively stable, plant pests and diseases result in a major loss of revenue to farmers and to companies in the agri-food sector (Oerke, 2006; Savary et al., 2006a). In order to protect crops, large quantities of pesticides are being used

(Nutter et al., 1993). This mass application is problematic as it leads to a reduction of biodiversity, to a contamination of soil and water resources, and potential risks to human health (EEA, 2023). Also, several plant pests have spread around the globe as a consequence of international travel and trade (Montgomery et al., 2023). What's more, some of these organisms have developed resistance to a wide range of pesticides, and several pesticides that are bioactive have been banned or restricted in recent years due to environmental or health concerns (Stokstad, 2018). As a consequence, the protection of crop plants against diseases and pests is becoming increasingly difficult (Savary et al., 2006b).

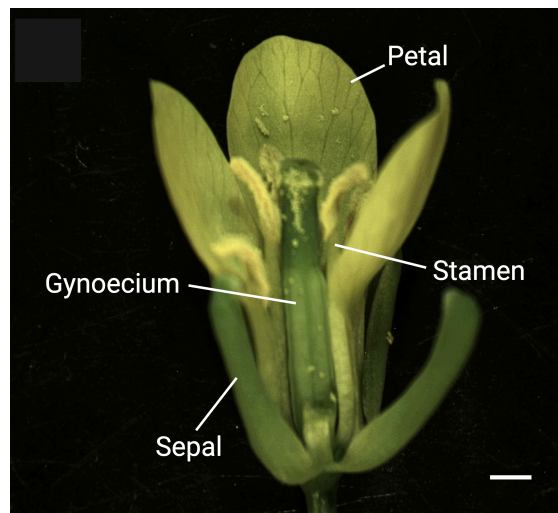


Figure 1.11: A flower of *Brassica rapa* var. Yellow Sarson (R-o-18).

Light microscope image of a *B. rapa* variety R-o-18 flower. The flowers are very similar in structure to those of *Arabidopsis*, consisting of four sepals in the first whorl, four yellow petals in the second whorl, six stamens in the third whorl and two carpels fused to form the gynoecium in the fourth whorl. Two sepals and two petals were removed from this flower to allow for visibility of the internal floral organs. Scale bar: 1 mm. Photo kindly provided by Dr Chistina Fitzsimons.

A possible solution to the problems outlined above is the use of new crop cultivars with enhanced resistance to pests and diseases that give stable yields and can be grown with reduced quantities of pesticides. One trait that has been specifically targeted in recent years is the formation of trichomes, which are known to aid in the defence against chewing or sap-feeding insects. In fact,

it has demonstrated that an increase in trichome density on leaves and stems of oilseed rape (*Brassica napus*) protects the plants from flea beetle attacks (Soroka et al., 2011). Because these plants were transgenic and hence not suitable for the European market, the research grant that financed my work aimed at generating new crop cultivars with supernumerary trichomes through non-transgenic approaches. To this end, the results from the experiments in *Arabidopsis* outlined in Section 1.7 were to be translated to crops of the genus *Brassica*, which comprises many important vegetable and oilseed cultivars such as cauliflower, turnip, kale, broccoli, cabbage and oilseed rape (**Figure 1.12**). *Brassica* crops were chosen for this work because they are among the most closely related crop species to *Arabidopsis*, as both genera are members of the Brassicaceae family. This relationship should facilitate the translation of the results from basic research to agriculture (Hendriks et al., 2023). Although *Arabidopsis* and the *Brassic*as are not within the most closely related of the 58 tribes of Brassicaceae, both genera are within the same subfamily and share morphological similarities, as demonstrated by **Figures 1.1 & 1.11** (German et al., 2023; Hendriks et al., 2023). Additionally, the *Brassic*as are arguably the closest relatives of *Arabidopsis* which have high agronomic value, which is why a more closely related species or genus was not considered to be of interest for this work.

1.9 Aims of the thesis

To achieve the goals of the research grant that financed my work, my research aimed at obtaining additional insights into the genetic control of trichome formation in flowers. To this end, I was to investigate the role of known regulators of floral trichome formation. Furthermore, my work aimed at generating and characterising mutant lines in *B. rapa* which could be used to generate crops with supernumerary trichomes. The work I conducted is summarised in three chapters, as follows:

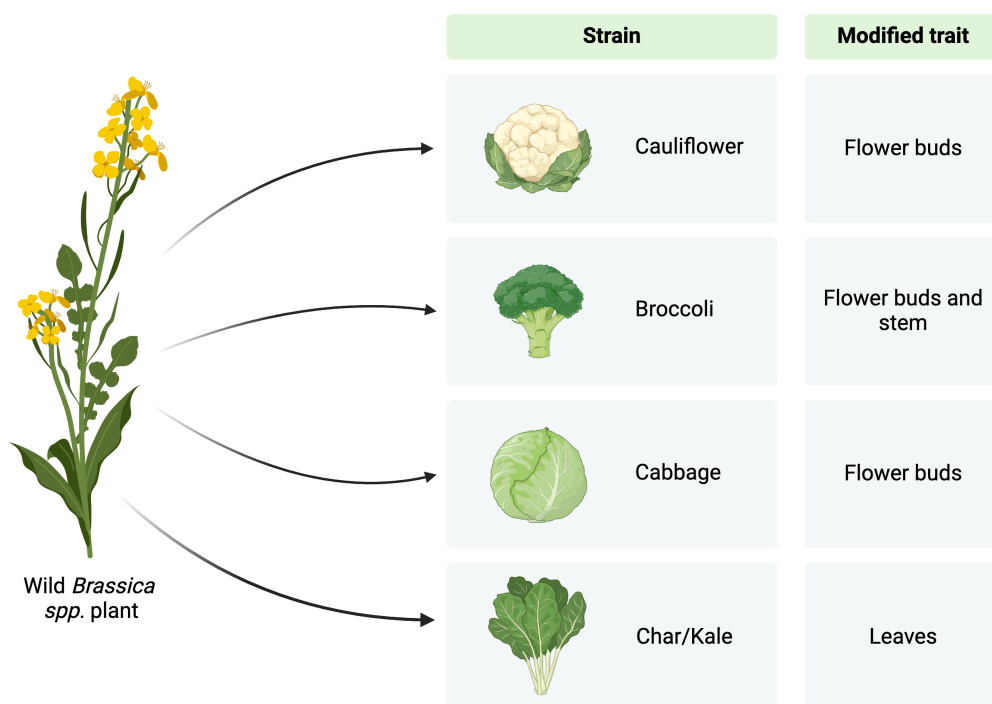


Figure 1.12: Selected *Brassica* cultivars used as vegetables.

On the left: schematic of a wild *Brassica* plant. On the right: selected cultivars and the traits that were modified by breeding efforts. Figure created with Biorender.com.

In Chapter 3, a novel investigation of the function of the R3R2-MYB family gene *TRIPTYCHON* (*TRY*) was undertaken, where its expression and function in the medial domain of the gynoecium was explored. Previous work from our laboratory suggested that in addition to being involved in the suppression of trichome formation, *TRY* may also function in the control of replum development in *Arabidopsis* fruits. My work therefore aimed at testing this hypothesis and to conduct experiments to explore the molecular basis of this activity. To this end, I generated multiple novel transgenic lines, including two novel null *try* alleles obtained using the CRISPR/Cas9 system. Rescue lines to restore *TRY* activity in a *try* mutant background and a *TRY* overexpression line were also generated. To untangle the genetic mechanisms behind this observed phenotype, the BAP experiments were first repeated on published lines and then carried out on the

rescue and overexpression lines to confirm that the cause of this phenotype was the loss of TRY function, and not an artefact of the transgenic allele. To follow on from this, two novel *try* were also treated with BAP and their responses quantified.

In Chapter 4 the focus was on identifying and characterising homologs of the *SHP1* and *SHP2* genes in *B. rapa*, which are known regulators of trichome formation on seed pods (see Section 1.7) but are also key regulators of seed dispersal (see Section 1.6). A series of crosses was carried out to isolate higher order mutant combinations, and the phenotypic consequences of a loss of *SHP* genes activities was then to be examined and compared to *shp* double mutant of *Arabidopsis*. Then, a number of molecular techniques were employed to characterise the functions of the genes identified, such as qRT-PCR, 3' RACE and generation of transgenic lines to complement the loss of function in a *shp1/2* mutant background in *Arabidopsis*.

Finally, the aim of Chapter 5 was to examine the role of *SHP1/2* in *Arabidopsis*, specifically focusing on their role in suppressing the formation of trichomes on the gynoecium. This investigation arose from a previous study published by our research group (Ó Maoiléidigh et al., 2018) which found that the *SHP* genes act in a partially redundant manner with AG in suppressing the formation of trichomes on the gynoecium. The specific aim of my work was to investigate the genetic interactions between the *SHP* genes and repressors of trichome formation. This was done to assess if this high order mutant was sufficient to produce trichomes on the gynoecium, without having to perturb AGAMOUS function, which is critical to normal reproductive organ development. This is also a potentially interesting avenue of research in crop sciences.

2 Materials and Methods

2.1 Strains and growth conditions

2.1.1 Plants lines used in this work

Arabidopsis thaliana

The following previously published plant lines were used during the course of this work:

- Landsberg *erecta* (L-er), Colombia (Col-0) and Wassilewskija (Ws) wild-type accessions
- *as1-1* (Ori et al., 2000)
- *ckx6-1* (Schmülling et al., 2003)
- *ful-1* (Roeder et al., 2003)
- *gl1-20990* (Tominaga2008iArabidopsisFormation)
- *rpl-1* (Alonso-Cantabrana et al., 2007)
- *shp1-1 shp2-1* (Liljegren et al., 2000)
- *try-29760* (Tominaga2008iArabidopsisFormation)
- *cpc-1* (Wada et al., 1997)
- *try-29760 cpc-1* (Ó Maoiléidigh et al., 2018)

- *ag-10* (Ji et al., 2011)

The lines that were generated during the course of this work are outlined in Table 2.1

Brassica rapa

Two wild-type accessions were used during this work:

- R-o-18 (donated by Dr. Emmanuelle Graciet, Maynooth University) (Stephenson et al., 2010)
- C-634 (obtained from Tohoku University Brassica Seed Bank) (Li et al., 2009)

Additionally, the TILLING mutant alleles which were purchased from RevGen UK are outlined in Table 4.6.

Table 2.1: *Arabidopsis* lines generated during the course of this study. Constructs and the mutant lines/wild-type accessions that were transformed are listed. Lines generated by crossing of existing lines are also listed. These lines are discussed in greater detail in 2.7

Transformed into <i>shp1-1 shp2-1</i> & L-er	
pAtSHP2::AtSHP2 CDS	p35S::AtSHP2 CDS
pBrSHP2::BrSHP2 CDS	p35S::BrSHP2 CDS
pAtSHP2::BrSHP2 CDS	pBrSHP2::AtSHP2 CDS
pBrSHP2::GUS CDS	BrSHP2 intron 1::GUS CDS
BrSHP2 intron 1::BrSHP2 CDS	
Transformed into <i>try-29760</i> & Col-0	
p35S::TRYg	pTRY::TRYg
CRISPR alleles genes generated in a Col-0 background	
<i>try-14</i>	<i>try-19</i>
Generated through crosses	
<i>ag-10 try-29760 cpc-1</i>	
<i>ag-10 shp1-1 shp2-1</i>	
<i>shp1-1 shp2-1 try-29760</i>	
<i>try-29760 rpl-1</i>	
<i>try-29760 as1-1</i>	

2.1.2 Plant growth media

On soil: seeds were sown on the surface of moist soil made up to a 3:1:1 ratio of compost:vermiculite:perlite (NAD).

On agar: For antibiotic screening or supplementation with gibberellic acid to promote germination, seeds were sterilised and plated on sterile plates containing 0.5x Murashige and Skoog (MS) medium - 2.2 g/L MS salts, adjusted to pH 5.7 using 1 M KOH, 6 g/L agar (Sigma Aldrich).

Seeds were sterilised by placing them in a closed chamber with a beaker containing 100 mL of domestic bleach and 3 mL of strong hydrochloric acid, to produce chlorine gas. Seeds were left to gas sterilise for 3-4 hours. Seeds were only sterilised immediately before plating on MS as to expose them to chlorine gas can reduce their longevity and viability.

2.1.3 Plant growth conditions

For *Arabidopsis*: In normal growth conditions, seeds were sown, either on the surface of moist soil in a tray or on MS medium in a Petri dish. Trays were covered with transparent plastic to increase humidity. Petri dishes were sealed with Micropore tape (3M) to maintain a sterile environment, while allowing for gaseous exchange. Trays or Petri dishes were placed at 4 °C in the dark for 3 days to synchronise germination. They were then placed under constant light at 20 °C, with the transparent cover of trays remaining in place for the first seven days of growth while seeds germinated.

For *B. rapa*: Seeds were sown approximately 2cm under soil in a 4-cell pot (Deker), with one seed per cell. 500 mL of water was poured into the bottom of the tray to keep the soil moist. The tray was covered with transparent plastic and placed at 4°C in the dark for 3 days. The seeds were germinated under constant light at 20 °C for 7-10 days, or until the seedling was touching the plastic covering. At this point, the plastic was removed and the seedlings were then

grown at 23 °C under long day conditions (16 h light, 8 h dark) for the remainder of the life cycle.

2.1.4 Crossing of plant lines

Sepals, petals and stamens were removed from flowers of maternal plant before anthesis to prevent self-pollination. A stamen from the paternal plant was used to manually pollinate the maternal stigma using a forceps.

2.1.5 Bacterial strains used in this work

- *Escherichia coli* strain stbl2 (F- endA1 glnV44 thi-1 recA1 gyrA96 relA1 D (lac-proAB) mcrA Δ (mcrBC-hsdRMS-mrr) λ) - (Invitrogen)
- *Escherichia coli* strain XL1 BLue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F'proAB lacIqZ Δ M15 Tn10 (Tetr)) - (Agilent)
- *Agrobacterium tumefaciens* strain pGV2260 (McBride & Summerfelt, 1990)

2.1.6 Bacterial growth media

All bacteria were grown in LB (Luria-Bertani) medium or on LB agar made up of 10 g/L NaCl (Fisher Scientific), 10 g/L Bacto-tryptone (Fisher Scientific), and 5 g/L Bacto- yeast extract (Fisher Scientific). To make LB agar plates, 6 g/L agar was added (Sigma Aldrich).

2.1.7 Preparation and transformation of competent *Escherichia coli* cells

Competent *E. coli* cells were generated using the protocol as described by Inoue et al., 1990. Transformations were carried out by incubating 100 μ L of competent cells with the 6 μ L plasmid on ice for 10 minutes. The cells were then incubated at 42°C for 45 seconds. They were placed back on ice for 2 minutes, followed

by incubation at 37°C with 900 μ L of LB medium (described above) for 60-90 minutes to allow the cells to recover. The cells were then pelleted at 4,000 xg for 5 minutes. 800 μ L of the supernatant was discarded, with the pellet being resuspended in the remaining 200 μ L of LB, which was then plated on an LB agar plate containing the relevant antibiotic(s) for selection and incubated at 37°C overnight.

2.1.8 Blue-white screening using XL1 Blue cells

The principle of blue-white screening is to detect the presence or absence of the lac operon within colonies of bacteria in order to determine if a specific segment of a vector has been successfully ligated in. LB agar must be supplemented with IPTG to induce expression of the lac operon, and X-Gal to provide a substrate. If the lac operon is present, the colonies will appear blue in colour due to the breakdown of X-Gal.

XL1 Blue cells are transformed with the plasmid of interest in an identical manner to that described in the section above. LB agar was supplemented with 100 mM IPTG diluted 1 in 1,000 to give a final concentration of 100 μ M and similarly with 40 mg/mL X-Gal, to give a final concentration of 40 μ g/mL.

2.1.9 Preparation and transformation of competent *Agrobacterium tumefaciens* cells

A 5 mL LB culture of *Agrobacterium* was grown at 28 °C with constant agitation at 220 RPM overnight. A 250 mL SOB culture supplemented with 0.2 g/L MgSO₄ was then inoculated with the 5 mL culture. This larger culture was grown at 28 °C with constant agitation at 220 RPM until an OD₆₀₀ of between 1 and 2 was measured.

The culture was centrifuged at 4,000 xg for 10 minutes at room temperature to pellet the cells. The pellet was resuspended in 20 mL ice-cold 10 mM CaCl₂ as

a washing step. The cells were then centrifuged at 4,000 xg for 10 minutes and the pellet was resuspended in 4 mL of ice-cold 10 mM CaCl₂.

Cells were aliquoted in 200 μ L volumes and snap- frozen in liquid N₂.

To transform, an aliquot of the cells was thawed and incubated with 6 μ L of the plasmid of interest for 5 minutes in liquid N₂. The cells were then thawed, 1 mL of LB medium was added and the cells were allowed to recover at 28 °C under constant agitation for 4 hours.

After recovering, the cells were centrifuged at 3,000 xg for 2 minutes, 800 μ L of the supernatant was removed and the pellet was resuspended in the remaining LB medium. Cells were then plated on LB agar containing rifampicin (50 μ g/mL), carbenicillin (100 μ g/mL) and the selective antibiotic for the plasmid being transformed. The plates were incubated at 28°C for 2-3 days.

2.2 Treatment of *Arabidopsis* with 6-Benzylaminopurine (BAP)

Inflorescences were treated with the synthetic cytokinin BAP approximately one week after bolting had occurred, under regular growth conditions. BAP was applied exogenously with a spray bottle (to decrease droplet size and increase surface covered) 5 times, each 24 hours apart (Zúñiga-Mayo et al., 2014).

Effects of BAP treatment were then examined and photographed under light microscope at regular intervals after treatment had ceased, to monitor the effects it had on various stages of development, as defined by Smyth et al., 1990.

2.3 Random impact test of *B.rapa*

The random impact test was first described by Morgan et al. (1998) and has remained largely unchanged as a method of measuring shatter resistance. This method was based off Lenser and Theissen (2014) and Stephenson et al. (2019)

and optimised for the materials available in this work.

In brief, dry siliques were harvested from plants and then incubated in a dessicator for at least 3 days. Then, individual siliques were placed in the FastPrep-24 (MP Biomedicals) within 2 mL tubes, each containing 10 1 mm diameter metal bearings. The siliques were agitated at a speed of 4 m/s for 5 seconds (where $4 < n < 8$). This step was repeated and then followed by 10 seconds, then 20 seconds, 40 seconds, 80 seconds and 160 seconds. After each agitation, the number of siliques broken open was recorded and once all siliques were opened the experiment was concluded.

The shatter resistance of each genotype was then quantified by first plotting the data as time vs. no. pods shattered. Then this graph was transposed into a linear graph by plotting the log values. Then, the cumulative shaking time required to shatter half of the siliques present was determined. This is denoted as the dehiscence halftime, or the RIT_{50} . This method of calculation was completed according to Lenser and Theissen (2014).

2.4 Plant imaging

All photographs of plants included in figures were taken using the Optika stereo microscope and accompanying software fitted with an OPTIKAM Pro Plus 3 mounted camera. All measurements of *Arabidopsis* replums were obtained at maximum digital and lens magnification with the digital calliper feature enabled for measurements and scale bars.

2.5 Prediction of binding sites

In the case of predicting transcription factor binding sites, JASPAR was used (Castro- Mondragon et al., 2022). JASPAR is an online database of transcription factor (TF) binding profiles. The database stores transcription factors binding profiles as position frequency matrices (PFMs). PFMs summarise the frequency

at which each nucleotide at each position in a given transcription factor binding site occurs. PFMs can be used to construct position weight matrices (PWMs). These PWMs can be used to scan a user-input DNA sequence to predict TF binding sites. JASPAR was used to predict transcription factor binding sites within *AG*, *SHP2* and the *B. rapa* homologs of *AG* and *SHP2* in Section 5.2.4.

2.6 Genotyping and molecular work

2.6.1 "Edward's preparation" genomic DNA extraction

Crude DNA extraction for use in PCR genotyping assays was carried out as described in Edwards et al. (1991). In short, a small disc of leaf tissue was collected using a standard hole punch. At this point, the tissue could be stored at -20 °C for several days until needed, or the extraction could be completed straight away.

In a 1.5 mL centrifuge tube, the leaf tissue was ground using a pestle in 400 μ L of extraction buffer (described in Appendix A1.1.2). After the sample was spun down at 14,000 RPM for 1 minute at room temperature, 300 μ L of the supernatant was transferred and mixed with 300 μ L isopropanol to precipitate the DNA. The DNA was pelleted by centrifugation at 14,000 RPM at room temperature for 10 minutes, and then washed with 70% (v/v) ethanol and re-centrifuged at 14,000 RPM for 3 minutes. The pellet was allowed to air dry to ensure no ethanol was remaining and then it was resuspended in 100 μ L of TE buffer (described in Appendix A1.1.5).

2.6.2 Cetyltrimethyl Ammonium Bromide (CTAB) genomic DNA extraction

When a cleaner genomic DNA extraction was required for use in cloning to amplify a gene or region of interest, the CTAB protocol was used. To do this, plant

tissue with a diameter of 1 cm was collected and flash frozen. The tissue was ground to a powder in liquid nitrogen before being incubated at 65°C with 500 μ L of 2% CTAB extraction buffer (described in A1.1.3) for 30-60 minutes. After incubation, 500 μ L of phenol/chloroform/isoamyl alcohol (Sigma Aldrich) was added to the tube and vortexed. The tube was spun down at 14,000 RPM at room temperature for 3 minutes, at which point the supernatant was added to a clean tube along with 400 μ L of isopropanol. This was gently mixed by inverting the tubes and incubated at room temperature for 10 minutes. The DNA was pelleted at 14,000 RPM at room temperature for 15 minutes. Finally, the pellet was washed with 70% (v/v) ethanol followed by air drying and resuspending the pellet in 50 μ L of TE buffer.

2.6.3 Quantification of DNA and RNA

The concentration and purity of both DNA and RNA were measured using the NanoDrop ND-1000 spectrophotometer. Firstly, the instrument was initialised using ultrapure deionised water. The instrument could then be blanked or zeroed using the solution that the sample was suspended in, for example RNA elution buffer or TE buffer. In all cases, 1.5 μ L of liquid was pipetted onto the NanoDrop to record a measurement.

2.6.4 DNA sequencing

All plasmids and PCR products were sequenced by Eurofins GATC (www.eurofinsgenomics.eu) using Sanger sequencing. All sequencing results were aligned with the wild-type gene sequences or expected construct sequence using the Align feature on A Plasmid Editor (ApE) software (<https://jorgensen.biology.utah.edu/wayned/ap/>).

2.6.5 Quantitative real-time polymerase chain reaction (qRT-PCR)

Tissue collection: *B. rapa* and *Arabidopsis* tissue were both collected using sterile forceps and then deposited into an RNase-free 2 mL tube that was immersed in liquid N₂ to snap freeze the tissue. Where necessary, floral or leaf tissue was dissected using either a sterile No. 4 jeweller's forceps or a No. 10 scalpel blade (Swann- Morton). The tissue was then stored at -80°C until ready for use.

Total RNA extraction: The Plant Total RNA kit (Sigma Aldrich) was used according to manufacturer's instructions to extract RNA from tissue collected. The resulting elution was stored at -80°C.

cDNA synthesis: Complementary DNA (cDNA) was generated by reverse transcription using RevertAid H Minus Reverse Transcriptase (ThermoFisher) and oligo(dT)18 primers (ThermoFisher) as per the manufacturer's instructions. RNA was quantified after extraction as described in Section 2.6.3. For each 20 µL cDNA reaction, 400-450 ng of RNA was used and the product was diluted 1 in 2, i.e. adding 20 µL ultrapure water before being used as template DNA.

qPCR: Taq-based SYBR Green Master I mix kit (Roche) was used in the Roche LightCycler 480. In short, a 10 µL reaction was set up in a 96-well plate consisting of 2 µL of template cDNA, diluted 1 in 2 from the product of the reaction described above. Primer pairs were designed to have an annealing temperature of between 60 °C and 63 °C and have no more than 1 °C difference between them. Primer pairs were designed to span an exon-exon junction to avoid amplifying genomic DNA.

One reaction consisted of 5 µL of 2x SYBR Green Master 1, 1 µL of 10 µM primer pair mixed, and 2 µL of PCR-grade water. An elongation time of 60 seconds per 1 kb of DNA was set, but amplicons were typically 75-250 bp. Annealing temperature was set to 60 °C. The enrichment was calculated relative to the reference gene. The relative enrichment of the cDNA for each amplicon was calculated as follows:

$$2^{CP(REF)-(GENE)}$$

For qRT-PCR in *B. rapa*, the reference gene primers CB187/188 (Bra002483 homolog of the *Arabidopsis EF1α* gene, AT1G07940) (see Table A1.3 in the Appendix) were used. For *Arabidopsis* qRT-PCRs, the reference gene primers DM242/243 (targeting At1G13320) (see Table A1.3 in the Appendix) were used.

The Cp is the crossing point as calculated by the LightCycler480. The Cp (REF) is the value for the chosen reference gene and the Cp (GENE) is the value for the gene of interest.

2.6.6 Reverse-transcription polymerase chain reaction (RT-PCR)

To examine the effects of a splice donor site mutation (described in Chapter 4), RT-PCR was used to examine the gene transcript and test if it was altered.

To do this, RNA extraction and cDNA synthesis were performed as described above. Then, a PCR amplifying the relevant gene was completed so the product lengths of the wild-type and splice mutant could be compared via agarose gel electrophoresis.

2.6.7 Double-mismatch allele-specific (DMAS) quantitative PCR genotyping

Completed as described in Lefever et al., 2019. The assay was used to genotype all described TILLING alleles in *Brassica rapa* from the John Innes Centres as alleles used were SNPs. In short, the assay involves running two parallel qPCR reactions on the same template DNA. Both reactions had a common reverse primer, while each of the two reactions either had a forward primer specific to the wild-type (WT) allele or the mutant (mut) allele. The primers used in these reactions were (see Table A1.3 in Appendix)

2.6.8 3' RACE PCR

Rapid Amplification of cDNA Ends (RACE) PCR was performed to determine the 3'UTR sequence of the mRNA of the *B. rapa* homologs of *SHP1/2*. To this end, a previously published protocol was followed (Scotto-Lavino et al., 2007).

Briefly, cDNA was generated using a modified oligo(dT) primer which contained restriction digest recognition sites. A PCR was carried out using this cDNA as a template with a gene-specific primer and the modified oligo(dT) primer to generate an amplicon from the gene of interest which contains the 3'UTR. The 3'RACE product along with pUC19 was digested with HindIII (ThermoFisher) restriction enzyme and the two were ligated together using T4 ligase (ThermoFisher). The pUC19 plasmid containing the 3' RACE fragment was sent to Eurofins for sequencing.

2.6.9 Isolation of plasmid DNA

OMEGA BioTek Plasmid MiniPrep kit was used according to the manufacturer's instructions.

2.6.10 Purification of PCR product

OMEGA BioTek Cycle Pure kit was used according to the manufacturer's instructions. In the case that a large product (>2 kb) was being purified, the DNA-binding columns and the elution buffer provided were warmed to 65 °C before completing the elution step to ensure a higher quantity of DNA was being dissolved in the buffer.

2.6.11 Oligonucleotide design

All primers were designed using the "primer finder" feature on the ApE software. Only oligos with GC content 40-60% and a melting temperature between

50 °C and 60 °C were used where possible. Off-target amplicons and hairpins were avoided by screening chosen primer sequences using NCBI's Primer BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

2.6.12 Agarose gel electrophoresis

Unless otherwise stated, 1% agarose gels were used to visualise the presence of plasmids and PCR products as well as determine their length. Gels were prepared using agarose powder (Sigma Aldrich) in a 1%(w/v) dilution in 1x TAE buffer (described in Appendix A1.1.6). Ethidium bromide (Invitrogen) was added to the agarose and TAE solution at a dilution factor of 10,000. Gels were cast in standard trays (Thermo Scientific)

5x DNA loading dye (described in Appendix A1.1.7) was added to PCR product to make the progress of the DNA through the gel visible, and increase density for ease of loading samples into wells. Loading dye was added to give a final concentration of 1x (a five-fold dilution in solution). For example, in a 50 μ L PCR reaction, 12.5 μ L 5x loading dye was added to give a final volume of 62.5 μ L. GeneRuler DNA Ladder Mix (Thermo Scientific) was used as a ladder to determine the band sizes obtained once the gel had run.

2.6.13 Extraction of DNA from an agarose gel

OMEGA BioTek Gel Extraction kit was used according to the manufacturer's instructions.

2.6.14 Colony PCR to isolate plasmid of interest

Single colonies were selected from the LB agar plate and resuspended appropriately. In the case of *E. coli* colonies, 20 μ L of liquid LB was used. For *Agrobacterium* colonies, 20 μ L of pH 8.0 lysis buffer was used (described in A1.1.4). In the case of *Agrobacterium*, the resuspended colony in lysis buffer was incubated

at 96°C for 10 minutes before being used as template DNA for a PCR, in order to break down the bacterial cells so the plasmid DNA is accessible for amplification.

2.6.15 GreenGate cloning

Completed according to Lampropoulos et al. (2013). The Green Gate kit was obtained from Addgene (Kit #1000000036; <https://www.addgene.org/kits/lohmann-greengate/#kit-contents-1>) and used in the generation of the transgenic lines of *Arabidopsis* outlined in **Table 2.1**.

The regions amplified by PCR to incorporate into entry vectors are outlined below and in Section 2.7. The entry reaction to achieve this consists of: 1.5 μ L 10X T4 Ligase Buffer (NEB), 1 μ L T4 Ligase (NEB), 1 μ L BsaI (NEB), 1.5 μ L 10mM ATP (ThermoFisher), 1.5 μ L 10X BSA (NEB), 100 ng of the entry vector, insert DNA and ddH₂O to a total volume of 15 μ L. The reaction conditions were [37°C 2 minutes, 16°C 2 minutes] x 30 cycles, followed by heat inactivation at 80°C for 5 minutes.

Using various combinations of the assembled entry vectors, the desired transgenic lines as listed in **Table 2.1** were generated by Green Gate destination reaction, which consists of: 150 ng of each entry vector and 100 ng of the empty destination vector, 1.5 μ L 10X CutSmart Buffer (NEB), 1 μ L T4 Ligase (NEB), 1 μ L BsaI (NEB), 1.5 μ L 10mM ATP (ThermoFisher) and ddH₂O to a total volume of 15 μ L. The reaction conditions were [37 °C 2 minutes, 16 °C 2 minutes] x 50 cycles, followed by heat inactivation at 80 °C for 5 minutes.

2.7 Generation of transgenic *Arabidopsis* lines

2.7.1 Transformation of *Arabidopsis* with construct-carrying *Agrobacterium tumefaciens* by floral dipping

Flowering plants were transformed with the C58 pGV2260 strain of *Agrobacterium tumefaciens* using the floral dip method according to Zhang et al. (2006).

In short, bolting plants were stripped of their siliques to prevent collection of seeds that were not transformed. The plants were then dipped in a 5% (w/v) sucrose solution containing *Agrobacterium* cells carrying the desired construct. Seeds were then collected and the T₁ generation were screened as transformants according to the antibiotic or herbicide resistance introduced with the vector. All constructs were assembled using the E9 RUBISCO terminator sequence as well as the p35S::Blp^R herbicide resistance cassette, both of which are included in the Green Gate kit. The only unique components of the destination reactions were the promoter and CDS modules used, which are outlined in the following sections.

2.7.2 pTRY::TRYg

This construct was assembled using the pTRY and TRYg entry vectors donated by another lab member, Dr Andrea Finocchio, by way of the destination reaction as described in Lampropoulos et al. (2013).

2.7.3 p35S::TRYg

The 35S entry module included in the GreenGate kit and the pre-existing TRYg entry module generated by Dr. Andrea Fionocchio mentioned in the previous section were used to assemble this construct.

2.7.4 p35S::AtSHP2 CDS

The 35S entry module included in the GreenGate kit was used as a promoter module. The coding sequence of the *Arabidopsis* gene *SHATTERPROOF2* was amplified from cDNA generated as described in 2.6.5. RNA was extracted from *Arabidopsis* gynoecium tissue at developmental stage 13 (Smyth et al., 1990).

2.7.5 p35S::BrSHP2 CDS

The 35S entry module included in the GreenGate kit was used as a promoter module. The coding sequence of the *B. rapa* homolog of *SHATTERPROOF2* was amplified by PCR from cDNA that was derived from RNA extracted from *B. rapa* stage 13 gynoecium tissue (Smyth et al., 1990).

2.7.6 pAtSHP2::AtSHP2 CDS

The promoter sequence was amplified by PCR from genomic *Arabidopsis* DNA. A region of 2.1 kb upstream of the start codon was amplified as this has been shown to be sufficient to drive GUS expression in the expected tissues (Liljegren et al., 2000). The coding sequence of the *Arabidopsis* gene *SHATTERPROOF2* was amplified from cDNA generated as described in Section 2.6.5. RNA was extracted from *Arabidopsis* gynoecium tissue at developmental stage 13 (Smyth et al., 1990).

2.7.7 pBrSHP2::BrSHP2 CDS

The promoter sequence was amplified by PCR from genomic DNA. A region of 2.1kb of the start codon was amplified to be consistent with the selected promoter region in *Arabidopsis*. The coding sequence of the *B. rapa* homolog of *SHATTERPROOF2* was amplified by PCR from cDNA that was derived from RNA extracted from *Brassica* stage 13 gynoecium tissue (Smyth et al., 1990).

2.7.8 BrSHP2 intron 1::BrSHP2 CDS

The entire first intron of *SHATTERPROOF2* in *B. rapa* was amplified by PCR from genomic DNA to use in a promoter module. This region was chosen as the large intron of *Arabidopsis AGAMOUS* has been shown to be necessary to drive expression. The coding sequence of the *Brassica rapa* homolog of *SHATTERPROOF2* was amplified by PCR from cDNA that was derived from RNA extracted from *B. rapa* stage 13 gynoecium tissue (Smyth et al., 1990).

2.7.9 pAtSHP2::BrSHP2 CDS

The promoter sequence was amplified by PCR from genomic *Arabidopsis* DNA. A region of 2.1 kb upstream of the start codon was amplified. The coding sequence of the *B. rapa* homolog of *SHATTERPROOF2* was amplified by PCR from cDNA that was derived from RNA extracted from *B. rapa* stage 13 gynoecium tissue (Smyth et al., 1990).

2.7.10 pBrSHP2::AtSHP2 CDS

The promoter sequence was amplified by PCR from genomic DNA. A region of 2.1kb of the start codon was amplified to be consistent with the selected promoter region in *Arabidopsis*. The coding sequence of the *Arabidopsis* gene *SHATTERPROOF2* was amplified from cDNA generated as described in Section 2.6.5. RNA was extracted from *Arabidopsis* gynoecium tissue at developmental stage 13 (Smyth et al., 1990).

2.7.11 Selection for transformed progeny using the BASTATM herbicide

All of the constructs listed above had a BASTATM (BAYER) resistance cassette incorporated into them. One week old seedlings were sprayed with BASTATM

three times, each treatment 3 days apart. Plants which survived this treatment would then be genotyped by PCR to confirm the presence of the construct of interest.

2.7.12 Generation of CRISPR/Cas9 lines targeting *TRIPTYCHON*

A twenty-three nucleotide Cas9 guide sequence was searched for in the coding sequence of *TRY* using the program CasDesigner (available at <http://www.rgenome.net/cas-designer/>). Subsequently, the sequence was evaluated for potential off-target effects using the potential off-target finder tool (available at <http://www.rgenome.net/cas-offfinder/>). The 20 bp guide sequence is depicted in **Figure 3.12**. The RNA guide sequence was incorporated into the vector carrying Cas9 by ordering an oligonucleotide pair in the format:

Fwd: 5'ATTG(N)₂₀

Rvs: 5'AAAC(N)₂₀

The vector contains an RFP cassette which was driven under the *Arabidopsis* At2S seed coat promoter. The purified fragment was ligated into the pHEE401E vector and transformed into chemically competent *stb12 E. coli*. This vector was a gift from the Goodrich lab (University of Edinburgh). It is based on the pHEE401 backbone and was used as vector containing the sgRNA cassette targeting the first exon of *TRIPTYCHON* which was assembled using a modified Golden Gate protocol (Wang et al., 2015).

Col-0 wild-type plants were transformed with the CRISPR construct as described in Section 2.7.1. This accession was used as it is the background for the commonly used T-DNA *try-29760* line.

2.7.13 Selection of transformed plants carrying a CRISPR construct

Transformed plants were identified by examining seeds collected from the dipped plants for the RFP-containing seed coat. This was done under a standard light microscope as the RFP could be detected without using the precise excitation wavelength and transformed seeds were visibly red compared to wild-type seeds. This is demonstrated in **Figure 3.12**.

2.8 Image manipulation and construction of figures

Adobe Illustrator and Adobe Lightroom were used to improve image contrast and visibility and re-size photographs. Figures were assembled in Adobe Illustrator. Graphs were produced using *Prism*, while schematics and illustrations were created in BioRender (<https://www.biorender.com>).

3 Investigating the role of *TRIPTYCHON* in replum development

3.1 Introduction

3.1.1 The role of *TRY* in trichome formation and flower development

As described in Chapter 1, *TRY* is a well-characterised trichome repressor that controls the spatial patterning of trichome cells across the epidermal surfaces of the plant. In *Arabidopsis* flowers, trichomes are prevented from developing on the gynoecium and most other floral organs through high expression of *TRY* (**Figure 3.1**) and other trichome repressor genes, in particular *CPC* (see Section 1.7).

In addition to its function in trichome patterning, *TRY* negatively controls endoreduplication in trichome cells (Szymanski and Marks, 1998). Endoreduplication is the process by which mitosis occurs apart from the final step of dividing into two daughter cells, resulting in a larger cell with double the amount of genetic material. It also controls the spatial patterning of trichome cells across the epidermal surface by way of the lateral inhibition model (**Figure 1.7**). Its activity is regulated by *TRY* travelling to neighbouring cells to ensure that trichomes are regularly spaced. This was discussed in more detail in Section 1.7.

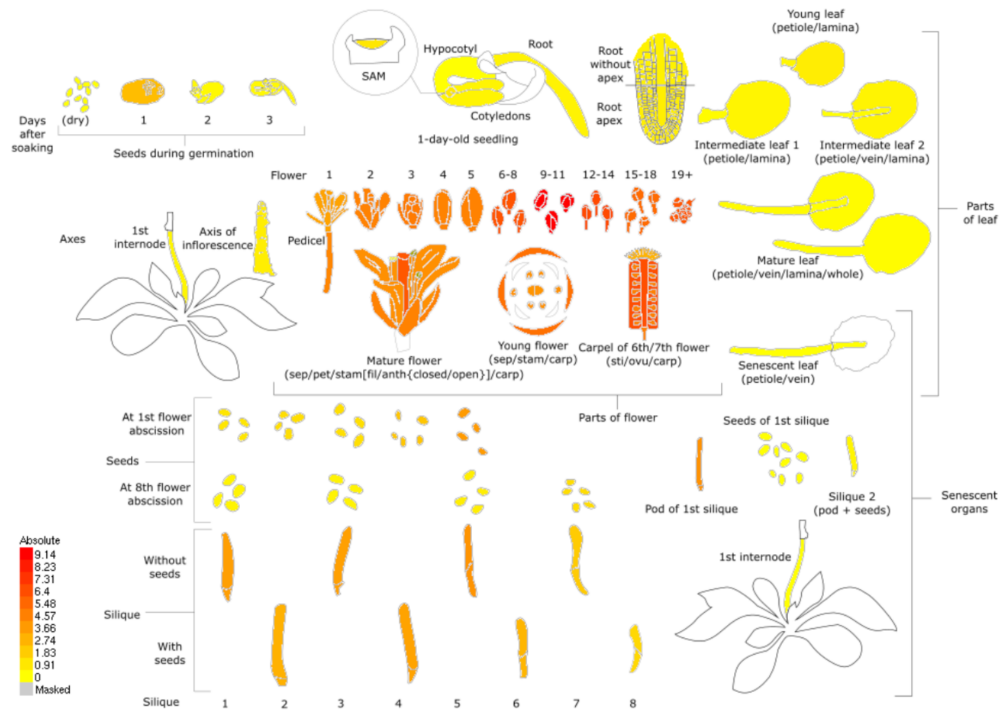
In *Arabidopsis*, trichomes are prevented from developing on the gynoecium

through *TRY* and *CPC* being expressed there to repress trichome development, as shown in **Figure 3.1**. The gynoecium is always glabrous in the wild type, that is to say without trichomes. As demonstrated in **Figures 1.8 and 1.10**, the C-class gynoecium regulator *AG* is also responsible for repressing trichome formation in parallel, through the suppression of CK signalling, which promotes trichome initiation. Hence, the additive effect of the increased number of trichomes on the gynoecium when *ag*, *try* and *cpc* are all perturbed (**Figure 1.8**)

When *TRY* function is lost, the trichomes on rosette leaves have a greater number of branches as a result of increased ploidy. Furthermore, the number of trichomes on the leaf surface of *try* mutant plants increases slightly (by approximately 5%) but they initiate abnormally and are often arranged in clusters rather than being evenly spaced as in the wild type (Schellmann et al., 2002). In contrast, in *cpc* mutant plants, the spatial patterning of trichomes is largely normal but the number of trichomes increases further (Schellmann et al., 2002). This phenotype is compounded when *try* and *cpc* mutants are combined, where there are many more trichomes than the wild type, arranged in dense clusters (**Figure 3.2**).

During the work carried out in our laboratory to characterise the control of trichome formation in flowers (see Section 1.7) it was noted that plants of the *try* mutant allele *try-29760* form gynoecia with repla that are narrower than in the wild type. This observation suggested that *TRY* may not only be involved in the suppression of trichome formation but might have other roles in the development of the plant. This was an attractive hypothesis especially in the context of the now accepted view that floral organs are derived from leaves (see Section 1.5). During floral evolution, the genetic program for leaf development must have been modified to carry out new functions needed for floral organ formation. However, while some floral regulators are also active in leaves (e.g. those that are involved in determining the polarity of organs), how their functions have been adopted, and whether they have been possibly altered for flower de-

A



B

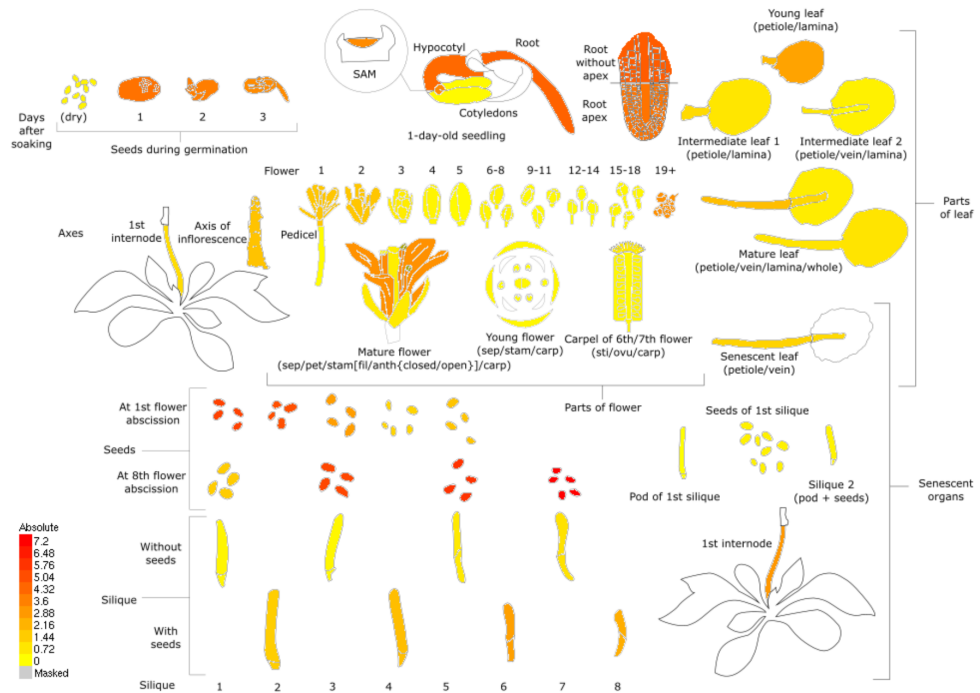


Figure 3.1: Expression patterns of *TRY* and *CPC* in *Arabidopsis*.

(A-B) Expression heat maps for (A) *TRY* and (B) *CPC*. Heat maps were generated using the *Arabidopsis* eFP Browser, based on available RNA-seq data (Klepikova et al., 2016).

velopment, is largely unknown. Investigating the function of TRY in replum development therefore provided an opportunity to obtain insights into an aspect of floral evolution that has not yet been studied in detail.

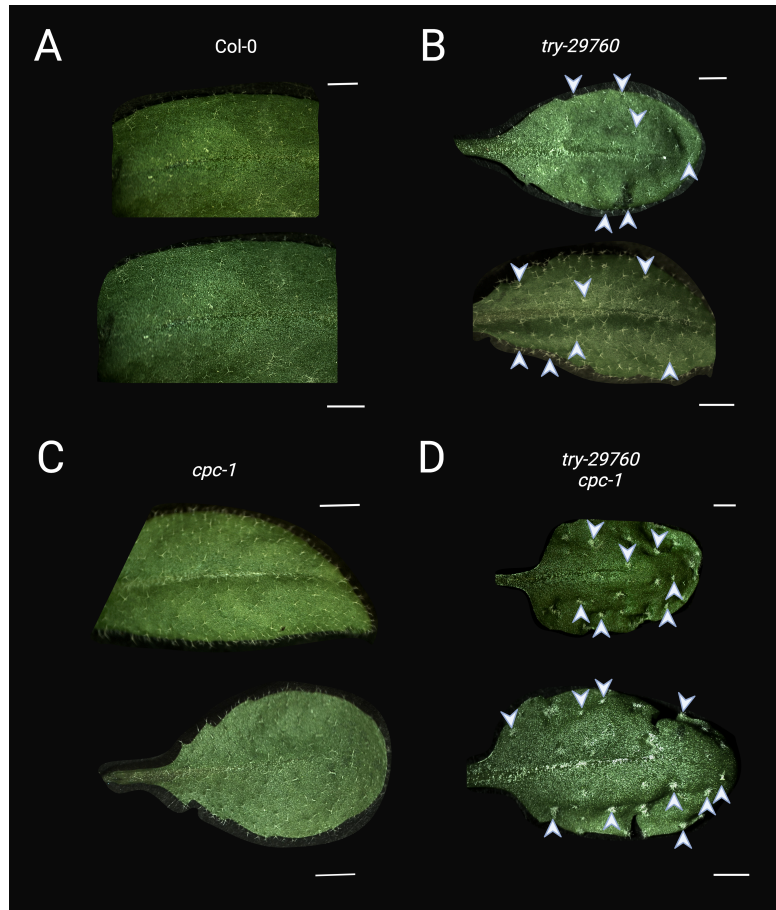


Figure 3.2: Phenotypes of trichome repressor mutants.

Leaves of a wild-type plant (Col-0) (A), *try-29760* (B) and *cpc-1* (C) single mutants, and of a *try-29760 cpc-1* double mutant (D) are shown. All images show the third rosette leaf of a plant. Arrow heads mark trichome clusters. Scale bars: 2 mm.

3.1.2 Replum development

As mentioned in Section 1.6, the replum is a specialised structure found in the fruit, known as a silique, and plays a crucial role in seed dispersal. During fruit development, the ovary wall differentiates into two distinct layers, the valve and the replum (Figure 1.5). As the silique matures, the replum undergoes specific

cell elongation and division, leading to the formation of a thin, central tissue region. This elongation causes the replum to protrude between the developing seeds, ultimately creating a characteristic partition that separates the two valves of the silique (**Figure 1.5**).

Following the development of the carpel margin meristem (CMM) as described in Chapter 1, the two ends of this septum formed in the gynoecium that are visible on the outside of the gynoecium are known as the replum. The two carpels which form the gynoecium are known as the valves, separated from each other by the replum. There is very finely tuned interplay and antagonism between the key regulators of the valves and replum to ensure the correct patterning and zoning of these tissues. Some key regulators and their interactions are depicted in **Figure 1.6** to visualise these antagonistic effects.

The medial domain of the gynoecium is established in stage 6-7 of flower development (Smyth et al., 1990). This gives rise to what becomes known as the septum, where the ovules are anchored - which becomes clearly defined by stage 10 (Smyth et al., 1990). There is a distinct zone of CK activity which is confined to the medial domain, which is known as the CMM during development of the septum. This CK activity allows for elongation and proliferation of the developing septum, while auxin maxima in the stigma and valves restrict this CK activity to ensure differentiation of the carpal structures. A key orchestrator of the control of organ differentiation within the medial domain of the gynoecium is *SEEDSTICK* (*STK*), a MADS-domain transcription factor which suppresses the meristematic activity of *SHOOT MERISTEMLESS* (*STM*) to allow for differentiation to occur.

Other upstream regulators of replum development are *JAGGED* (*JAG*) and *FILAMENTOUS FLOWER* (*FIL*) (González-Reig et al., 2012). They are key for the differentiation of the different tissue types at the CMM, where there is an auxin efflux from approximately stage 12 onwards (Smyth et al., 1990; Marsch-Martínez et al., 2012). *JAG* and *FIL* drive the differentiation of tissues at the valve

margin. This is discussed in Chapter 4.

3.1.3 The role of cytokinin in gynoecium development and trichome formation

During the course of previous work in our lab to understand the control of trichome formation in *Arabidopsis* flowers, different trichome mutants, including *try-29760*, were exogenously treated with a solution containing the synthetic CK BAP to examine its effect on trichome initiation on floral organs (**Figure 1.9**). It was observed that *try-29760* mutant gynoecia appeared to have wider replum than BAP-treated wild-type plants. This suggested that the effect of TRY on replum development may be CK-dependent.

As discussed in Chapter 1, CK has not only been demonstrated to promote trichome formation but has also been identified as being essential for the formation of the replum. The interplay between CK and auxin signalling pathways plays an important role in the growth and definition of the replum. In previous studies, it has been shown using reporter lines that during gynoecium development, there is a broad dispersal of CK across the entire gynoecium in its early stages of development from approximately stages 5-6, which then shifts to be observable almost exclusively in the septum from stages 7-12 as the CMM and replum are growing (Marsch-Martínez et al., 2012; Smyth et al., 1990). Then during stages 13 and 14, auxin signalling shifts to being localised in the medial domain only (McLaughlin et al., 2024; Smyth et al., 1990). This is further demonstrated by the detection of the auxin efflux carrier PINFORMED3 (PIN3) at the valve margin around the time of this transition in auxin localisation, which promotes transport of auxin into the medial domain (Gómez-Felipe et al., 2023).

3.1.4 CRISPR/Cas9 genome editing

The above-mentioned observation of abnormal replum formation after inactivation of *TRY* was limited to a single mutant allele (*try-29760*) and had not been reported in the literature for other *try* mutants. We therefore thought it possible that the replum phenotype of *try-29760* may be due to a second-site mutation and might not be directly related to the mutation in *TRY* carried in this allele. Furthermore, other published *try* mutant alleles appear weaker than *try-29760* in their effects on trichome formation and may thus not be null alleles. Furthermore, they were generated in different genetic backgrounds (i.e. different *Arabidopsis* accessions), hampering a direct comparison to the *try-29760* mutant allele, which is in a Col-0 background. To test whether the *try-29760* replum phenotype is indeed caused by the mutation in *try-29760*, my project aimed at generating additional *try* mutant alleles in the same genetic background as *try-29760*. To this end, a gene editing approach was to be employed.

The introduction and use of the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR/Cas9) technology in research over the past decade has revolutionised reverse genetics experiments and many other genetic analyses because of the efficiency with which targeted changes can be made to the genome (Charpentier and Doudna, 2013). It enables the inductions of targeted mutations to a gene of interest, which can subsequently be observed for phenotypic changes to gain knowledge about the function of the gene being studied (Charpentier and Marraffini, 2014).

Techniques using specially designed sequence-specific nucleases such as zinc finger nucleases and transcription activator-like effector nucleases were highly effective when commonly in use, but protein design and synthesis proved time-consuming and technically difficult (Doudna and Charpentier, 2014). This can be preferable to the use of RNA interference (RNAi) - depending on the gene of interest - which prevents expression at the transcriptional level but may

still result in some background expression. RNAi results in a knock-down effect as opposed to the knock-out of function seen in CRISPR/Cas9 (Boettcher and McManus, 2015).

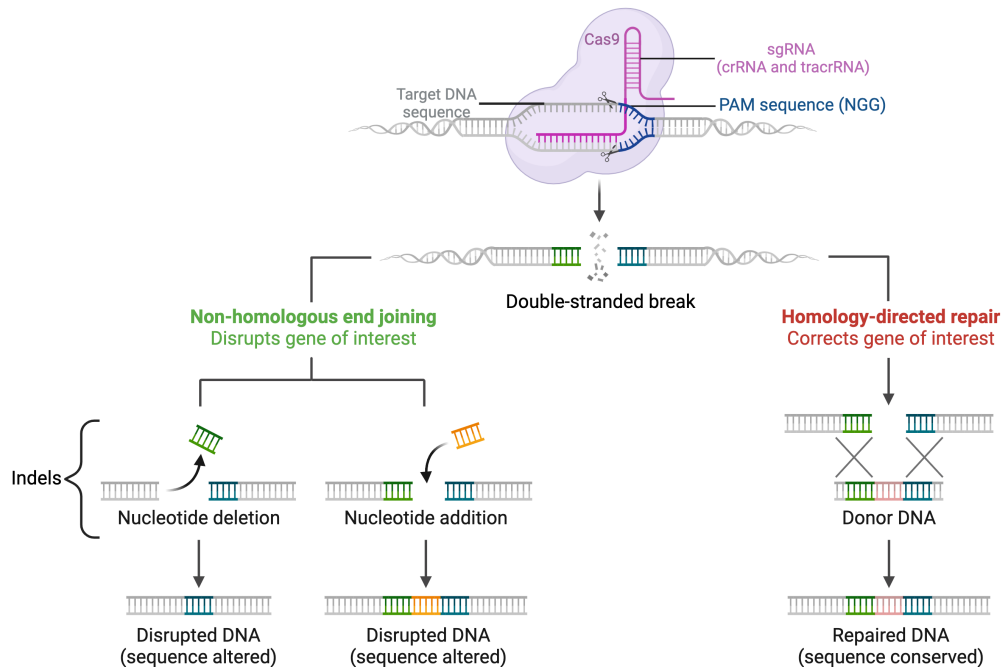


Figure 3.3: Schematic overview of the fate of DNA cleavages induced by the CRISPR/Cas9 machinery.

The designed sgRNA which is associated with Cas9 is shown at the top. This is capable of inducing a double-stranded DNA break at the site targeted by the sgRNA. The fate of a double-stranded break is outlined by the flow chart. There are two main methods of DNA repair in eukaryotes, non-homologous end joining and homology-direct repair. This is explained in further detail in the text. Figure created with Biorender.com

The CRISPR/Cas9 system exploits a bacterial defence mechanism, which recruits various Cas proteins to its genome when it detects that foreign fragments have been integrated (Charpentier and Doudna, 2013). It is able to remove these pathogen's Protospacer-Adjacent Motifs (PAM) - which a pathogen introduces into the bacterial genome to enable itself to replicate within the cell - by recognising the PAMs interspaced between CRISPR RNAs (crRNAs) which then recruits Cas proteins to the site of the invading DNA (Jinek et al., 2012). The Cas9 nuclease of *Streptococcus pyogenes* has two hallmark domains: a HNH nu-

cleave domain, and a RuvC-like domain (Garneau et al., 2010). The two domains bind one strand of DNA each at the target site, cleaving one strand each which leads to the resulting double-stranded break (**Figure 3.3**) (Garneau et al., 2010). There are also two guide RNA structures involved in recruiting and guiding Cas9 to the target site: CRISPR RNA (crRNA), and a trans-activating CRISPR RNA (tracrRNA) which binds a mature crRNA to form a double RNA structure responsible for guiding Cas9 (Brouns et al., 2008). The tracrRNA molecule activates Cas9 to become an active nuclease, and then crRNA guides it to the target site for cleaving (Deltcheva et al., 2011). The crRNA sequence binds its complementary strand at the target site, recruiting the HNH domain of Cas9 which cleaves this strand of the DNA (Brouns et al., 2008). The RuvC-like domain is then responsible for cleaving the DNA strand identical to the crRNA molecule (Chen et al., 2014). Both crRNA and tracrRNA may be tethered together by forming a hairpin-like structure, giving a single guide RNA (sgRNA), which is what is most commonly employed in research (Charpentier and Marraffini, 2014). This is due to the technical simplicity of only needing to generate one RNA molecule when cloning a guide sequence.

The double-stranded, blunt-ended DNA cleavage induced by the two domains of Cas9 triggers the endogenous DNA repair mechanism, non-homologous end joining (NHEJ) (**Figure 3.3**) (Jinek et al., 2012). This repair system is extremely error-prone and often introduces small insertions or deletions known as “indels” in the genome at the target site (Lieber, 2010). The errors in NHEJ commonly induce a frameshift mutation in the target gene, creating a knockout of function and protein truncation (Lieber, 2010).

3.1.5 Aims of this chapter

As described above, previous observations in our laboratory suggested that *TRY*, in addition to its function as a trichome repressor, is involved in the control of replum development and that this function may be linked to CK. Because these observations were preliminary and based on just one *try* mutant allele, my work aimed at confirming the replum phenotype and then to conduct experiments to find out whether the defect in replum development is indeed caused by a disruption to *TRY* function.

To this end, I aimed at carrying out rescue experiments and at generating and characterising additional *try* mutant alleles. If the results of these experiments were to confirm our hypothesis, additional work was to be conducted to investigate the possible link between *TRY* and CK. Furthermore, through the use of genetic analysis, I would aim at characterising the position and function of *TRY* in the gene regulatory network controlling replum development.

3.2 Results

3.2.1 The localisation of TRY and CPC in the gynoecium

As stated in Section 3.1, *TRY* and *CPC* are expressed in the gynoecium to prevent trichome initiation. However, very little work to date has been done on the precise tissues and the stages in which they are expressed. To begin to address this, we have provided our collaborator, Dr Nathanaël Prunet (University of North Carolina), with pTRY::TRYg-GFP *try-29760* and pCPC::CPCg *cpc-1* lines generated previously in our laboratory by Dr Andrea Finocchio. Confocal imaging by Dr Prunet showed TRY (**Figure 3.4 (A)**) and CPC (**Figure 3.4 (B)**) localisation to the medial domain of the gynoecium. A similar finding was previously reported, where it was demonstrated via GUS staining that the second intron of *TRY* is required to drive expression in this domain (Cui et al., 2022). These results together suggest that TRY is active in the medial domain of gynoecia and that a loss of *TRY* function may have effects on replum development.

3.2.2 Establishment of experimental conditions for replum measurements

To validate the previous observation that replum width is affected in *try-29760*, I first sought to establish experimental conditions suitable for replum width measurements. These conditions had to permit the analysis of a large number of siliques to yield a sample size sufficient for statistical analysis. At the same time, the method had to allow accurate measurements to minimise the variability of the data obtained. Based on the equipment available in our laboratory, I established a protocol (see Section 2.4 for details) where I dissected siliques from plants and imaged them under a stereomicroscope. Pictures of the replum were then taken and the replum width was measured using an image processing software (**Figure 3.5**). Three measurements were taken on any individual silique as

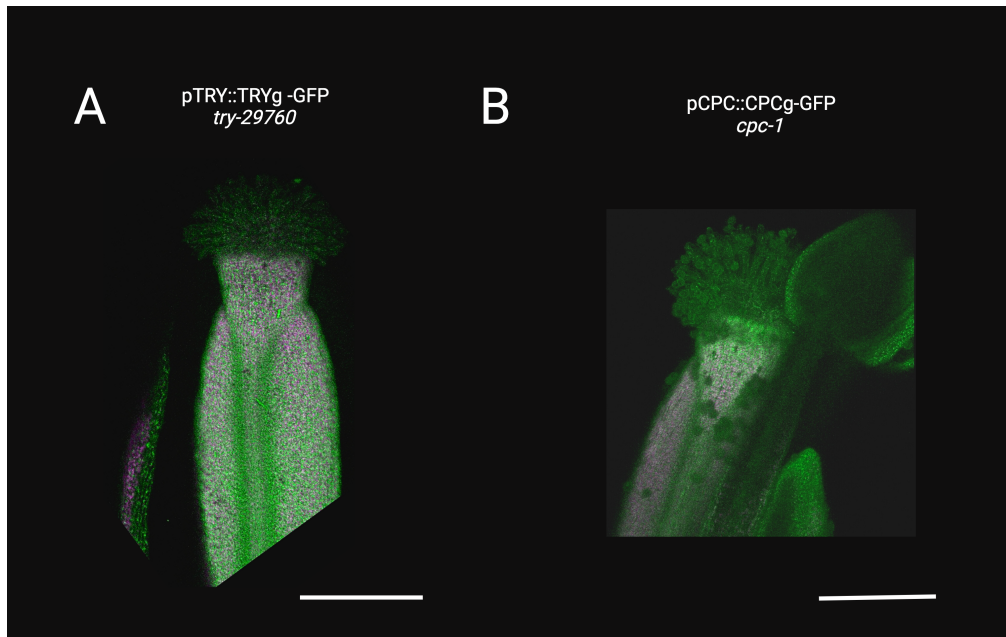


Figure 3.4: Confocal images of TRY and CPC localisation.

(A) pTRY::TRYg-GFP in a *try-29760* background. Note the strong localisation at the medial domain. (B) pCPC::CPCg-GFP in a *cpc-1* background. In both panels, green depicts GFP while magenta indicates the presence of chlorophyll. Green signal in stigmatic tissue and anthers is likely due to auto-fluorescence. Images kindly provided by Dr Nathanaël Prunet (University of North Carolina). Scale bars: 200 μ m

technical replicates. The measurement data were then exported and analysed using the statistical software *Prism*.

Using this method, it became apparent in preliminary experiments that the width of the replum in wild-type siliques varies both along the length of a silique and possibly also depending on the stage of the plant. In order to standardise the measurements for each of the experiments presented in this chapter and to obtain reliable data, I sought to identify conditions that would minimise this variability.

To this end, I measured replum width for siliques of both *try-29760* and Col-0 wild-type plants in three different regions (basal, apex and midpoint) as well as for different flower positions (5th, 6th, and 10th flower formed by a plant on the primary shoot). From the results of these measurements (**Figure 3.6**), it appeared that for plants of both genotypes, the least amount of variability

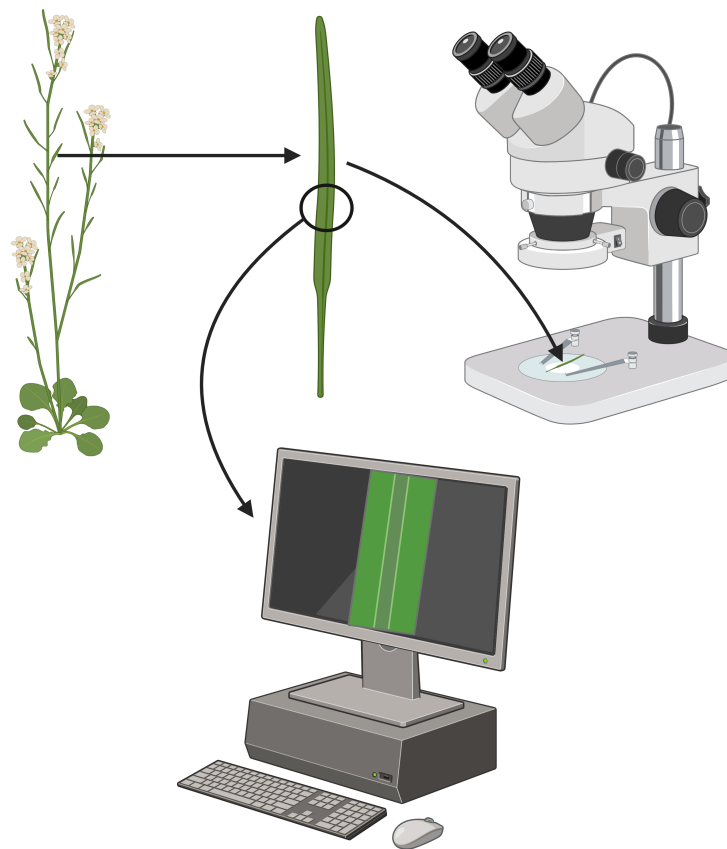


Figure 3.5: Schematic representation of the method of replum measurement. See text for details. Figure created with Biorender.com.

occurred at the midpoint of siliques where it was largely independent of the position of the flower analysed. Replum measurements were therefore carried out using the midpoint of siliques from the 5th to the 10th flower formed by a plant on the primary shoot. Three technical replicates of each measurement of a silique were also taken, to account for any variability.

3.2.3 Reduction in replum width in the *try-29760* mutant

Using the experimental conditions outlined above, I sought to verify the preliminary observation by previous members of our laboratory that the replum is narrower in *try-29760* when compared to the wild type. To this end, I measured replum width in both *try-29760* mutant and Col-0 wild-type plants. In

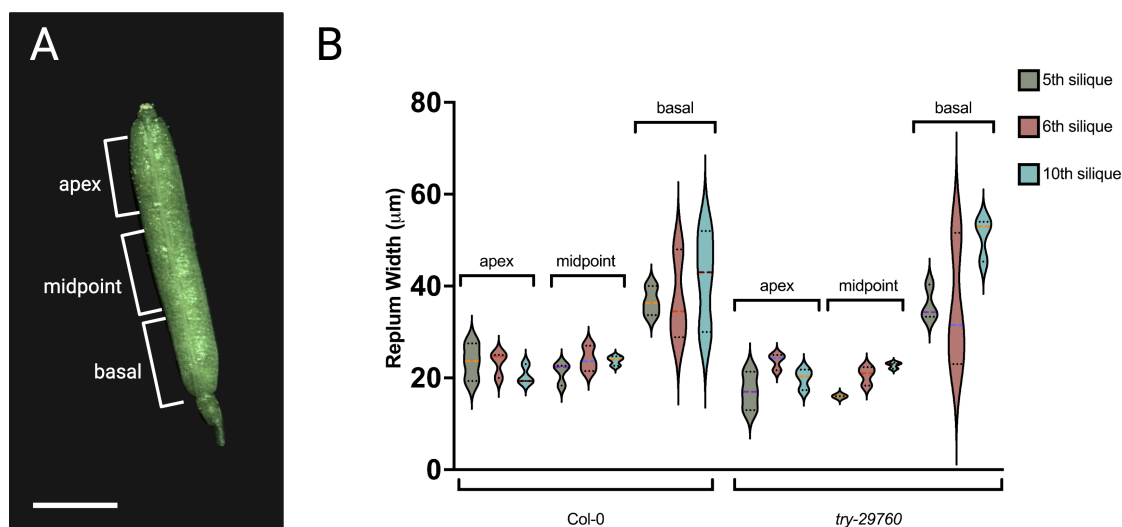


Figure 3.6: Stage-specific and spatial differences in replum width.

(A) Silique of a wild-type plant showing how the gynoeceum was zoned for the purpose of replum measurements. Scale bar: 2 mm. (B) Violin plots showing the results of replum measurements for siliques of Col-0 wild-type and *try-29760* mutant plants in three different regions (basal, apex and midpoint) as well as for different flower positions (5th, 6th, and 10th flower formed by a plant on the primary shoot). Data are based on three biological replicates. Dashed lines represent medians, while dotted lines indicate upper and lower quartiles.

agreement in the previous observation, I found that the width of the replum in *try-29760* is significantly reduced when compared to Col-0 (Figure 3.7).

3.2.4 Complementation of the *try-29760* mutant

In order to establish whether the reduction in replum width in *try-29760* was due to a disruption in *TRY* activity or was caused by a second-site mutation, I first attempted to complement *TRY* function in the *try-29760* mutant background. To this end, I transformed *try-29760* mutant plants with a construct (pTRY::TRYg) that contained the genomic region of *TRY* including its regulatory region (Figure 3.8 (A)), as previously described (Cui et al., 2022; Lampropoulos et al., 2013).

Transformants were selected and PCR-genotyped to confirm the presence of the transgene (see Section 2.7). While *try-29760* mutant plants consistently form trichome clusters on the adaxial side of leaves, trichome patterning on leaves of *try-29760* plants that carried the complementation construct resembled,

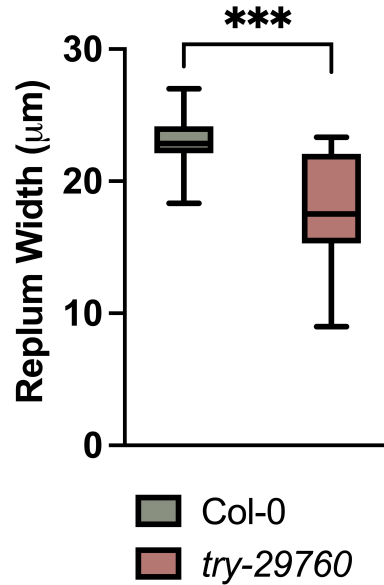


Figure 3.7: Reduction of replum width in *try-29760* mutant plants.

Replum width of stage 17 untreated siliques of *try-29760* and Col-0 plants was measured as outlined in the text. Box and Whisker plots represent the results of five biological and three technical replicates. Whiskers indicate the 5th to 95th percentile range of the data sets. Statistical significance determined using Welch's *t*-test. "***": $0.0002 < P < 0.002$.

in most of the independent transformants I obtained (14 out of a total of 17) that of the wild type (**Figure 3.8 (B)**). Thus, the pTRY::TRYg construct appeared to be able to rescue the leaf trichome phenotype of the *try-29760* mutant, suggesting that the construct is functional. Transgenic lines displaying a wild-type trichome phenotype were carried through to the T₂ generation for further experimentation.

To test the effects of *TRY* over-expression on the *try-29760* mutant, I generated a construct that mediates the expression of *TRY* under the control of the strong Cauliflower Mosaic Virus (CaMV) 35S promoter (**Figure 3.9 (A)**; see Section 2.7) in a *try-29760* background. Primary transformants carrying this construct were in most cases (4 individuals of 5 obtained) completely glabrous (**Figure 3.9 (B)**), in agreement with previous observations showing that *TRY* over-expression suppresses trichome formation (Schellmann et al., 2002). Thus, this construct appeared to be functional as well.

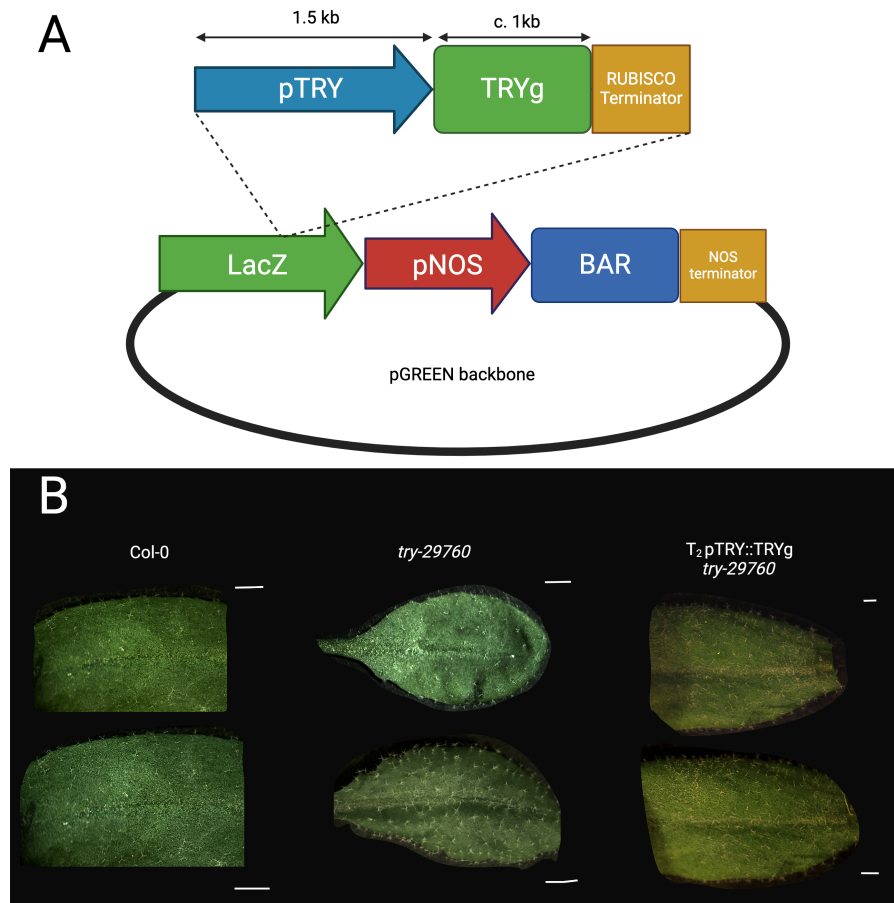


Figure 3.8: Complementation of *TRY* function in a *try-29760* mutant background.

(A) Map of the construct containing the genomic region of *TRY* fused to a transcriptional terminator sequence (RUBISCO terminator). This construct was inserted into a GreenGate plant transformation vector (based on a pGreen vector backbone). Panel created with Biorender.com. (B) Images of the adaxial surfaces of rosette leaves of wild-type Col-0, *try-29760* and T₂ generation pTRY::TRYg *try-29760* plants (as indicated). Scale bars: 2 mm.

I next tested whether the complementation constructs could also rescue the replum defect of the *try-29760* mutant. To this end, I examined selected transgenic lines that exhibited a clear effect on trichome formation and where the transgenes appeared to be active. The results of replum width measurements showed that *try-29760* plants carrying either pTRY::TRYg or p35S::TRYg had significantly wider repla than *try-29760* mutants (**Figure 3.10**). Thus, these constructs rescued the replum phenotype of *try-29760* plants, suggesting that it is indeed caused by a loss of *TRY* function.

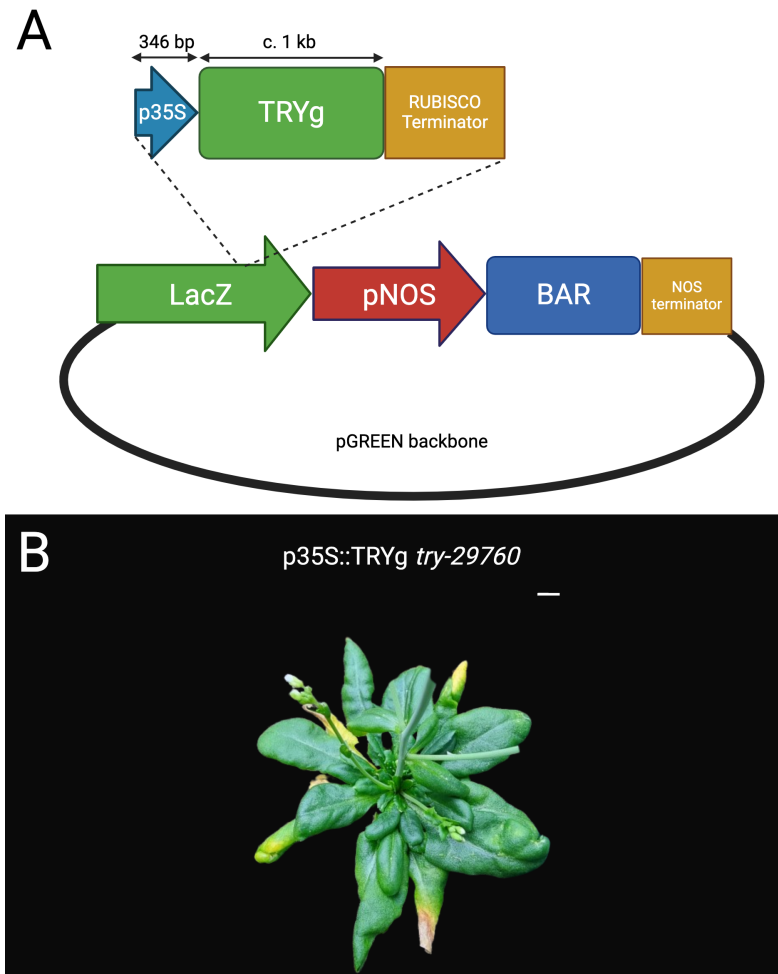


Figure 3.9: Effects of *TRY* over-expression on trichome formation in a *try-29760* mutant background.

(A) Map of the construct containing the CaMV 35S promoter and the genomic region of *TRY* fused to a transcriptional terminator sequence (RUBISCO terminator). This construct was inserted into a GreenGate plant transformation vector (based on a pGreen vector backbone). Panel created with Biorender.com. (B) Image of a T₁ p35S::TRYg *try-29760* plant. Note that all leaves are glabrous. Scale bar: 10 mm.

In addition to transforming the complementation constructs into *try-29760*, I also introduced them into a wild-type (Col-0) background, to test whether extra copies of *TRY* would affect replum development. Replum widths in these lines didn't seem to be significantly different to the transgenic lines in a *try-29760* background (Figure 3.10). The repla of wild-type plants carrying p35S::TRYg in particular, appeared to be slightly wider than that of the wild-type, but not to a

significant level.

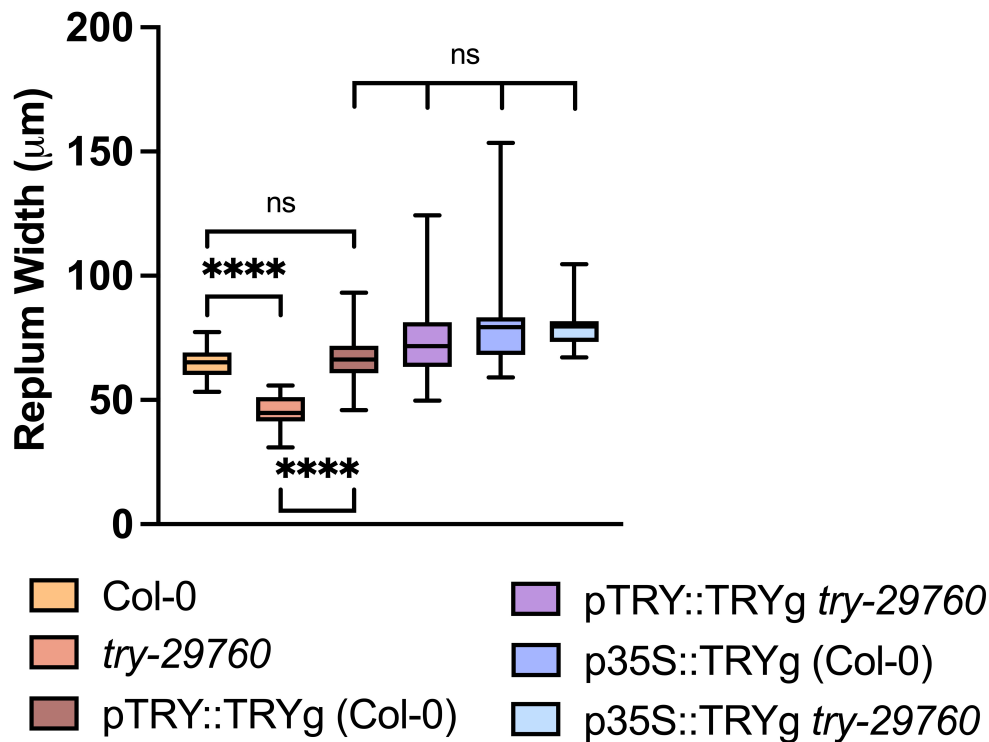


Figure 3.10: Effects of the *TRY* complementation constructs on replum width. Values shown are the mean (line within box) of 20 independent siliques of each genotype. Three technical replicates of each measurement were completed. Error indicated by the limits of the box is the standard deviation of the sample. Whiskers outside the box of each data point show the 5th to 95th percentile range of the data sets. Lines situated outside the limit of whiskers, where present, indicate outlier data points. Statistical significance determined by Welch's *t*-test. "ns": $P \geq 0.05$; "****": $P < 0.0001$.

Finally, to complete the characterisation of these novel transgenic lines, an RT-qPCR experiment was performed to test for the presence of *TRY* mRNA as a further measure to determine that the transgenic lines generated were functional (Figure 3.11). While the p35S::TRYg *try-29760* lines showed, as expected, high levels of *TRY* transcripts, surprisingly, in the pTRY::TRYg *try-29760* line, *TRY* expression could not be detected. While the reason for this is not clear, perhaps earlier expression of *TRY* in the gynoecium is sufficient to control replum width. Additionally, this line was demonstrated to phenotypically rescue *try-29760*, so there must be sufficient *TRY* expression to achieve this (Figure 3.8).

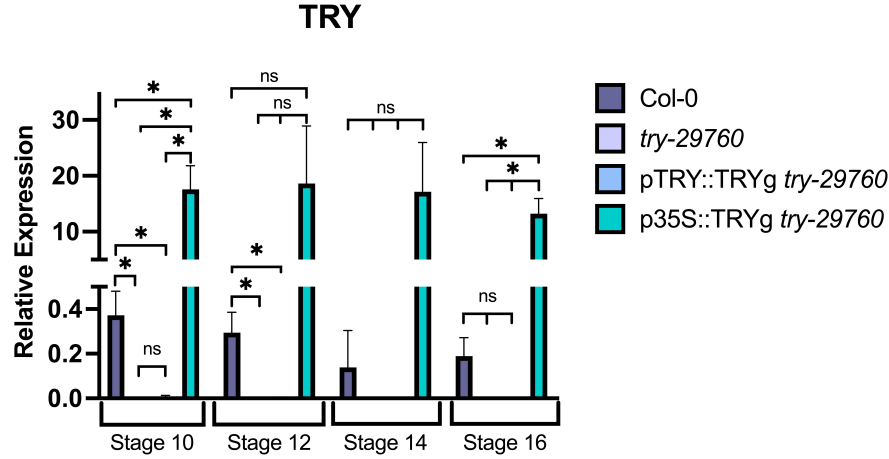


Figure 3.11: Characterisation of the novel lines generated in this work. RT-qPCR results detecting *TRY* mRNA levels in the gynoecium of Col-0, *try-29760*, pTRY::TRYg *try-29760* and p35S::TRYg *try-29760* genotypes at stages 10, 12, 14 and 16 (Smyth et al., 1990). Values are relative to the reference gene *REF1* (AT1G13320). Values shown are the mean of three (or two for *try-29760* stage 10) biological replicates for each genotype. Two technical replicates were performed for each sample. Error bars represent the standard deviation of the replicates. Statistical significance determined by Welch's *t*-test. "ns": $P \geq 0.05$; "*": $0.0332 < P < 0.05$.

3.2.5 Generation of novel *try* mutant alleles through genome editing

The results of the complementation experiments shown above suggested that the reduction in replum width in *try-29760* is indeed caused by a disruption in *TRY* activity. To further test this, I employed a CRISPR/Cas9 genome editing approach to generate novel *try* alleles in the same genetic background as *try-29760* (i.e. the Col-0 accession). To this end, I made a sgRNA that targets the first exon of *TRY* (**Figure 3.12 (A)**) using a modified version of the vector pHEE-401 (**Figure 3.12 (B)**) and a modified GoldenGate protocol (see Section 2.7) (Wang et al., 2015). This vector carries a copy of red fluorescent protein (RFP) which is driven by a seed coat-specific promoter. This allowed for simplified selection of transformants due to the formation of red-coloured seeds (**Figure 3.12 (C)**).

To identify transformants with lesions in *TRY*, 25 plants from the T₁ gen-

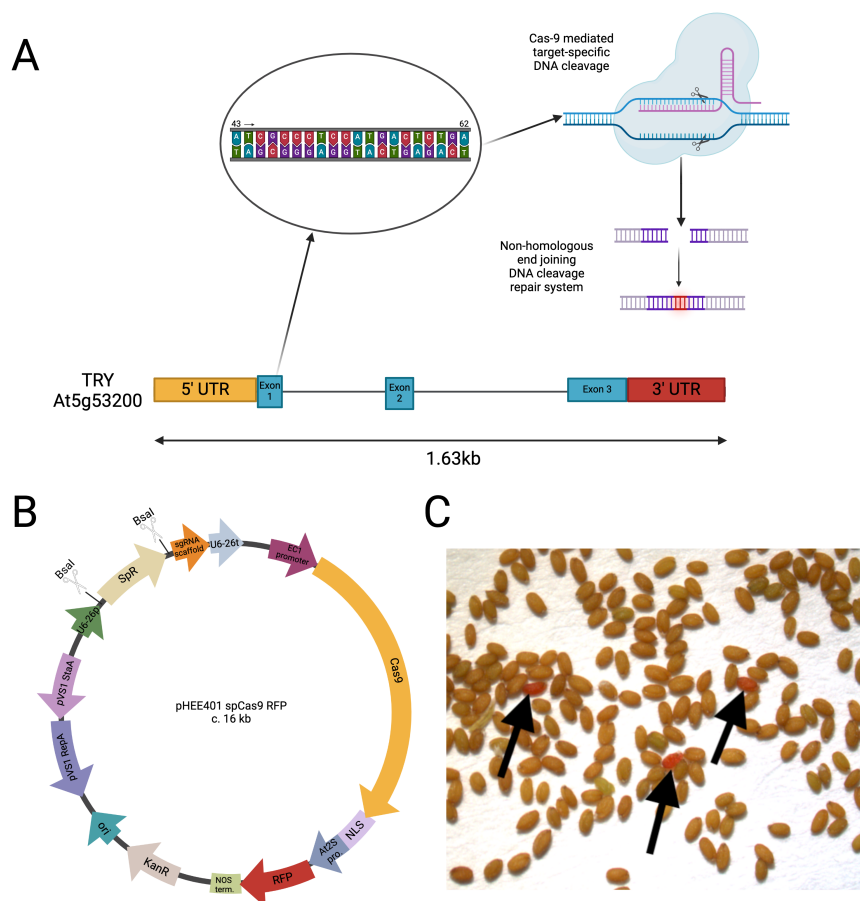


Figure 3.12: Generation of novel *try* null mutant lines through CRISPR/Cas9. (A) Schematic of the design of the sgRNA used to target *TRY*. The structure of the *TRY* locus and the pairing of the sgRNA to its complementary genomic sequence are shown. (B) Map of vector pHEE401, carrying Cas9 driven by an egg cell promoter, and RFP driven by the At2S promoter. (C) Selection of transgenic seeds harvested from T₀ plants. Arrows indicate seeds displaying a red seed coat phenotype, indicating they are transgenic and harbour the CRISPR/Cas9 cassette. Panels A and B created with Biorender.com.

eration were assessed by PCR-amplification of the region of *TRY* targeted by the sgRNA and subsequent Sanger sequencing. This work led to the identification of two independent mutant alleles for *TRY* (Figure 3.13). These plants were then taken to the T₂ generation and progeny homozygous for the mutations in *TRY* were isolated. Furthermore, the plants were genotyped to ensure that they no longer contained Cas9 to prevent an induction of secondary mutations. One of the two novel alleles identified, termed *try-19*, has a 1 bp insertion at the beginning of the coding sequence, leading to a frame shift and a premature stop codon

so that the resulting protein product would be composed of only 18 amino acids. The second allele, termed *try-14*, has a 14 bp deletion in the *TRY* coding region and also induces a premature stop codon, leading to an even shorter protein fragment than in the case of *try-19* (**Figure 3.13**). Given the likely effects on the synthesis of TRY protein, these alleles were predicted to be amorphic. In agreement with this idea, plants homozygous for both CRISPR alleles formed leaves with trichome clusters and thus resembled *try-29760* mutant plants (**Figure 3.14**).

3.2.6 Replum defects in the novel *try* mutant alleles

I next tested whether the novel *try* mutant alleles showed similar replum defects to the one observed in *try-29760*. When I measured replum widths in these two lines, I found that as in *try-29760*, their repla were narrower than in the wild-type (**Figure 3.15**). This effect appeared to be somewhat weaker in *try-14*, while the replum widths in *try-19* and *try-29760* were very similar.

Taken together, the results from the analysis of the CRISPR alleles and from the complementation experiments strongly suggest that the reduction in replum width first observed in *try-29760* is indeed due to a loss of *TRY* function. Thus, *TRY* appears to be not only a repressor of trichome formation but to have an additional function as a regulator of replum development.

3.2.7 Effect of *CPC* function on replum development

As described in Section 3.1, *TRY* acts in a largely redundant manner with *CPC* in the suppression of trichome formation on leaves and floral organs. Given this shared function, I decided to investigate whether *CPC* is also involved in the control of replum development. To this end, I measured replum widths in a *cpc-1* single mutant as well as in a *try-29760 cpc-1* double mutant and compared the results to the values for the wild type and for the *try-29760* single mutant (**Figure 3.16**). This comparison showed that the replum in *cpc-1* is only slightly narrower than in the wild type. A similar phenotype was observed in the *try-*

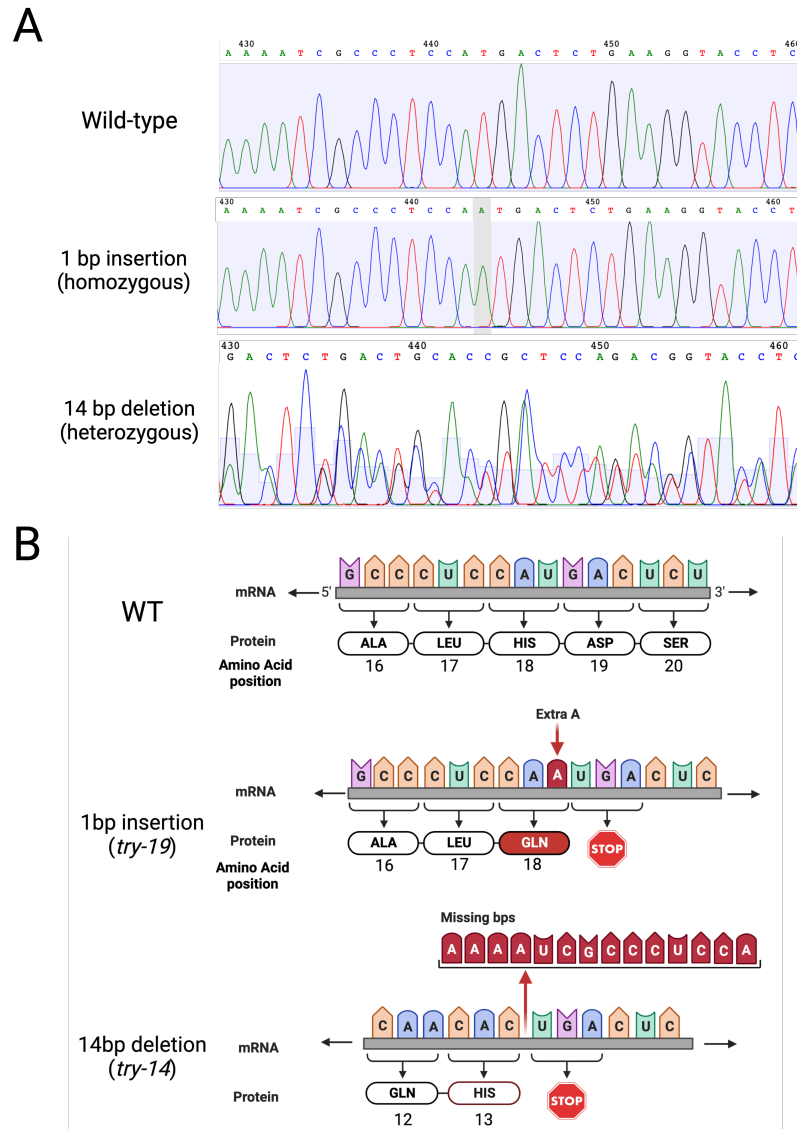


Figure 3.13: Lesions in the novel *try* mutant alleles generated by CRISPR/Cas9.

(A) Chromatograms of the wild-type sequence of *TRY*, the homozygous 1 bp insertion and the heterozygous 14 bp deletion (labelled). (B) Schematic describing mutations identified and their effect on the amino acid sequence of *TRY*, with the adopted nomenclature annotated. Panel (B) created with Biorender.com.

29760 *cpc-1* double mutant, where the replum was narrower than in the wild type, but wider than in the *try*-29760 single mutant. These results suggest that *CPC* does not act in functionally redundant manner with *TRY* in the control of replum development and may in fact partially suppress the effects of a loss of

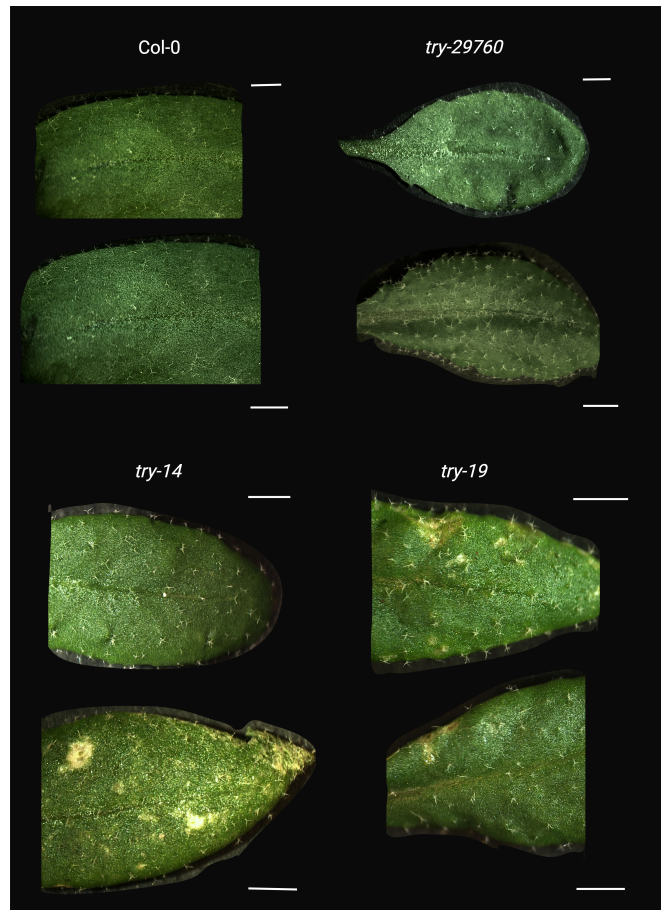


Figure 3.14: Trichome phenotypes of the two novel homozygous *try* lines generated through CRISPR/Cas9.

All images were taken of the third rosette leaf of the plant. Leaves of wild-type (Col-0) and *try-29760* shown as controls. Scale bars: 2 mm.

TRY function.

3.2.8 Relationship between *TRY* and CK in replum development

In Section 3.1, I described previous work done in our laboratory which suggested that *try* mutants are hypersensitive to the exogenous application of the synthetic CK, BAP. To confirm this observation and to understand the relationship between *TRY* and CK in replum development, I conducted experiments in which I treated different mutants with a solution containing 100 μ M BAP or with a mock solution for 5 consecutive days and measured replum widths of

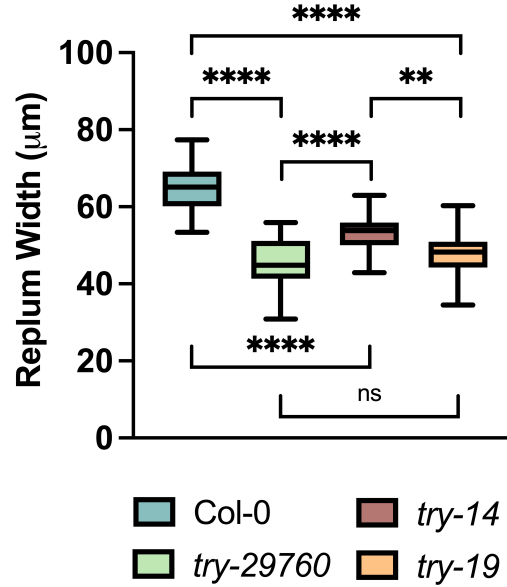


Figure 3.15: Replum widths of two novel *try* mutants lines generated using CRISPR/Cas9 genome editing.

Values shown are the mean (line within box) of 20 independent siliques of each genotype. Three technical replicates of each measurement were completed. Error indicated by the limits of the box is the standard deviation of the sample. Whiskers outside the box of each data point show the 5th to 95th percentile range of the data sets. Statistical significance determined by Welch's *t*-test. "ns": $P \geq 0.05$; "***": $0.0021 < P < 0.0332$; "****": $P < 0.0001$.

gynoecia 14 days after treatment (Zúñiga-Mayo et al., 2014; Ó Maoiléidigh et al., 2018). As previously reported, BAP treatment of wild-type plants led to a significant increase of replum width (**Figure 3.17 (A)**) (Marsch-Martínez et al., 2012; Zúñiga-Mayo et al., 2014). In the case of *try-29760*, the defects of replum development described above were completely rescued by BAP treatment and the average replum width appeared larger than that of BAP-treated wild-type plants. For *cpc-1* single mutants and *try-29760 cpc-1* double mutants, BAP treatments increased replum widths to a similar degree as in the wild type (**Figure 3.17 (B)**).

I also tested the effects of BAP treatment on the different complementation lines described in Section 3.2.4 above (**Figure 3.18**). With the exception of the pTRY::TRYg line in a Col-0 wild-type background, no significant differences in

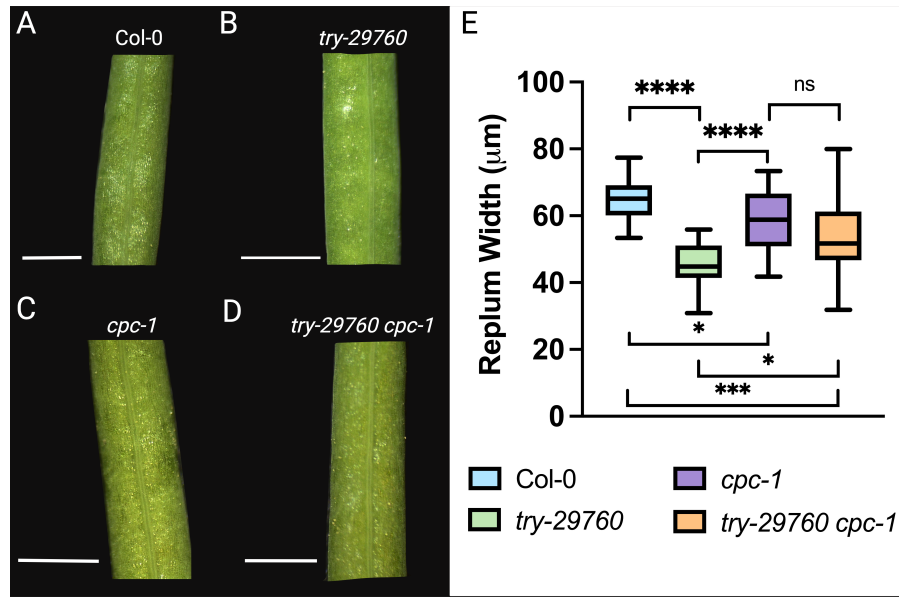


Figure 3.16: Replum width in *cpc-1* single and *try-29760 cpc-1* double mutants. (A) to (D) Stage 17 siliques of Col-0 (A), *try-29760* (B), *cpc-1* (C) and *try-29760 cpc-1* (D) plants (Smyth et al., 1990). (E) Replum width was measured as outlined in the text. Box and Whisker plots represent the results for 20 siliques per genotype. All measurements were carried out in triplicate. Whiskers indicate the 5th to 95th percentile range of the data sets. Statistical significance was determined using Welch's *t*-test. "ns": $P \geq 0.05$; "*": $0.0332 < P < 0.05$; "***": $0.0002 < P < 0.002$; "****": $P < 0.0001$.

replum width were detected in response to BAP. This may be simply a consequence of the slightly wider repla in the untreated plants (Figure 3.10) so that the difference between BAP-treated and mock-treated repla decreases relative to what I observed in the wild type.

Taken together, these results confirm previous observations showing that exogenous CK treatments increase replum width in the wild type and show that normal replum development can be restored in *try-29760* by BAP. The wider repla in BAP-treated *try-29760* plants when compared to the BAP-treated wild type suggest that *try-29760* may have some degree of hypersensitivity to CK. This would be in agreement with previous observations from our laboratory showing hypersensitivity of *try-29760* to BAP in trichome initiation of gynoecia (Figure 1.9) (Ó Maoiléidigh et al., 2018).

I then went on to treat the novel *try* mutant lines generated using CRISPR/Cas9,

as described in Section 3.2.5. This was done, as previously outlined, to determine if the response of *try-29760* to BAP was due to a lack of *TRY* function or a secondary mutation present in the line. These lines showed a similar effect to that seen in *try-29760* (**Figure 3.19 (A)**). While *try-14* shows a slightly weaker phenotype than *try-29760* in the mock-treated sample, *try-19* is very much in keeping with the results of *try-29760*. Additionally, both lines demonstrate a similar increase in replum width in response to BAP treatment to *try-29760* (**Figure 3.19 (B)**).

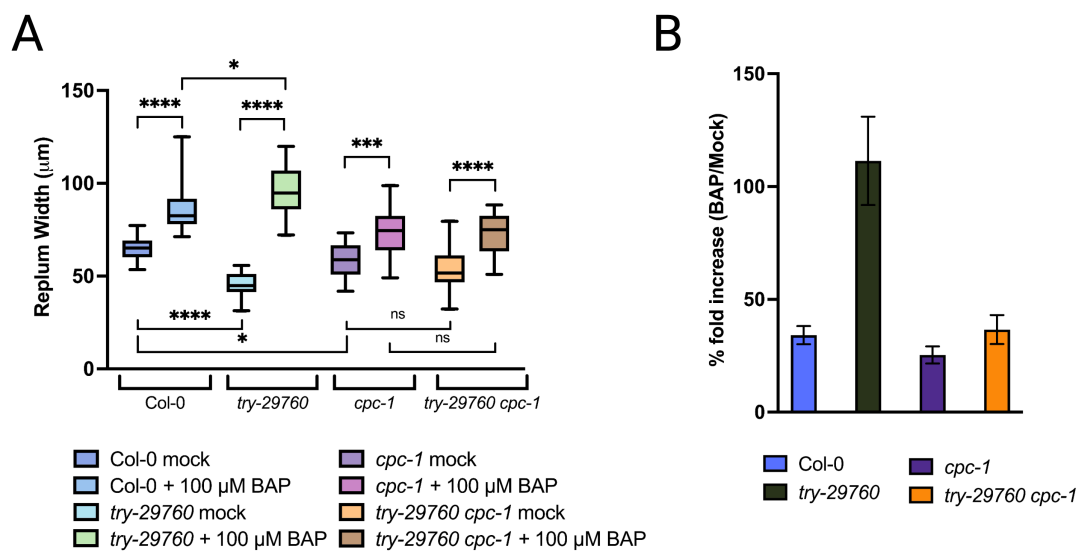


Figure 3.17: Effects of BAP treatment on replum width in *try* and *cpc* mutant plants.

(A) Col-0, *try-29760*, *cpc-1* and *try-29760 cpc-1* plants were treated with a mock solution or with a solution containing 100 μM BAP. Box and whisker plots showing the results of replum width measurements for 20 siliques per genotype. Whiskers indicate the 5th to 95th percentile range of the sample data. Statistical significance determined by Welch's *t*-test: "ns": not significant, $P \geq 0.05$; "**": $0.0332 < P < 0.05$; "***": $0.0002 < P < 0.002$; "****": $P < 0.0001$. **(B)** Percentage increase in replum width in BAP-treated samples versus mock-treated samples. Percentages were calculated by dividing the mean value of BAP-treated by the mean value of mock-treated samples. Error bars represent the 95% confidence interval.

These data help to demonstrate the response of *TRY* to exogenous BAP, as well as the effect of the absence of *TRY* expression on BAP response. **Figure 3.20**

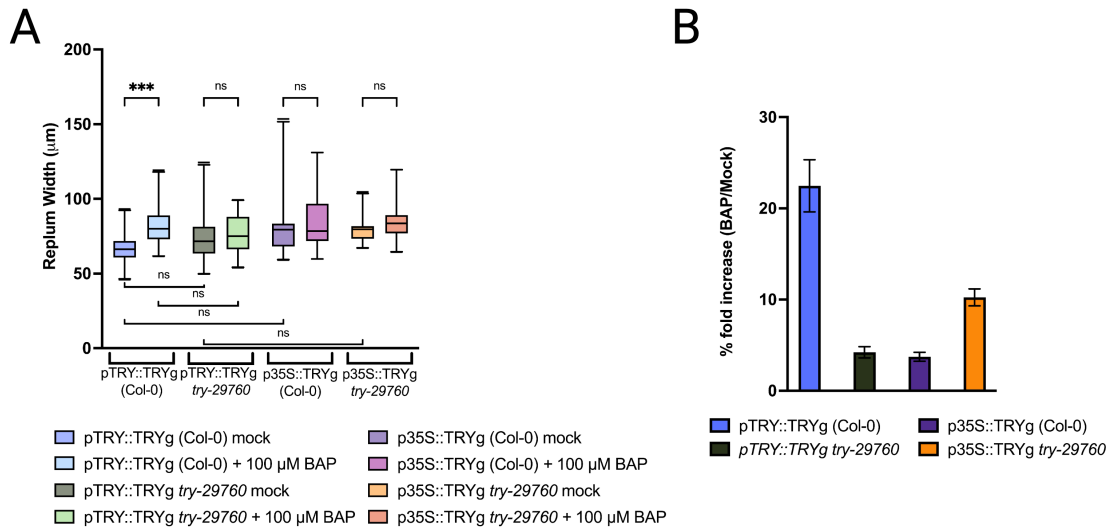


Figure 3.18: Effects of BAP treatment on replum width in *TRY* complementation and overexpression lines.

(A) Results of replum width measurements in pTRY::TRYg (Col-0), pTRY::TRYg *try-29760*, p35S::TRYg (Col-0) and p35S::TRYg *try-29760* lines after treatment with a mock solution or a 100 μM BAP solution. Twenty siliques were analysed per sample. Whiskers outside the box of each data point show the 5th to 95th percentile range of the data sets. Statistical significance determined by Welch's *t*-test. "ns": not significant, $P \geq 0.05$; "****": $0.0002 < P < 0.002$. **(B)** Calculation of the percentage increase of the width of the replum of each genotype in BAP-treated versus mock-treated samples. Percentage increase was calculated by dividing the mean values for BAP-treated and mock-treated samples. Error bars represent a 95% confidence interval.

demonstrates the similarity of the response to BAP in the novel *try* mutants to the existing *try-29760* line, as well as demonstrating that this effect is rescued (and even diminished) in the complementation lines.

3.2.9 Effect of cytokinin on other regulators of replum development and their genetic interactions with *TRY*

The results of the experiments described above suggested that *TRY* is a regulator of replum development and that the replum defects observed in *try* mutants can be rescued by exogenous CK treatment. To obtain further insights into the role of *TRY* and BAP in replum formation, I decided to examine mu-

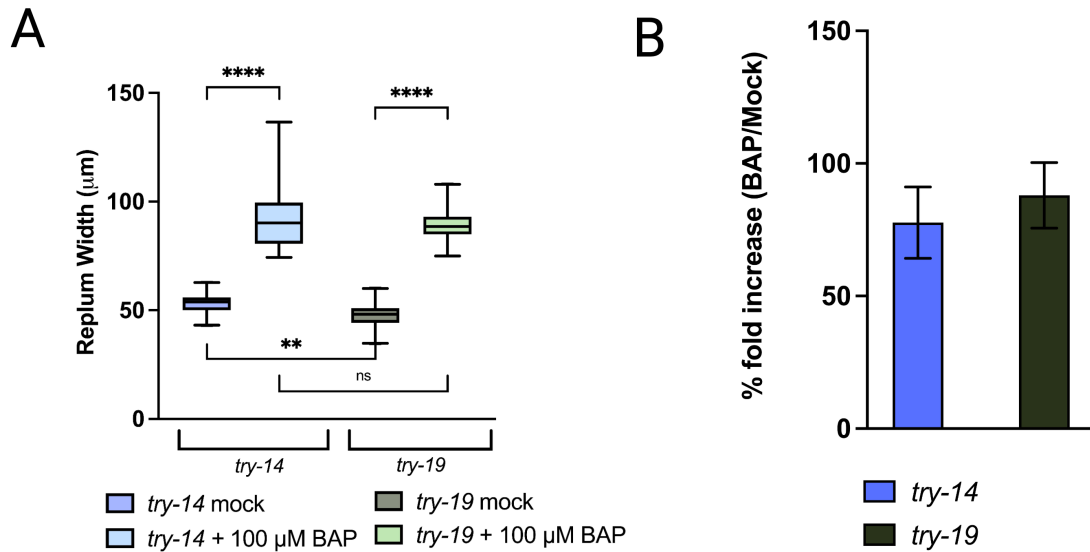


Figure 3.19: Effects of BAP treatment on replum width in novel *try* mutant lines.

(A) *try-14* and *try-19* plants were treated with a mock solution or with a solution containing 100 μM BAP. Box and whisker plots showing the results of replum width measurements for 20 siliques per genotype. Whiskers indicate the 5th to 95th percentile range of the sample data. Statistical significance determined by Welch's *t*-test: "ns": not significant, $P \geq 0.05$; "***": $0.0021 < P < 0.033$; "****": $P < 0.0001$. (B) Percentage increase in replum width in BAP-treated samples versus mock-treated samples. Percentages were calculated by dividing the mean value of BAP-treated by the mean value of mock-treated samples. Error bars represent the 95% confidence interval.

tants of known regulators of replum development that had been previously characterised and where pertinent, combine them with *try* mutant alleles. Because some of these mutants were isolated in different *Arabidopsis* accessions, I first determined whether these accessions exhibited any difference in response to exogenous BAP treatment. This experiment did not reveal any significant accession-specific effects on replum width across the three accessions tested (Figure 3.21), suggesting that the different mutants I chose for analysis could be compared directly.

Treatment of these mutant lines with a BAP-containing or with a mock solution was done as described in Section 2.2. Among the mutants used was *gl1*, which disrupts the function of the main activator of trichome formation,

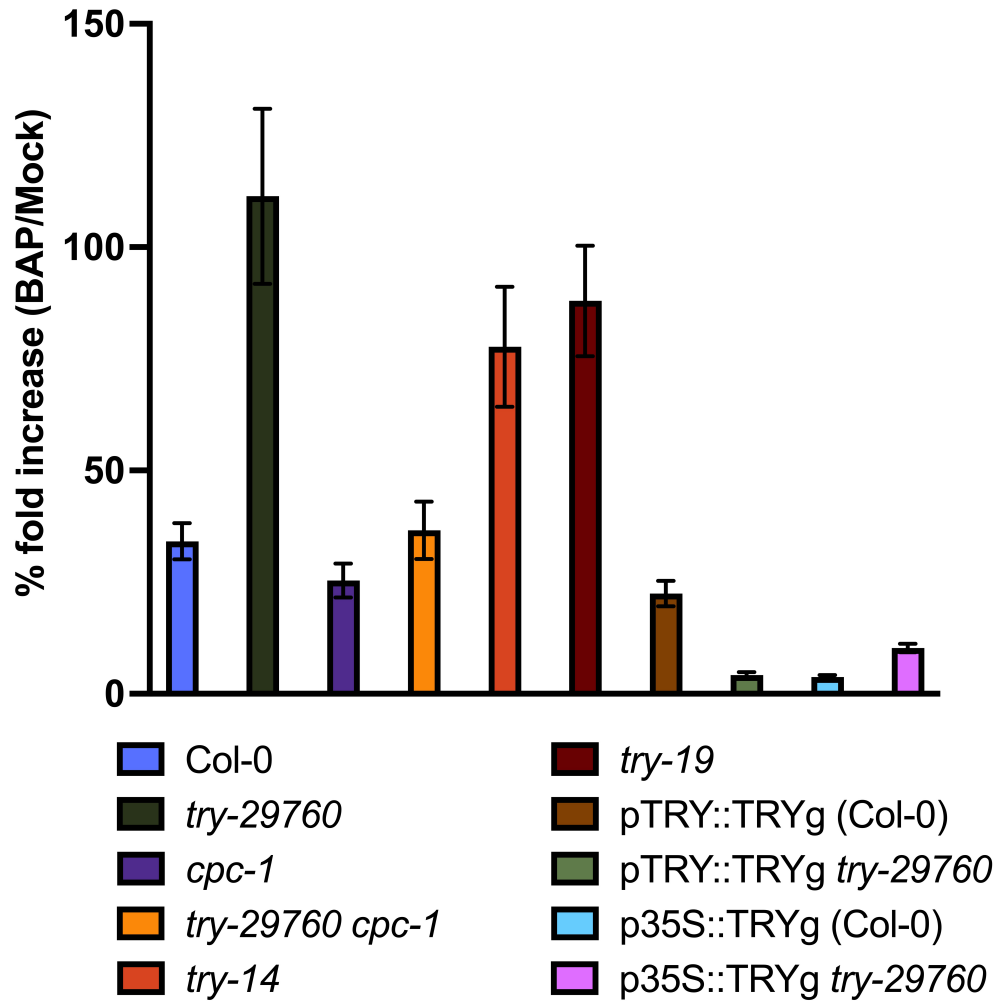


Figure 3.20: The effect of BAP treatment on the replum of the lines generated in this work, compared to the existing mutant lines. Percentage increase in replum width in BAP-treated samples versus mock-treated samples. Percentages were calculated by dividing the mean value of BAP-treated by the mean value of mock-treated samples. Error bars represent the 95% confidence interval.

GLABRA1 (see Section 1.7, **Figure 1.7**). This mutant line was included to test whether other trichome regulators may be involved in the control of replum development. As discussed above, my experiment showed that a loss of *CPC* function has no major effects in replum formation but may be able to partially suppress the *try* mutant phenotype (**Figure 3.16**). Replum width in the *gl1* mutant was not significantly different from the wild-type, independently of whether BAP had been applied or not (**Figure 3.22**). Thus, *GL1* does not appear to have a secondary function in replum development.

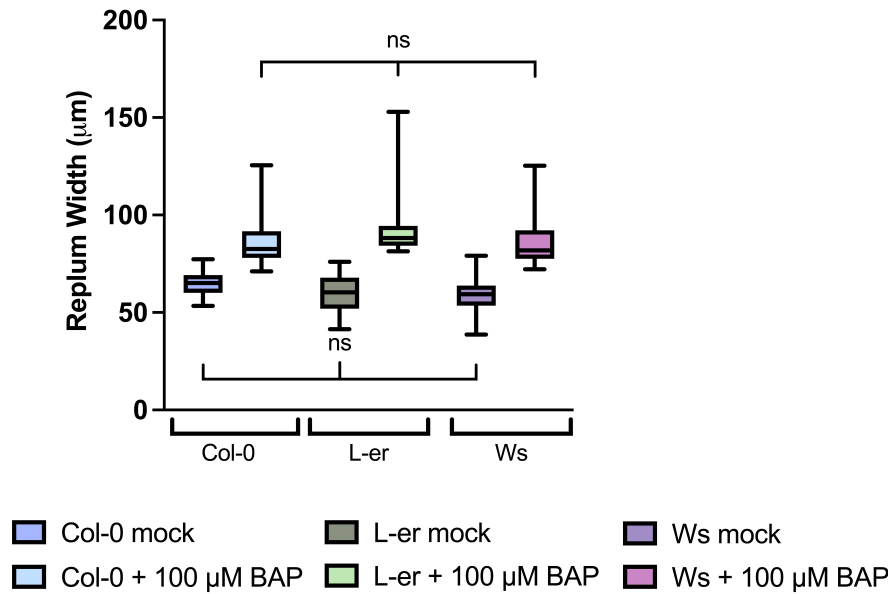


Figure 3.21: Effects of BAP treatment on replum width in different *Arabidopsis* accessions.

Results of replum width measurements in three *Arabidopsis* accession (Col-0, L-er and Ws) after treatment with a mock solution or a 100 μ M BAP solution. Twenty siliques were analysed per sample. Whiskers outside the box of each data point show the 5th to 95th percentile range of the data sets. Statistical significance determined by Welch's *t*-test. "ns": not significant, $P \geq 0.05$.

As previously described, I found that replum width in *ful* mutants is dramatically increased when compared to the wild type (**Figure 3.22**) (Gu et al., 1998). BAP treatment did not increase replum width significantly, suggesting that *ful* mutants are largely insensitive to exogenous CK application. Similarly, in a *ckx6-1* background, BAP treatment did not have a significant effect on replum width (**Figure 3.22**). In this mutant background, endogenous CK is not being broken down as efficiently as in the wild type due to impaired CK metabolism (Werner et al., 2001; Schmülling et al., 2003). The increase in replum width observed in mock-treated *ckx6-1* relative to the mock-treated wild type may therefore be a direct consequence of increased CK levels. Furthermore, the mock-treated width of *ckx6-1* is similar to that of the BAP-treated wild-type, which also suggests the width of *ckx6-1* repla is due to increased endogenous CK levels. The fact that BAP treatment does not further increase replum width in this mutant

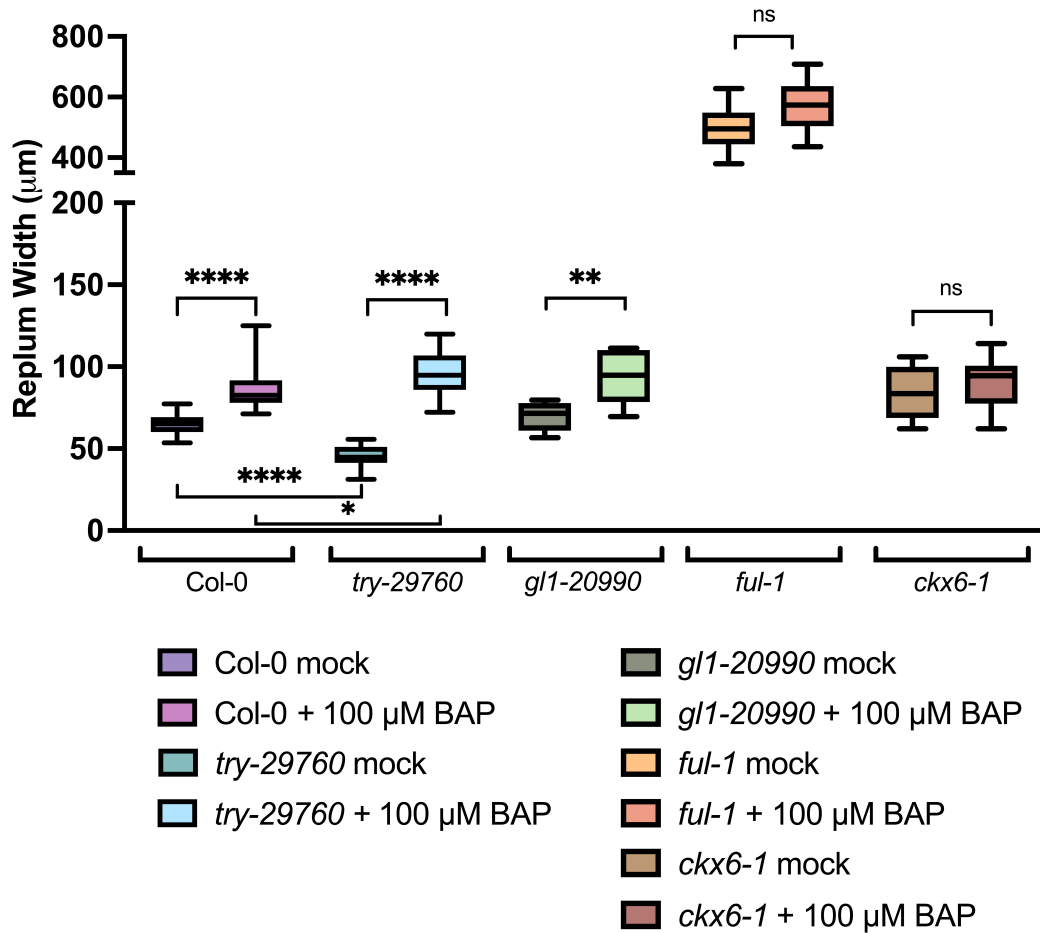


Figure 3.22: Effects of BAP treatment on replum width in various mutants with known or potential defects in replum formation.

Results of replum width measurements in different mutant backgrounds (as indicated) after treatment with a mock solution or a 100 μM BAP solution. Twenty siliques were analysed per sample. Whiskers outside the box of each data point show the 5th to 95th percentile range of the data sets. Statistical significance determined by Welch's *t*-test. "ns": not significant, $P \geq 0.05$; "****": $0.0332 < P < 0.05$; "***": $0.0021 < P < 0.033$; "*****" $P < 0.0001$.

line suggests that a "saturation point" may have been reached, wherein additional CK cannot further activate CK signalling and thus promote cell growth and proliferation in the replum.

Using double mutant analyses, I further found that the addition of *try*-29760 to *rpl-1* and *as1-1* mutant backgrounds led to double mutants that are hypersensitive to BAP treatment when compared to BAP-treated *rpl-1* and *as1-1* single mutants (Figure 3.23). The mock-treated single *rpl-1* mutant displays a

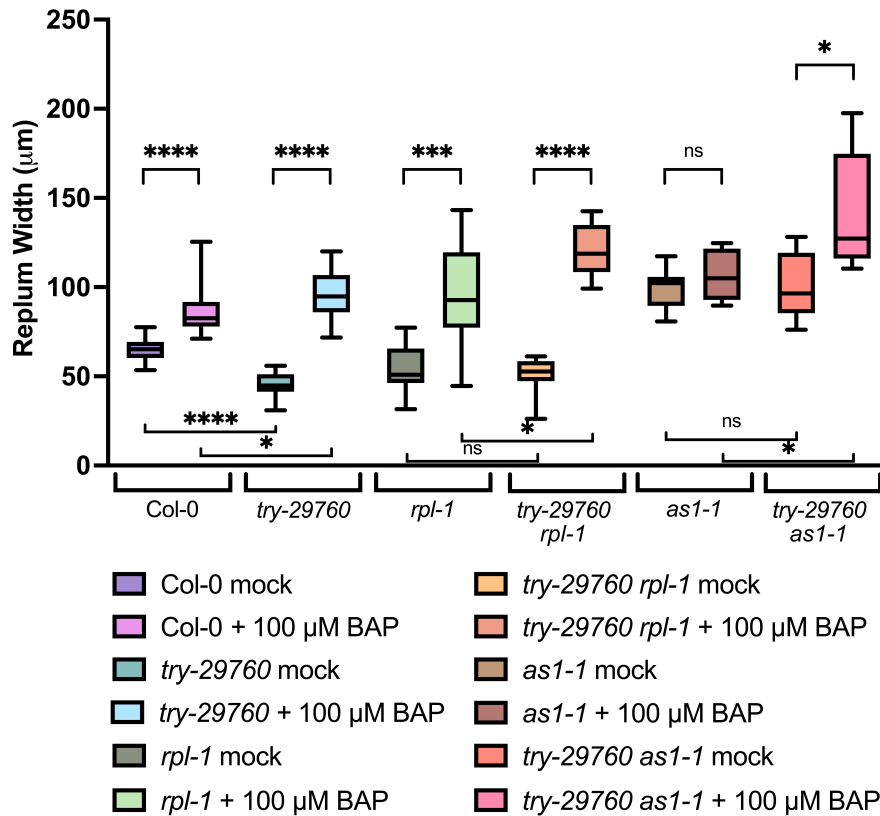


Figure 3.23: The effect of BAP treatment on single and double mutants involving known replum regulators.

Results of replum width measurements in different mutant backgrounds (as indicated) after treatment with a mock solution or a 100 μM BAP solution. Twenty siliques were analysed per sample. Whiskers outside the box of each data point show the 5th to 95th percentile range of the data sets. Statistical significance determined by Welch's *t*-test. "ns": not significant, $P \geq 0.05$; "***": $0.0332 < P < 0.05$; "****": $0.0002 < P < 0.002$; "*****" $P < 0.0001$.

narrow replum phenotype, similar to *try-29760*. However, the *rpl-1* single mutant when BAP-treated displays a similar replum width to the wild type after BAP treatment. When *rpl-1* and *try-29760* are combined, there is a slight decrease in the replum width, although not significant. When this double mutant is treated with BAP, the increase in replum width is significantly increased, compared to the *rpl-1* single mutant (Figure 3.23). The response to BAP is similar to that seen in *try-29760* single mutants. This suggests that *TRY* may in fact act upstream of *RPL* in directing replum development, as if *TRY* was downstream of *RPL*, we would expect to see a similar BAP response in both *rpl-1* and *rpl-1*

try-29760.

Furthermore, the *as1-1* mutation appears to completely suppress the narrow replum phenotype of *try-29760* in the absence of exogenous BAP as the replum width of *as1-1 try-29760* is increased relative to the wild-type and is in fact similar to that of *as1-1* single mutants (**Figure 3.23**). The response to BAP is slightly increased in the *as1-1 try-29760* double mutant compared to the *as1-1* single mutant, but this is far less striking than the result in *rpl-1 try-29760* and may simply be due to sample variance. Thus, *AS1* may be epistatic to *TRY* due to the single *as1-1* mutant phenotype and double *as1-1 try-29760* mutant phenotype being highly similar.

3.2.10 Response of regulators of CK signalling and replum formation to *TRY* perturbation

The results discussed above strongly suggest that *TRY* controls replum development in a CK-dependent manner. To better understand the role of *TRY* in replum formation, I therefore monitored, using RT-qPCRs, the expression of genes that are either involved in CK response or are known regulators of replum development (see Section 3.1). For these experiments, I collected gynoecia at four different floral stages from Col-0 wild type and *try-29760* single mutant plants, as well as from the pTRY::TRYg *try-29760* and p35S::TRYg *try-29760* complementation and overexpression lines, and extracted total RNA for analysis. This would test the effectiveness of the transgenic lines generated as well as testing the effect on the expression of selected genes under normal *TRY* function, loss of *TRY* and excessive *TRY* expression. The stages of the siliques chosen included two (stages 10 and 12) prior to fertilisation and two (stages 14 and 16) post-fertilisation (Smyth et al., 1990).

The purpose of these experiments was to attempt to address the question of how *TRY* and CK may be influencing one another within the replum. It may also address the question of if *try* mutants have narrow repla due to a lack of

CK, is this being mediated through decreased CK synthesis or increased CK degradation. Additionally, the experiments aimed to elucidate any effects the presence or absence of TRY may have on genes known to regulate replum development.

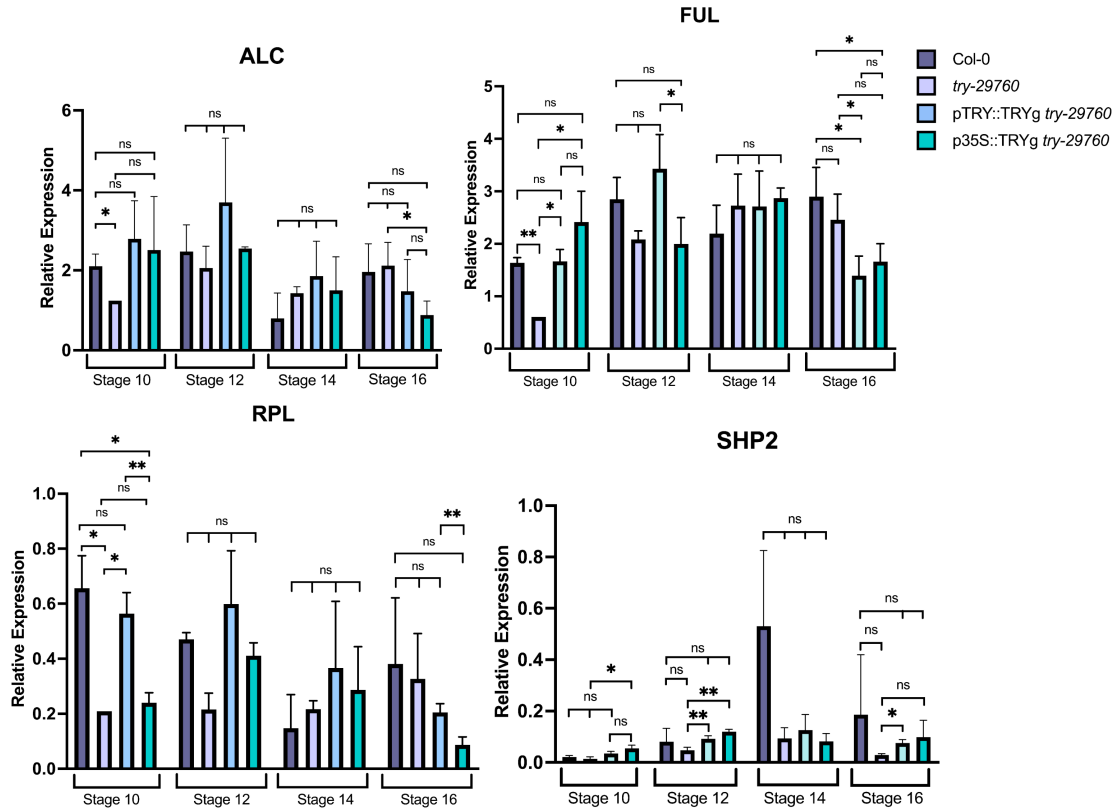


Figure 3.24: Characterisation of the response of replum regulators *ALC*, *FUL*, *RPL* and *SHP2* to the lines generated in this work.

RT-qPCR results of mRNA levels of replum-related genes (labelled) in the gynoecium of Col-0, *try-29760*, *pTRY::TRYg try-29760* and *p35S::TRYg try-29760* genotypes at stages 10, 12, 14 and 16 (Smyth et al., 1990). Values are relative to the reference gene *REF1* (At1G13320). Values are the mean of three (or two for *try-29760* stage 10) biological replicates for each genotype. Two technical replicates were performed for each sample. Error bars represent the standard deviation of the replicates. Statistical significance determined by Welch's *t*-test. "ns": $P \geq 0.05$; "*": $0.0332 < P < 0.05$; "***": $0.0021 < P < 0.0332$.

For the four regulators of replum formation tested in these experiments (i.e. *ALC*, *FUL*, *RPL* and *SHP2*), significant transcript changes between the wild type and *try-29760* were primarily observed in siliques prior to fertilisation (**Figure**

3.24). This was most pronounced for *FUL* and *RPL*, which were reduced in expression in *try-29760*. This occurred to some extent in all four genes in the pre-fertilisation stages, but was most consistent in these two genes. In the case of *RPL*, the complementation lines did not show a consistent restoration of expression to wild-type levels as would be expected based on phenotypic rescue seen in these lines (see Section 3.2.4 above). Thus, it appears from the results of these experiments that at least *RPL* and *FUL* expression are controlled by *TRY* activity to some extent. The main effect of *TRY* on the expression of these genes is seen pre-fertilisation, when these tissues are emerging (**Figure 3.24**).

Similar to the regulators of replum formation, for the three regulators of CK response tested (i.e. *CKX6*, *IPT8*, and *SPL9*), transcript alterations between the wild type and *try-29760* were primarily observed in siliques prior to fertilisation (**Figure 3.25**). Arguably the most pronounced difference detected in this experiment came from the analysis of *CKX6*, which seems to be repressed in the *try-29760* mutant. This was unexpected as a reduction of *CKX6* expression in *try-29760* should lead to an increase in CK, which not in agreement with the observed replum phenotype in *try-29760* and the fact that it can be rescued by exogenous BAP treatment (**Figure 3.17**). *IPT8* is expressed at such low levels in these samples that no meaningful analyses could be performed. *SPL9* expression increases with the presence of *TRY*, similarly to *CKX6*.

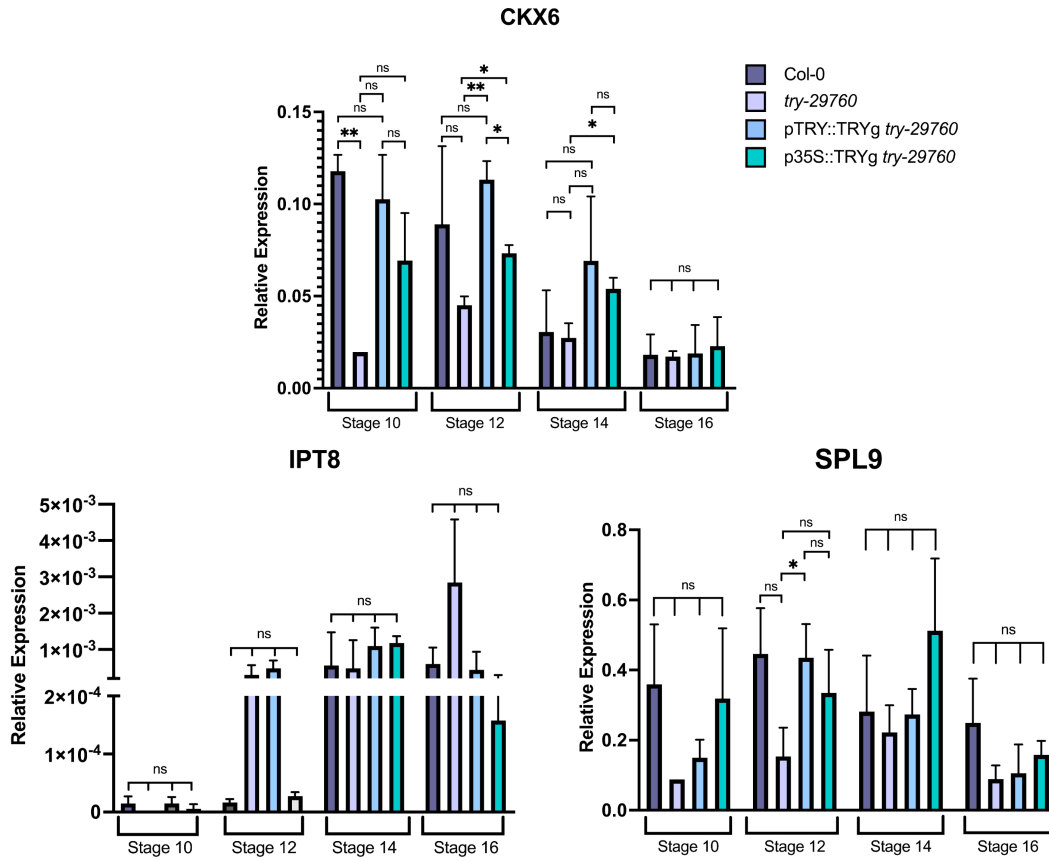


Figure 3.25: Characterisation of the response of CK regulators to the lines generated in this work.

RT-qPCR results of mRNA levels of CK-related genes (labelled) in the gynoecium of Col-0, *try-29760*, pTRY::TRYg *try-29760* and p35S::TRYg *try-29760* genotypes at stages 10, 12, 14 and 16 (Smyth et al., 1990). Values are relative to the reference gene *REF1* (AT1G13320). Values shown are the mean of three (or two for *try-29760* stage 10) biological replicates for each genotype. Two technical replicates were performed for each sample. Error bars represent the standard deviation of the replicates. Statistical significance determined by Welch's *t*-test. "ns": $P \geq 0.05$; "**": $0.0332 < P < 0.05$; "***": $0.0021 < P < 0.0332$.

3.3 Discussion

While *TRY* has been studied for many years for its role as a trichome repressor, its functions in gynoecium development are not yet fully understood. Previous research in our laboratory showed that it may play a role in the formation of the replum and the work outlined in this Chapter sought to verify this observation. Then, I sought to further investigate *TRY* function during gynoecium development, and the stages of flower and fruit development at which this takes place.

In a first line of experiments, I investigated the possible replum phenotype of *try-29760* mutants. This was based on the observation that *try-29760* had a narrower replum in comparison to wild-type siliques, and that the *try* mutant replum appeared to be hypersensitive to exogenous CK application. Through developing a protocol for reproducibly measuring replum width without the need for advanced microscopy techniques, I was indeed able to show that the replum of *try-29760* plants are significantly narrower than that of the wild type. Because this observation was initially limited to a single *try* mutant allele and in principle, could have been a consequence of second-site mutations in its background, I generated two novel *try* mutant alleles through the use of CRISPR/Cas9 genome editing. When I analysed these novel alleles, I found that they had replum narrowness that closely resembled that of *try-29760*. To further show that the replum phenotype is due to a disruption in *TRY* function, I generated two different complementation lines in which the replum phenotype of *try-29760* was rescued. Together with the results of confocal imaging, showing *TRY* protein in the medial domain of gynoecia, these data together confirm the idea that *TRY* is a regulator of replum development.

While *CPC* acts with *TRY* in a partially redundant manner in the suppression of trichome formation in leaves and the gynoecium, the results of my work suggest that *CPC* may not play any role in the control of replum development. In fact, my data even suggest that *CPC* may antagonise *TRY* in the replum to

some extent. Although CPC is shown to localise to the medial domain in a similar manner to TRY, this could be in the context of trichome repression. Likewise, when I assessed replum formation in the *gl1* mutant, which is affected in the main activator of trichome formation, it appeared to be unaffected. From these results it seems unlikely that other core components of the regulatory mechanism for the control of trichome formation, besides TRY, have been co-opted for replum formation during the evolution of flowers. This suggests that this secondary function of TRY in repla may have evolved independently of the other components of trichome formation.

Similarly to TRY, CK has also been previously implicated in the regulation of trichome initiation and replum formation. Because previous work from our laboratory showed that *try* mutant plants show hypersensitivity to CK treatment in trichome formation, I investigated the possible link between TRY and the phytohormone. I found that exogenous CK treatments restored the replum defects in *try* mutants and even that the repla are wider than in the BAP-treated wild type, suggesting some degree of hypersensitivity.

To further investigate this, I focused on genes known to be involved in replum and CK regulation and to determine if any of these genes showed evidence of being affected by the perturbation of TRY expression. These experiments allowed for the identification of genes of interest, which appear to be impacted by TRY expression, and that would be candidates for further studies. It would also further inform the relationship between CK and TRY in replum development, as from the data thus far the role of TRY in the replum appeared to be CK-dependent.

I began by choosing genes which have been previously established to function in the mediolateral patterning of the gynoecium, and probing whether they may display evidence of interacting with TRY in any way. Then, based on my results which showed that a *try* mutation may dysregulate - in some way - the ability of the medial domain to regulate CK signalling effectively, I sought to

investigate the impact of *TRY* perturbations on genes responsible for both CK synthesis and degradation. These experiments demonstrated that it is possible that CK degradation is somewhat impeded in a *try* mutant. Through generating double mutants of *try* combined with other mutants which display defective replum phenotypes, I was able to show that there is evidence of interactions involving *TRY* and replum regulators, namely *AS1* and *RPL*. This indicated that *TRY* is an integral component of the gene regulatory network controlling the patterning of the mediolateral axis of the gynoecium. Thus, I attempted to deduce how these phenotypes and expression patterns place *TRY* within the context of this regulatory network.

Through the work in this Chapter, I have identified genes which *TRY* interacts with during replum development. I have also been able to show how *TRY* expression impacts some of these genes differently at different stages, such as when auxin efflux at the replum occurs and medial tissues are differentiated. This provides novel insights into the regulation of replum development, as well as proposing a novel function for *TRY*.

3.3.1 Proposed model of the mechanism of *TRY* in the gynoecium

The model of fruit development in *Arabidopsis* is based upon the concept of an antagonism of the medial and lateral patterning factors, i.e. the antagonism between replum factors *BP* and *STM* and the upstream regulators of the valve and valve margin tissues, *JAG* and *FIL* (González-Reig et al., 2012). In a *bp* mutant, the replum is extremely narrow and underdeveloped, whereas in *jag fil* double mutants the replum overproliferates, as the replum identity genes are not being repressed to the same extent as in the wild type (Alonso-Cantabrana et al., 2007). The model of the mediolateral axis definition is also informed by the hormonal interplay that occurs throughout gynoecium development, as previously discussed in Section 3.1.3. Before the CMM is defined, CK drives the formation

of the valves, which are then being differentiated in the presence of auxin, while CK is active in the CMM as the replum is defined, up to stage 12 (see Section 3.1.3) (Smyth et al., 1990). This suggests an antagonism between these two processes.

The purpose of the experiments conducted in this Chapter was to uncover the manner in which *TRY* may be participating in these processes, and whether the effect of *TRY* expression on medial domain regulation would be altered in other mutant backgrounds affecting replum development.

In an effort to investigate the role that *TRY* is involved in as the replum is developing, I generated multiple lines altering *TRY* expression (see Section 3.2.4). Although in the rescue line, *TRY* mRNA levels were not restored to wild-type quantities (**Figure 3.11**), a phenotypic evaluation showed that the trichome and replum phenotypes of *try-29760* is rescued in this line with replum width being similar to that in the wild type (**Figure 3.8 (B)**, **Figure 3.10**). The *TRY* over-expression line was clearly active, as it substantially increased the transcription level of *TRY*, completely suppressed trichome formation on all plant surfaces and displayed an increased replum width (**Figure 3.9 (B)**, **Figure 3.10**).

As outlined above, from the investigation of mutant lines with defective repla, it appears that *TRY* interacts with *AS1* and *RPL*. This is seen by examining the phenotypes of the double mutants generated (**Figure 3.23**). The interaction with *AS1* is particularly interesting in this context, as it seems to be epistatic where the *try* mutant phenotype is completely suppressed by the *as1* phenotype. Thus, *TRY* may act upstream of *AS1* and may be involved in its repression. A method of testing this hypothesis would be to conduct RT-qPCRs to determine *AS1* expression in the lines with perturbed *TRY* expression, which were used in this Chapter for RT-qPCRs. Also, RT-qPCRs could be conducted to determine *TRY* expression in the wild type, *as1* single mutant as well as the *as1 try* double mutant.

As mentioned above, *RPL* positively regulates replum development, while

AS1 represses *RPL* and itself drives valve development. *TRY* appears to interact in some manner with both of these regulators of mediolateral patterning, which are acting antagonistically to each other. This poses further questions as to the mechanism by which *TRY* is activated or repressed in the replum. Without conducting further experiments, it is unknown whether *TRY* may act directly on *RPL*, or if *TRY* may be acting further upstream, regulating *BP* or *STM*, for example (**Figure 3.26**). Moreover, *RPL* has been shown to repress *SHP1/2* in order to restrict the domain of the valve margin, but these data suggest that *TRY* may be acting against that and activating valve margin development, as *SHP2* expression increases when *TRY* is present (**Figure 3.26**) (Roeder et al., 2003).

The RT-qPCR data show significant alterations in *RPL* expression at stage 10 (Smyth et al., 1990). This is when the septum has been defined and the replum begins to emerge. The data suggest that when *TRY* is functional, *RPL* levels increase (**Figure 3.24**). *RPL* expression in the wild type gradually decreases as the flower approaches fertilisation. However, in stage 10, *RPL* expression in *p35S::TRYg try-29760* is similar to the *try-29760* mutant (Smyth et al., 1990). This counters what the other genotypes suggest. Given that both *FUL* and *SHP2* are somewhat upregulated in this sample, although not significantly, perhaps this effect is sufficient to downregulate *RPL* in an indirect manner. Both *FUL* and *SHP2* are known to suppress replum formation, as discussed above.

The RT-qPCR data also show that, in the early stages of replum development (stages 10-12), *ALC* levels are positively correlated with the presence of *TRY* (**Figure 3.24**) (Smyth et al., 1990). In later stages, when the replum is differentiating (stages 14-16), the converse is true (Smyth et al., 1990). This could possibly demonstrate that *TRY* is restricting *ALC* expression to the appropriate stages when it is present through an unidentified mechanism. Thus, when *TRY* expression is absent *ALC* expression may continue into later-stage gynoecium tissue.

When examining the effect of *TRY* perturbation on CK signalling-related

genes, there is a trend in the data wherein *SPL9* expression is positively correlated with *TRY* expression, although not statistically significant. *SPL9* drives meristematic activity and cell elongation and is associated with CK signalling (Wang et al., 2021). The expression of *IPT8*, which is involved in CK synthesis, is largely unaffected in all genotypes. However, across all samples, the level of expression was incredibly low which made any meaningful analyses impossible. Although further analyses need to be completed, the expression patterns observed for *SPL9* and *IPT8* here suggest that *TRY* function is associated with CK signalling rather than CK synthesis during replum development (**Figure 3.25**). Consequently, whether this is specific to CK perception, response, and/or transport will also need to be investigated.

The positive correlation with *TRY* seen in *SPL9* is also true for *CKX6*, especially at earlier stages of development. When *TRY* is present, more *CKX6* is present. This was a surprising result as it would suggest that more CK is being broken down. This could be due to an increased level of CK in these lines. When applying this information to the *try* mutant, this shows downregulation of *CKX6*. This implies that less CK is being broken down. Thus, it could be suggested that the impact of the *try* mutation on replum width could be that either CK synthesis is being impeded and there is less CK present to degrade, or that *CKX6* is being directly impacted and CK degradation is altered. Due to the BAP-treated phenotype and the knowledge that CK is vital for normal replum development, it is more logical to suggest that in the *try* mutant CK synthesis or signalling is interrupted, leading to there being less CK present in the first place. In all, these data suggest that *TRY* is possibly involved in fine-tuning CK levels during replum development by mediating *CKX6* activity. Whether *TRY* regulates *CKX6* directly or indirectly has yet to be clarified. To further elucidate the relationship between *TRY* and *CKX6* as well as other CK signalling factors, it would be important to assess the phenotype of the *ckx6-1 try-29760* double mutant. This may provide further insight into the effect that a loss of *TRY* as well

as increased endogenous CK levels may have on replum width, and its response to exogenous BAP. The double mutant was generated during this work, but was not isolated from the F₂ population with sufficient time to conduct experiments on. As shown in the GFP images (**Figure 3.4**), TRY is strongly localised specifically at the valve margin around stage 13, which is also when CK is localised to this same region (Marsch-Martínez et al., 2012; Smyth et al., 1990).

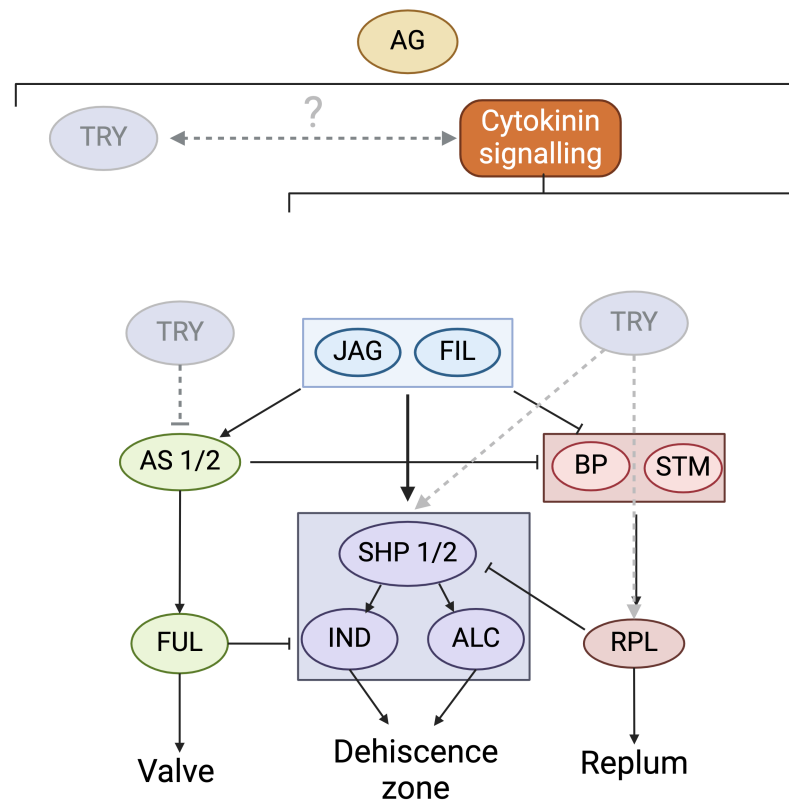


Figure 3.26: A proposed model of TRY interacting with gynoecium development genes.

Based on the existing model of gynoecium patterning from the literature previously shown in Figure 1.6 (with AG at the top as a master regulator) with TRY displaying potential interactions with dashed lines, based on the data obtained from this work. TRY is displayed here as being upstream of RPL, however it is not known if this is up- or downstream of BP/STM activity. The nature of the connection between TRY and CK signalling remains unclear, which is depicted by "?". Figure created with Biorender.com.

3.3.2 The localisation of TRY to the valve margin

As shown in **Figure 3.4**, there is a strong localisation signal of TRY to the medial domain. In the literature, when replum width is being analysed, it is usually done using SEM images (Alonso-Cantabrana et al., 2007; González-Reig et al., 2012; Marsch-Martínez et al., 2014). Additionally, the replum width is rarely quantified, and instead SEM images comparing the wild type to various mutant lines are presented together to demonstrate differences in replum sizes. However, this does not allow for any statistical analyses. To address this, as outlined in Section 3.2.2, a reliable and reproducible method had to be established due to the lack of known protocols. When measurements are conducted in the literature, they tend to encompass the valve margin (**Figure 1.6**) i.e. the measurements span from valve to valve (Marsch-Martínez et al., 2014). One reason for this may be the scale of the measurements, and the fact that it is very technically difficult to differentiate the replum and valve margin accurately and reproducibly. Given the measurements being taken are typically below 100 μm , even minor inaccuracies could impact the statistical power of these experiments. For these reasons, in the preceding sections, the measurements presented were taken from the edge of each valve, in line with the literature.

However, according to **Figure 3.4**, there appears to be more localisation of TRY at the valve margin than the replum itself (**Figures 1.6, 3.4**). I wanted to compare the replum width to the width of the entire medial domain and to also compare these widths in response to BAP treatment (**Figure 3.27**). This was done to establish whether the increase in BAP sensitivity observed in the *try* mutant causes the medial domain width to increase in the replum or if it concentrated to the valve margin.

When measuring the replum tissue only, the mean width in the Col-0 wild type is approximately 24 μm ($n = 9$), compared to almost 65 μm when the valve margin is included in the measurement ($n = 20$). In *try*-29760, the replum alone

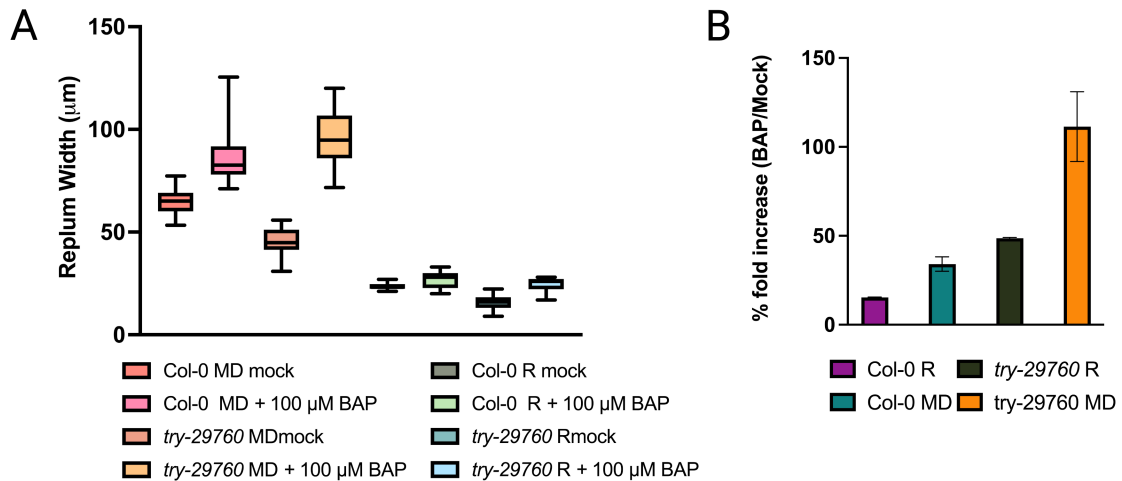


Figure 3.27: Comparing measurements taken of the replum and the medial domain.

As described in the text above, measurements were taken of replum tissue only “R”, or the entire medial domain (from valve to valve) “MD”. For the replum, $n = 9$, while for the medial domain, $n = 20$. **(A)** Box and Whisker plots showing the widths of mock-treated and BAP-treated samples of Col-0 and *try-29760* genotypes. Whiskers illustrate the 5th and 95th percentiles of the data set. **(B)** The percentage increase in width due to BAP treatment, as calculated by mean BAP divided by mean mock. Error bars indicate 95% confidence interval.

measures approximately 14 μm ($n = 9$), which increases to just over 45 μm when measuring the valve margin also ($n = 20$). By examining these mean measurements, while the replum is 42% narrower in *try-29760* than in Col-0, the valve margin is 25% narrower. Upon BAP treatment, the replum of Col-0 increases to approximately 30 μm, which is only a 20% increase compared to the mock treatment. Including the valve margin, BAP-treated Col-0 medial domains measure just over 87 μm. The valve margin width increased by almost 22%. BAP-treated *try-29760* has a replum width similar to mock-treated Col-0, about 25 μm. The replum width of *try-29760* increases by about 44% with BAP treatment. With the valve margin included, the medial domain of *try-29760* BAP-treated siliques measures over 96 μm. The valve margin width increases by over 56%. This suggests that while the replum of *try-29760* is more sensitive to BAP than the wild type, this effect is more pronounced within the valve margin, where TRY is localising (**Figure 3.4**).

Taken together, these data and the other experimental data presented in this Chapter demonstrate that *TRY* is likely involved in the development of multiple (if not all) abaxial gynoecium tissues (**Figure 3.26**). One possible explanation of this is *TRY* regulating the CK signalling pathway which, as discussed in Section 3.1, is key for the orchestration of the structure of the gynoecium throughout various stages. As suggested by the RT-qPCR data in **Figure 3.25**, the impact of *TRY* expression varies throughout replum development.

Additional untangling of the genetic mechanisms of replum development would be possible through similar experimentation being conducted on *JAG* and *FIL*, as they are upstream regulators of these three domains (**Figure 1.6**). If *TRY* is affecting or being affected in some way by *JAG* and/or *FIL* function, this may help to explain the apparent role of *TRY* in multiple gynoecium tissues (**Figure 3.26**). The one aspect of this model which would need to be addressed through examining *JAG* and *FIL* is that they are positive regulators of valve development, through activation of *AS1/2*. The data I have presented in this Chapter suggest that, in fact, *TRY* may act competitively with *JAG* and *FIL* to repress *AS1/2* (**Figure 3.23**). *JAG* and *FIL* negatively regulate replum patterning by suppressing *BP* and *STM*. Furthermore, my data suggest that *TRY* may in fact be positively regulating *RPL*, which acts downstream of *BP/STM* to develop the replum (**Figure 1.6**). Alternatively, as stated above, *TRY* could be regulating *BP* or *STM* which in turn affects *RPL* expression.

All together, the data accumulated thus far suggest that *TRY* may be acting in a converse manner across different tissue types (**Figure 3.26**). This may be, in part, explainable by the role of *TRY* being directly involved in CK signalling rather than having any direct interaction with these aforementioned genes. However, *TRY* has been shown to suppress its own expression, which may also need to be considered in this model (Tominaga and Wada, 2017). Again, characterisation of the effect of *TRY* expression on the function of *JAG* and *FIL* is paramount to gaining further insight into the mechanism of these interactions. Addition-

ally, while a relationship between *TRY* and *AS1* has been established through examination of the double mutant, the expression of *AS1* in response to *TRY* expression perturbation has not been determined. Furthermore, to understand the epistatic relationship of these genes, expression characterisation experiments or protein interaction methods (such as yeast two-hybrid,) in Col-0, *try-29760*, *as1-1* and *as1-1 try-29760* may illuminate the manner in which these two genes are impacting the patterning of the gynoecium.

When we consider the valve margin response to BAP of *try-29760* as well as the RT-qPCR data for *CKX6* presented here (**Figure 3.25**), there is also a case to be made for the possibility of CK degradation being dysregulated in the absence of *TRY*. The sensitivity to BAP in *try* loss of function mutants may be, in part, due to a lack of CK degradation. It has been demonstrated before that, as tissues age in *Arabidopsis* and less CK is present, *CKX* activity tends to decrease in response to the lack of CK in the environment (Werner et al., 2001). Perhaps this is the case to some extent in *try* mutants, where there may be reduced CK in this genotype, and as a result the *CKX* genes may not be functioning fully in these tissues. This makes sense when we also consider the downregulation of *SPL9* in the *try* mutant, which is indicative of downregulated meristematic activity in the gynoecium.

3.4 Future Work

Additional imaging of the TRY-GFP and CPC-GFP lines at multiple stages would be insightful in establishing how TRY is transported throughout gynoecium development, as well as determining stages where TRY is most abundant. This work is currently underway. Some other mutants of known trichome regulators should additionally be subjected to replum measurements and BAP treatment, to validate whether *TRY* is the only identified trichome gene involved in the replum.

More double mutants should be generated, especially in combination with a number of the candidate genes discussed throughout this Chapter, namely *ALC*, *SPL9* and *SHP1/2*. Additionally, characterisation of the expression of *BP* and *STM* in the lines used in Section 3.2.10 as well as the generation of a triple mutant of *try* and these two genes would allow the elucidation of the nature of the interaction between *TRY* and *RPL*. These experiments could assist in determining whether *TRY* may be directly regulating *RPL* and if *BP* and *STM* have an impact of *TRY* function. Another crucial set of experiments would be to generate *jag try* and *fil try* double mutants, as well as a *jag fil try* triple mutant given the redundancy of *JAG* and *FIL* in restricting the proliferation of the replum. *JAG* and *FIL* expression in response to perturbations of *TRY* expression should also be characterise. Taken together, these experiments will provide valuable insight into the role of *TRY* in replum development.

Of particular interest to this work would be the BAP treatment of a *ckx6-1 try-29760* double mutant, which as previously mentioned was generated but not subjected to treatment or measurement. This should allow for the observation of the replum in a background where *TRY* function is lost, but the endogenous level of CK is increased. This could also be completed in a CK synthesis mutant background to conduct the same experiments in a line where endogenous CK levels are decreased.

A CK quantification assay would be valuable to this study, if conducted in the lines used for the RT-qPCR experiments. Alternatively, a CK fluorescent tag line (such as TCS::GFP) could be introduced to the four lines used in the RT-qPCR experiments. This would demonstrate whether the localisation of CK within the gynoecium correlates with TRY localisation, or indeed if one precedes the other. Through examining both the TRY::GFP and TCS::GFP lines at multiple stages, it would be possible to determine the relationship between CK localisation and TRY localisation and the stages at which these events occur.

A central question that this work could not address is how *TRY* was co-opted into the regulatory network for replum development during evolution. Possibly, this happened through changes in the regulatory region of the gene, which put it under control of replum or CK regulators. It would therefore be interesting to understand which TFs bind to *TRY*, through Chromatin Immunoprecipitation (ChIP) in the medial domain and whether this occurs via *cis*-regulatory elements with gynoecium-specific activity.

4 Identification and characterisation of the homologs of *SHATTERPR- OOF1/2* in *Brassica rapa*

4.1 Introduction

In Section 1.6, I have outlined the main events of fruit development, primarily in *Arabidopsis*. As mentioned there, the dehiscence zone (DZ) is crucial to the dispersal of seeds and thus the propagation of the following generation of plants. In this Chapter, I will describe in deeper detail the orchestration of the DZ, with specific reference to the roles of *SHATTERPROOF1/2* in this process. Moreover, this information will be translated to *Brassica rapa* and its fruit development processes through the identification and characterisation of the counterparts of the *SHATTERPROOF* genes in this species.

4.1.1 The function of the *SHP* genes in *Arabidopsis*

There are two *SHATTERPROOF* (*SHP*) genes in *Arabidopsis*, namely *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF2* (*SHP2*), which encode MADS-domain transcription factors (Ma et al., 1991; Savidge et al., 1995; Liljegren et al., 2000). These genes are expressed in the valve margin, which is the region where the fruit (silique) eventually opens to release the mature seeds (Liljegren et al., 2000). The main role of *SHP1/2* in *Arabidopsis* is to define the lignified layer and the de-

hiscence zone (DZ), which form the border between the replum and the carpel valves of the silique (**Figure 1.5**). This can be demonstrated by the pattern of expression of *SHATTERPROOF1/2*, as seen in **Figure 4.1**. This occurs in the later stages of fruit development, to enable dehiscence of the silique to allow for seed dispersal (Liljegren et al., 2000).

When the fruit is fully mature and begins to dry out in *Arabidopsis*, the lignified layer degrades which allows for the separation of the carpel valves from the medial domain so that seeds can be dispersed by the wind to allow for proliferation of the following generation. In the absence of functional *SHP* genes, the valve margin cells fail to develop properly, and the DZ does not differentiate correctly (Liljegren et al., 2000). As a result, the silique remains closed as the replum remains fused to the valves and seeds are retained within the fruit, which severely affects seed dispersal and propagation of the plant.

The *SHPs* are known to interact with *FUL* and *ALC*, among others, to form a complex regulatory network that controls various aspects of fruit development, including replum formation and dehiscence (**Figure 1.6**) (Ferrándiz et al., 2000; Rajani and Sundaresan, 2001). When initially discovered, *SHP1/2* were proposed to be ovule development regulators and were then classified as D-class flower development genes, which are discussed briefly in Section 1.2 (Flanagan et al., 1996). However, further study of their functions showed that *SHP1/2* were involved in more than ovule development and were in fact key regulators of the DZ (Liljegren et al., 2000). It should also be noted that a *shp1/2* double mutant is still capable of forming healthy, viable seeds (Liljegren et al., 2000).

As previously mentioned in Section 1.4, floral MADS-domain transcription factors are proposed to form tetramers in order to regulate gene expression of downstream effectors to precisely control the spatiotemporal activity of organ identity factors (Melzer and Theissen, 2009; Theissen and Saedler, 2001). It has been previously shown that the *SHP1/2* genes as well as *AG* - also required for correct gynoecium development - can form in different combinations of protein

tetramers in order to regulate different regions of gynoecium development, allowing for the specification of these various tissue types (Espinosa-Soto et al., 2014; Hugouvieux et al., 2018).

While there is an agreement in the literature that SHP1 & 2 are functionally redundant, it has been shown that SHP2 is likely carrying out the majority of the function, as can be visualised in **Figure 4.1**, where the expression domain is less restricted than in *SHP1*. *SHP2* has also been used as the model in characterising the function of these genes, such as performing transcript localisation experiments (Savidge et al., 1995; Liljegren et al., 2000).

4.1.2 The dehiscence zone

As discussed in Section 1.6, the external surface of the two carpels are known as the valves (**Figure 1.5**). They are anchored by a narrow replum which sits in the medial domain. The development of the septum and replum are discussed in Section 3.1. The border of these different tissues is referred to as the valve margin, which post-fertilisation will form the DZ and lignified layer, as orchestrated by the SHATTERPROOF TFs.

FRUITFULL (*FUL*) is a key MADS-domain TF which is expressed in the valves and regulates the medial domain and restricts the expression of *SHP1/2* in the DZ by antagonism (**Figure 1.6**) (Ferrándiz et al., 2000). This ensures that the DZ is restricted in size. Upstream of *FUL* activity, *JAGGED* (*JAG*) and *FILAMENTOUS FLOWER* (*FIL*) are both key players in activating valve margin-related genes to ensure the auxin maxima drive the expression of the relevant genes for differentiation of the above-mentioned tissue types in the mature silique (González-Reig et al., 2012).

There is little meristematic activity or auxin flux seen on the abaxial side of carpels, but this is in contrast to the adaxial side which contains many important tissues for reproduction, such as the placenta and ovules (Girin et al., 2009). This allows for expression of *FUL* and *SHP1/2* in the appropriate domains. In

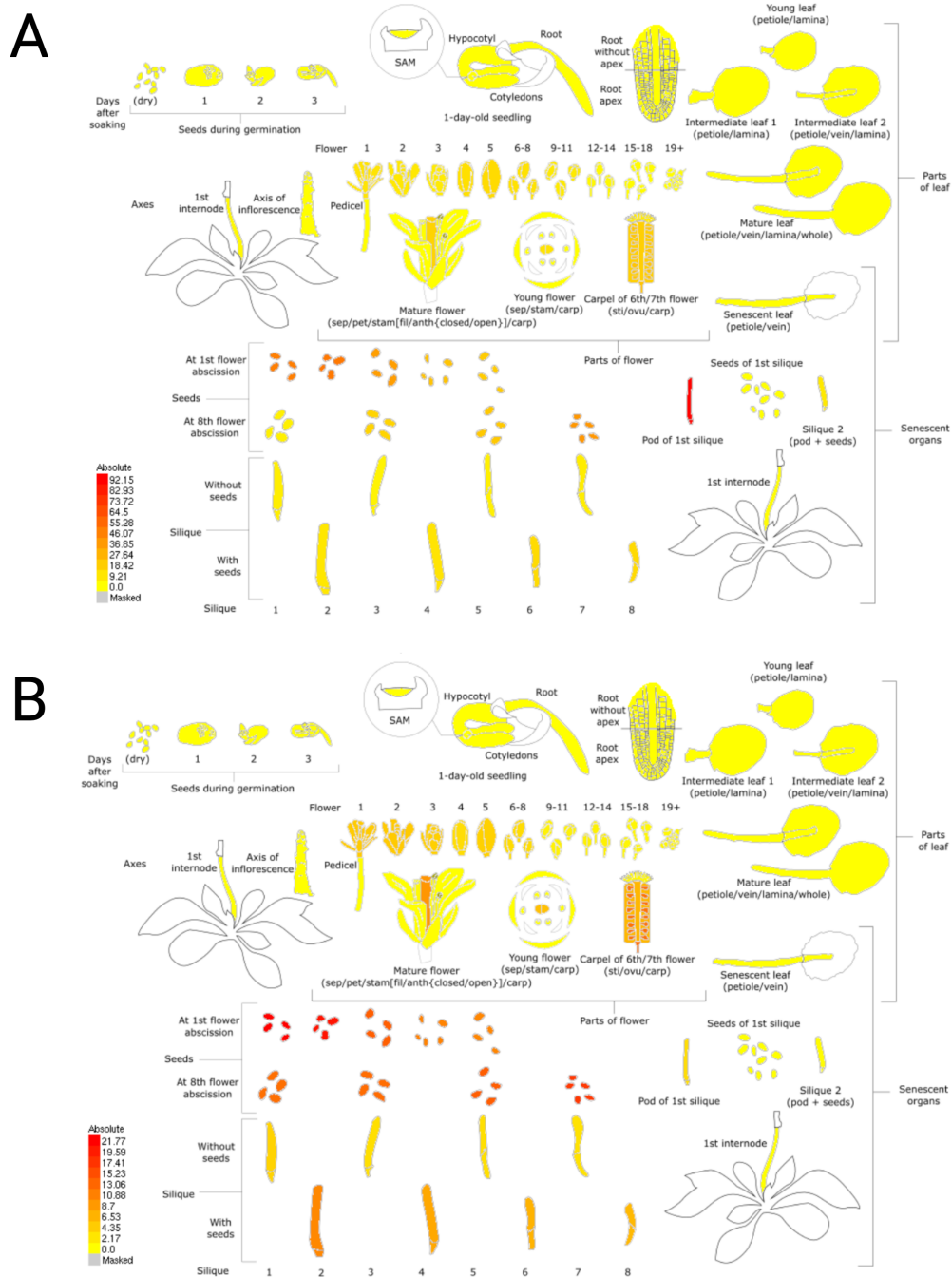


Figure 4.1: The expression domains of the *SHATTERPROOF1* & 2 mRNAs in *Arabidopsis*.

(A) The expression heat map of *SHP1* from the *Arabidopsis* eFP Browser based on RNA-seq data (Klepikova et al., 2016). **(B)** The expression heat map of *SHP2* from the *Arabidopsis* eFP Browser based on RNA-seq data (Klepikova et al., 2016).

a *jag fil* double mutant, the expression domain of *SHP1/2* is only diminished at the gynoecium apex, but largely unaffected at the basal end of the gynoecium, suggesting more robust expression patterns. However, *FUL* expression is largely dysregulated in the *jag fil* background (González-Reig et al., 2012).

4.1.3 Polyploidy within the *Brassica* genus

Arabidopsis is a member of the Brassicaceae family, which are more commonly referred to as the crucifers. The *Brassica* genus is also a member of this family and thus the species are quite closely related. The two genera are proposed to have diverged from each other approximately 29.5 million years ago (Song et al., 2020).

Within the 41 species of the *Brassica* genus, there are 6 which are widely used crop species (IPNI, 2024). Some are autopolyploid diploids, while some are allotetraploid as the result of the hybridisation of two diploid species, as demonstrated in Nagaharu, 1935. This is outlined in **Figure 4.2**. The first allotetraploid species is *B. napus*, which resulted from the hybridisation of *B. rapa* and *B. oleracea*. The second is *B. juncea*, hybridised from *B. rapa* and *B. nigra*. The last allotetraploid species is *B. carinata*, which was hybridised from *B. oleracea* and *B. nigra*. While this model was proposed in 1935, it has since been shown to be accurate (Song et al., 2020).

Polyploidy describes when two or more genomes are combined and fuse within one nucleus (Wendel, 2000). This is an incredibly common event throughout evolution and speciation, and many modern crops and domesticated plants have arisen through the hybridisation of species which results in the formation of a stable polyploid genome. Autopolyploid genomes are the result of a single genome duplicating (i.e. both parent genomes from the same species) (Wolfe, 2001). In the case of most modern *Brassica* species, chromosomes were merged from different parental species, giving rise to allopolyploid genomes (Wolfe, 2001). An example of this is the hybridisation of *B. rapa* and *B. oleracea*, which

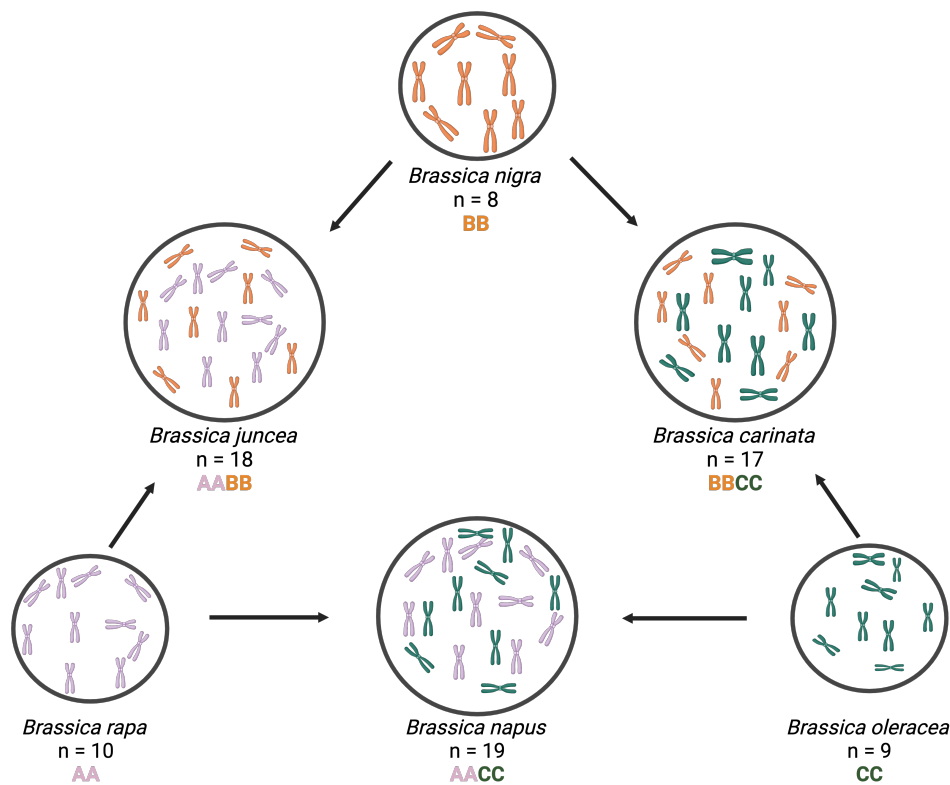


Figure 4.2: The triangle of U. Schematic representing the Triangle of U (Nagaharu, 1935). Shown are the six major species of the genus *Brassica*. The allotetraploid *B. napus* (AACC) is derived from hybridisation of the diploid species *B. rapa* (AA) and *B. oleracea* (CC). *B. juncea* (AABB) is derived from hybridisation of the diploid species *B. rapa* (AA) and *B. nigra* (BB), while *B. carinata* (BBCC) is derived from hybridisation of the diploid species *B. oleracea* (CC) and *B. nigra* (BB). The number of chromosomes for each species is indicated. Figure created with Biorender.com.

gave rise to the speciation of *B. napus*.

When a genome is duplicated in an autopolyploidy event, all genes are duplicated as a result. The additional gene copies usually fall into one of four possible fates. These duplicated gene copies are known as paralogs. Firstly, the additional copy can be lost due to multiple deleterious mutations as it is redundant with its paralog (Clark and Donoghue, 2018). If the paralog is retained and there are two copies of a gene, there can be a dosage alteration, subfunctionalisation or neofunctionalisation events (Birchler and Yang, 2022). This is when the protein function is split between the two (or more) gene copies. In the case of gene dosage, both copies have the same function, but both are required for there

to be sufficient protein translated to carry out its function. One copy may carry more function than the other, so the two paralogs are redundant, but a single copy may be sufficient to obtain almost wild-type levels of protein. It has been found that genes with regulatory functions and those involved in DNA replication are more likely to have their additional copy retained (Blanc and Wolfe, 2004; Jiang et al., 2013). During a subfunctionalisation event, the two paralogs work together to form the parent protein (Birchler and Yang, 2022). There is no redundancy as both copies are required to obtain normal protein function. A neofunctionalisation event is the rarest outcome in polyploidy. In this case, one copy retains the original function of the parent gene, and the second copy is no longer restricted by selection and is free to adapt to a novel function (Panchy et al., 2016).

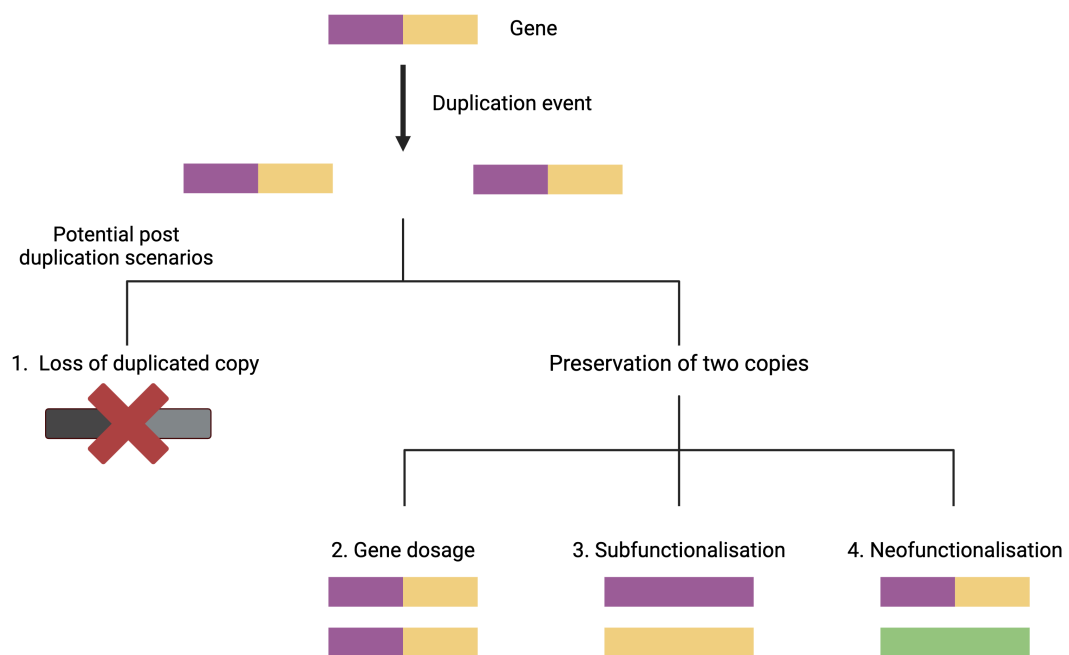


Figure 4.3: The possible fates of duplicated genes. A summary of the possible fates of duplicated genes. 1., The second copy can be lost. 2., In gene dosage both copies are intact and required for full function. 3., The original function of the parental gene is split between the two daughter genes 4., One daughter gene retains the function of the parental gene whilst the other gains a novel function. Figure created with Biorender.com.

4.1.4 Potential of a shatter-resistant phenotype in *Brassic*as

As discussed in Chapter 1, various *Brassica* species are used widely in agriculture, particularly in Europe. *Brassica napus* is commonly bred for seed size and seed oil content so that the seeds can be harvested to produce oil which is widely used in cooking as well as serving as a biofuel. This is commonly referred to as oilseed rape. Some cultivars of *B. rapa* are also bred for use as oilseed rape.

A significant source of yield loss is seed loss due to silique maturation and drying, as well as adverse weather conditions such as strong winds. These can account for approximately 20% of seed yield loss and up to 50%, respectively (Price et al., 1996). This can also have an impact on the propagation of a crop species, where the seeds need to be harvested in order to sow the subsequent generation. If a fused or partially fused silique was capable of preventing unwanted silique shattering, then seed yield could potentially be increased both for propagation of the crop as well as oil production.

One major advantage of isolating shatter-resistant plants via the *SHP* genes, as observed in *Arabidopsis*, is that unlike perturbing the function of other MADS-box TFs involved in flower development (such as *AG*) there is no effect of fertility or viability of the plants even in a double null mutant genotype (Liljegren et al., 2000). This is critical to take into consideration when identifying genes of interest to target in crop species as phenotype which results in stress tolerance or yield increase must not be obtained in lieu of fertility of the plant.

4.1.5 Aims of the chapter

As described above in Section 4.1.1, an *Arabidopsis* double mutant of *shp1/2* results in fused silique phenotype. This requires physical force to be applied to the silique in order for the seeds to be collected or dispersed. In the wild-type, wind or shaking would be sufficient for the silique to dehisce and allow seeds to fall.

The first aim of this work was to undertake a thorough characterisation of any and all homologs of the *SHATTERPROOF* genes in *B. rapa*. This was important both to establish the genes that would need to be targeted for mutagenesis, as well as determining whether the functions of these genes and thus the resulting phenotypes could be directly translated from research in *Arabidopsis*. To achieve this, transgenic methods and gene expression analyses were employed *in vivo*. Additionally, bioinformatic methods and analyses were used *in silico* to identify candidate genes and examine gene and protein structure.

The second aim was to isolate mutant lines of each of these homologs in order to identify the phenotype obtained in *B. rapa* when their function was perturbed. Furthermore, to characterise these phenotypes an additional aim was to conduct a test of shatter resistance. This would allow for the determination of the effect on shatter resistance of each gene perturbation, as well as assessing them in combination with each other to observe any additive effects.

4.2 Results

4.2.1 Identification of homologs of *SHP1/2* in *B. rapa*

To identify homologs of *Arabidopsis SHP1/2* (AT3G58780 and AT2G42830, respectively; referred to as *AtSHP1* and *AtSHP2*, hereafter) in the *B. rapa* genome, I used the Basic Local Alignment Search Tool (BLAST) algorithm from NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the *Brassica* Database (BRAD) (<http://brassicadb.org/brad/>), as well as EnsemblPlants (<http://plants.ensembl.org/>). The gene identifiers of the database hits and their chromosomal locations (**Figure 4.4**) were compared to the results from two previously published datasets on MADS-box genes identified in *B. rapa* (Saha et al., 2015; Duan et al., 2015) and were found to be in agreement with these studies. For *AtSHP1*, three homologous genes were identified, while a single homolog was identified for *AtSHP2* (**Table 4.1**).

Table 4.1: Proposed nomenclature for the *B. rapa* homologs of *Arabidopsis SHP1/2*

Unique gene identifiers from the BRAD database, their chromosomal locations, and the corresponding genes from *Arabidopsis* are shown. Also shown are the names given to the homologs, which are used throughout this chapter and thesis.

BRAD unique identifier	Given name	Chromosome	<i>Arabidopsis</i> homolog
Bra014552	<i>BrSHP1a</i>	A04	<i>AGL1/SHP1</i>
Bra003356	<i>BrSHP1b</i>	A07	<i>AGL1/SHP1</i>
Bra007419	<i>BrSHP1c</i>	A09	<i>AGL1/SHP1</i>
Bra004716	<i>BrSHP2</i>	A05	<i>AGL5/SHP2</i>

To confirm the homology to *AtSHP1/2* of the four *B. rapa* genes identified, their coding (**Figures 4.5 & 4.6**) and amino acid sequences (**Figures 4.7 & 4.8**) were aligned to the *Arabidopsis* genes/proteins, confirming a high degree of similarity between them. This observation was further supported by the results of

pair-wise comparisons of sequence conservation between the homologs (**Tables 4.2 & 4.3**), which was determined using the BLAST algorithm. While on average, homologous genes of *Arabidopsis* and *B. rapa* share around 70% sequence identity (Quiros et al., 2001), in the case of the *SHP* homologs this number was around 90%. Thus, the *SHP* homologs share an above-average level of sequence identity, suggesting that they may not only be homologs but *bona fide* orthologs. Additionally, the fact that all four genes are located on different chromosomes means that the genes are not linked (**Figure 4.4**). This is advantageous for combining mutant lines of these genes, as the mutant alleles for each gene can be crossed with the others without recombination affecting the inheritance of each mutant allele (Bateson et al., 1904).

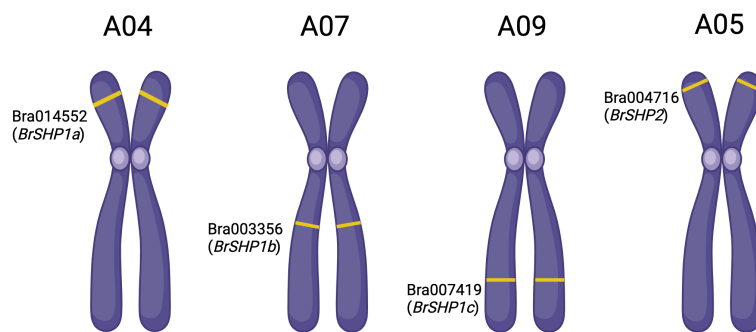


Figure 4.4: Chromosomal locations of the putative *BrSHP* genes.

The approximate positions of the *BrSHP* genes along the corresponding chromosome, indicated by yellow lines. Note all genes are located on different chromosomes, so are unlinked. Figure created using BioRender.com.

A notable difference in sequence was detected for the *AtSHP1* homolog

BrSHP1b (Bra003356), which appears to have an 40 amino acid extension at the N-terminus of the protein (Figure 4.7). While it is unclear what the function of this extension is (if any), it does not disrupt the sequence of the neighbouring MADS domain, which is normally found at the N-terminus of these proteins. Thus, this extension is not likely to affect protein function.



Figure 4.5: Alignment of the CDS of putative *SHP1* homologs identified in *B. rapa*.

Analysis completed using the *Geneious Prime* software. Each base (A, T, G, C) is colour-coded for easier visualisation (red, green, yellow and blue, respectively). When the nucleotides are shaded in colour, this indicates that they are identical across both sequences at this position. The *AtSHP1* CDS sequence was used as a reference sequence.

When members of MADS-box gene family were identified in *B. rapa* inde-

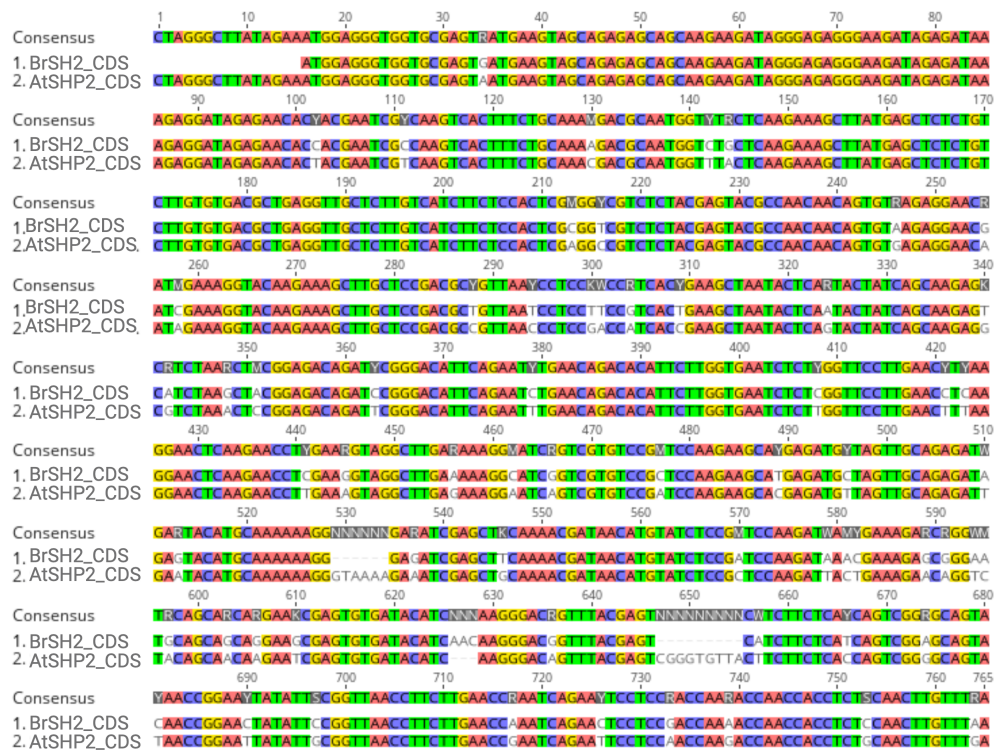


Figure 4.6: Alignment of the CDS of putative *SHP2* homolog identified in *B. rapa*.

Analysis completed using the *Geneious Prime* software. Each base (A, T, G, C) is colour-coded for easier visualisation (red, green, yellow and blue, respectively). When the nucleotides are shaded in colour, this indicates that they are identical across both sequences at this position. The *AtSHP2* CDS sequence was used as a reference sequence.

pendently by two groups (Saha et al., 2015; Duan et al., 2015), the individual genes were named differently, resulting in inconsistencies in nomenclature in the literature. Therefore, for this study, I adopted a simple naming convention for the *SHP* homologs which is outlined in **Table 4.1** to overcome this issue.

I additionally examined and aligned the genomic sequences of the *BrSHP* genes with each other and their *Arabidopsis* homologs (data not shown). This allowed me to assess conservation within introns, which was low. It also allowed me to ensure the annotation of the exons of the genes identified was as accurate as possible, as well as determining the exon/intron structure of the four genes (**Figure 4.9**). In all cases, I observed a large first intron which is also present in the *SHP* genes in *Arabidopsis*. The slightly different exon/intron struc-

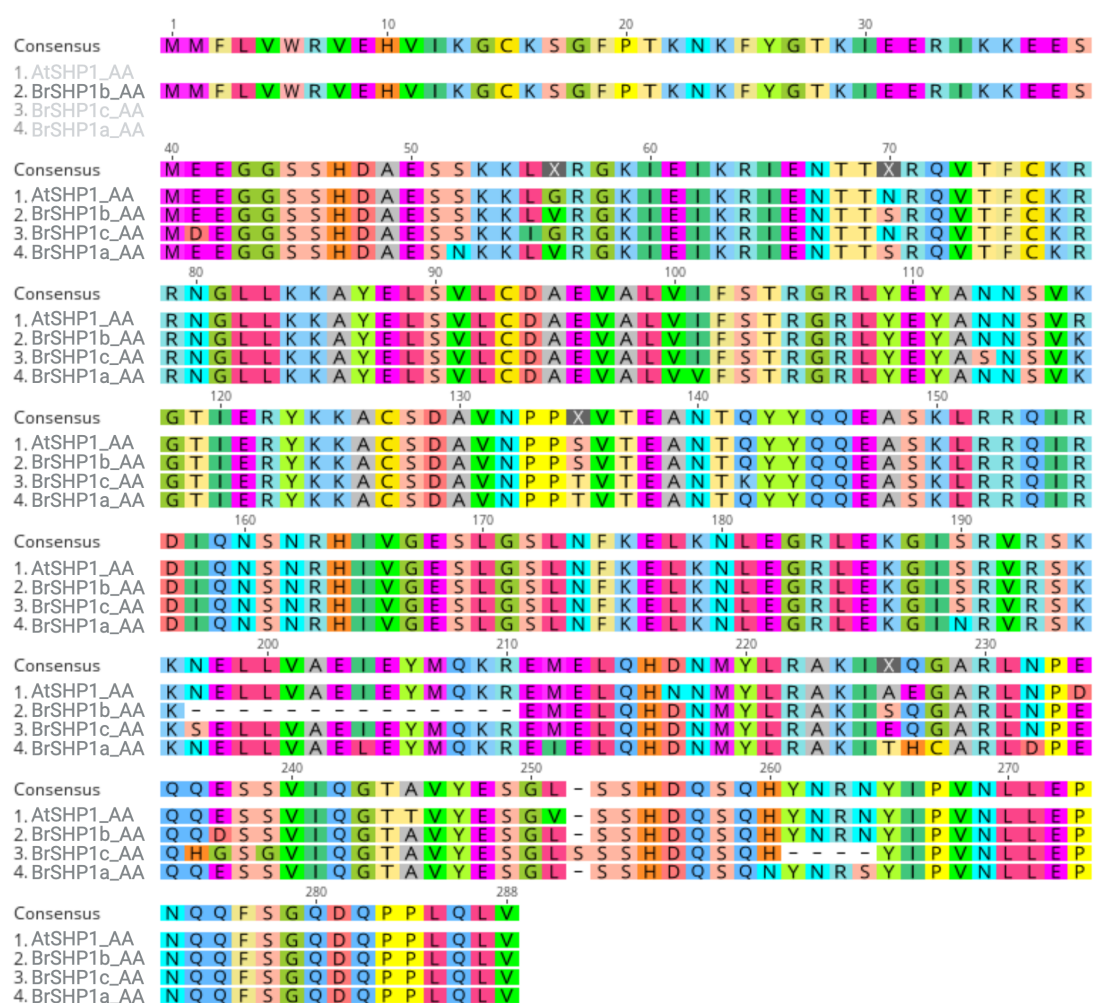


Figure 4.7: Alignment of the amino acid sequences of the putative *SHP1* homologs.

The amino acid sequences of AtSHP1 and the three homologous proteins from *B. rapa* were aligned using the *Geneious Prime* software.

Table 4.2: Sequence conservation across the *SHP1* homologs.

Percentage values for sequence identities between pairs of homologs are shown in the following matrix for coding sequences as well as for amino acid sequences.

	CDS				Amino acid			
	1	2	3	4	1	2	3	4
1. <i>AtSHP 1</i>	100	90.76	94.15	90.40	100	92.34	90.36	91.57
2. <i>BrSHP 1a</i>	90.76	100	91.57	89.73	92.34	100	89.52	89.96
3. <i>BrSHP 1b</i>	94.15	91.57	100	93.71	90.36	89.52	100	87.95
4. <i>BrSHP 1c</i>	90.40	89.73	93.71	100	91.57	89.96	87.95	100



Figure 4.8: Alignment of the amino acid sequence of the putative *SHP2* homolog.

The amino acid sequences of AtSHP2 and the homologous protein from *B. rapa* were aligned using the *Geneious Prime* software.

Table 4.3: Sequence conservation in the *SHP2* homologs.

Percentage values for sequence identities between pairs of homologs are shown in the following matrix for the coding sequences as well as for amino acid sequences.

Percentage identity	
CDS	90.03
AA	92.31

ture of *BrSHP1b* is due to the insertion at the beginning of the transcribed sequence which forms the first exon, but otherwise this gene is also highly similar in structure and sequence to the other *BrSHP* genes as well as *Arabidopsis SHP1/2* (Figure 4.9).

4.2.2 Synteny analysis

To investigate the *SHP* homologs from *B. rapa* further, I examined them for synteny. Synteny refers to the conservation of gene order on segments of a chromosome throughout evolution. Given the high degree of sequence identity between *AtSHP1* or *AtSHP2*, a synteny analysis appeared to be especially important to

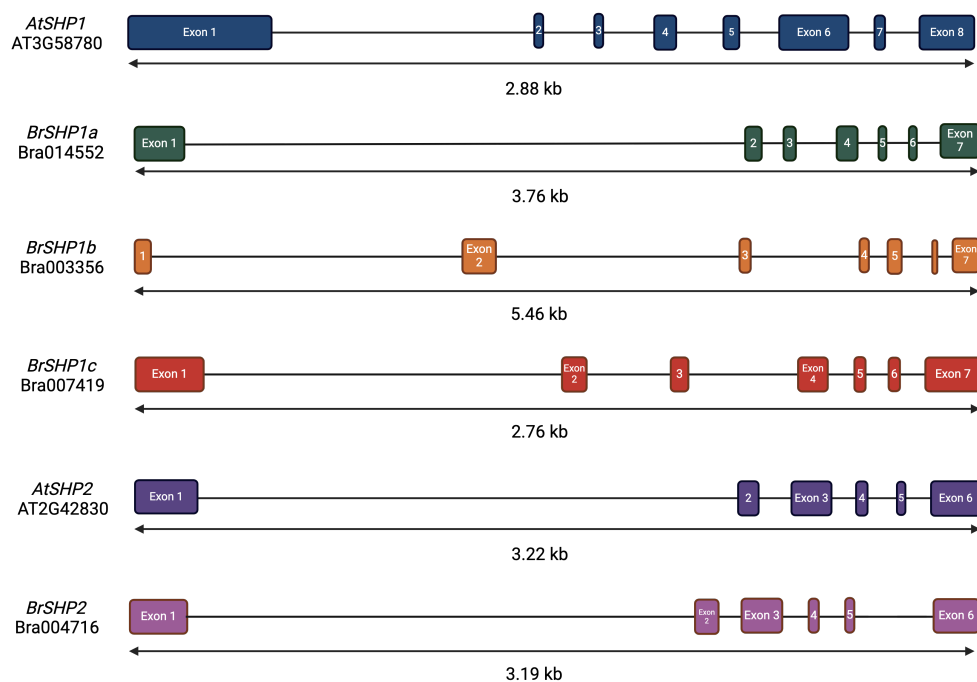


Figure 4.9: Schematic representation of the exon/intron structures of the *Br-SHP* genes.

Schematics to-scale of each of the putative *BrSHP* genes, with the *Arabidopsis* copies at the top as a reference point. Figure created using BioRender.com.

confirm whether a given *B. rapa* homolog was a copy of *AtSHP1* or *AtSHP2*.

For the synteny analysis, the Genome Viewer function on the NCBI Gene library was used to identify genes flanking *AtSHP1* and *AtSHP2*. An equivalent feature is available for *B. rapa* on EnsemblPlants. The genes immediately upstream or downstream of the identified *B. rapa* homologs are shown in **Tables 4.4 & 4.5**. It should be noted that in the case of *AtSHP1*, the genomic region surrounding its locus is less well characterised than in the case of *AtSHP2*. There are also long non-coding RNAs (lncRNAs) and putative mRNAs present in that region, which may or may not be evolutionarily conserved across species, and were therefore omitted from the analysis. Nevertheless, the results of the analysis showed clear evidence of syntenic conservation for all *SHP* genes from *B. rapa* as all had genes in their vicinity that are also neighbouring *AtSHP1* or *At-*

SHP2, respectively. The results further confirmed that the *B. rapa* genes had been correctly assigned as homologs to either *AtSHP1* or *AtSHP2*.

Table 4.4: Synteny analysis for *BrSHP1* homologs.

Uncharacterised proteins and lncRNAs in the region were omitted from analysis. The genes and their positions (upstream (+) or downstream (-)) relative to a given *SHP1* homolog are indicated. Shared genes are highlighted in the same colour.

Position	<i>AtSHP 1</i>	<i>BrSHP 1a</i>	<i>BrSHP 1b</i>	<i>BrSHP 1c</i>
-5	At3G58710	At4G18730	At3G58690	At3G58690
-4	At3G58730	At3G58710	At3G58730	At3G58710
-3	At3G58740	At3G58730	At1G31740	At3G58730
-2	At3G58750	At3G58750	At5G20710	At3G58750
-1	At3G58760	At3G58760	At3G58750	At3G58760
+1	At3G58790	At3G58850	At3G58790	At3G58790
+2	At3G58800	At3G59010	At3G58830	At3G58800
+3	At3G58810	At3G59020	At3G58840	At3G58850
+4	At3G58820	At3G59220	At3G59020	At3G58970
+5	At3G58830	At3G59280	At3G59030	At3G59010

Table 4.5: Synteny analysis for the *BrSHP2* homolog.

The genes and their positions (upstream (+) or downstream (-)) relative to a given *SHP2* homolog are indicated. Shared genes are highlighted in the same colour.

Position	<i>AtSHP 2</i>	<i>BrSHP 2</i>
-5	At2G42770	At2G42750
-4	At2G42790	At2G42790
-3	At2G42800	At2G42800
-2	At2G42810	At2G42810
-1	At2G42820	At2G42820
+1	At2G42840	At2G42840
+2	At2G42850	At2G42850
+3	At2G42870	At2G42870
+4	At2G42880	At2G42610
+5	At2G42890	At2G42600

This analysis shows syntenic conservation of the *SHP* genes in that these chromosomal regions analysed, spanning 40-50k bases, have been conserved and therefore the genes can be considered homologs. This is particularly the case

in *SHP2* (Table 4.5). As an additional measure of confirmation, the homologs were each amplified and sequenced to confirm the databases corresponded with what was observed *in vivo*. The sequences shown in Figures 4.5 & 4.6 were validated through this experiment.

4.2.3 Characterisation of the identified *SHATTERPROOF* homologs

After the identification of the *BrSHP* genes, I employed quantitative real-time PCR (qRT-PCR) to assess their spatio-temporal expression. For this work, I tested two different reference genes, namely *Elongating factor1 alpha* (*EF1 α*) and *TUBULIN* (*TUB*), and determined the expression levels of the *BrSHP* genes relative to the expression of these reference genes. For this analysis, I used RNA extracted from a range of different tissue/organ types. These included leaves, floral buds of different stages, the four floral organs and different stages and parts of gynoecia where I expected the *BrSHP* genes to be expressed based on the expression pattern of the *SHP* genes in *Arabidopsis*.

The results of these experiments (Figures 4.10 – 4.13) were mostly consistent for the two reference genes used. However, a notable discrepancy was detected in the samples representing open (stage 13) flowers where *BrSHP* gene expression appeared to be very high when *EF1 α* was used as reference while the expression seemed much reduced with the *TUB* reference gene (Smyth et al., 1990). A comparison of the data for the two reference genes in the different tissues showed that there is some variation in Cp values across different tissue types and that any discrepancies between the two data sets are likely due to the slight variations in reference gene expression (Figure 4.14).

As expected, the results of the experiments showed that the *BrSHP* genes are strongly expressed in flowers, especially at later stages, and in particular in the medial domain of gynoecia (Figures 4.10-4.13). In most tissues, *BrSHP2* expression appeared higher than that of any of the *BrSHP1* genes. Of the three

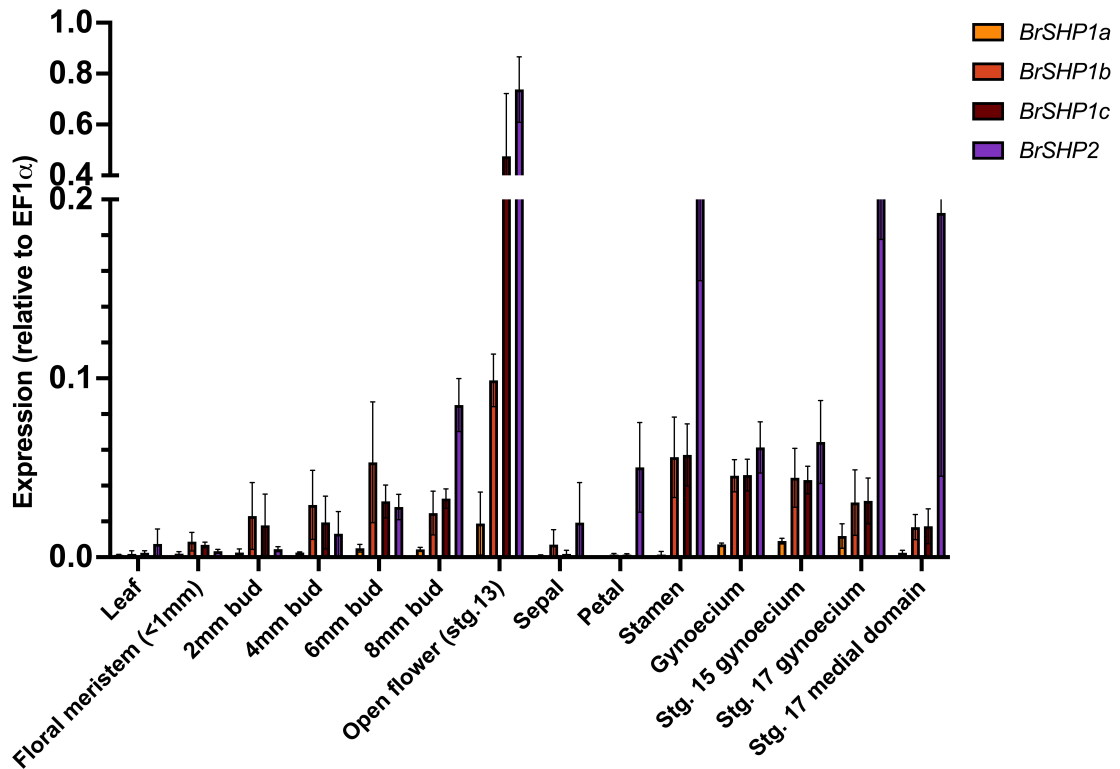


Figure 4.10: Expression of *BrSHP* mRNAs relative to *EF1α*.

RT-qPCR results measuring the level of *BrSHP* mRNA in various tissue types (labelled) of the R-o-18 wild-type accession of *B. rapa*. Values are relative to the reference gene *Elongating Factor 1 Alpha* (*EF1α*). Values shown are the mean of three biological replicates for each tissue type. Two technical replicates were performed for each sample. Error bars represent the standard deviation of the replicates.

BrSHP1 genes, *BrSHP1a* seems to be expressed at much lower levels than either *BrSHP1b* or *BrSHP1c*.

An unexpected result was that the *BrSHP* genes displayed a high level of expression in stamens which is not seen for the *SHP* genes in *Arabidopsis* (Figure 4.1). To verify this observation and to obtain further insights into the nature of *BrSHP* expression in stamens, I collected whole stamens from floral buds of different stages, as well as separately anthers and filaments of stamens of two different stages. This was done to determine at which stage and in which part of the stamen the expression of *BrSHP* genes occurs.

After extracting RNA from these samples, I conducted a qRT-PCR analysis

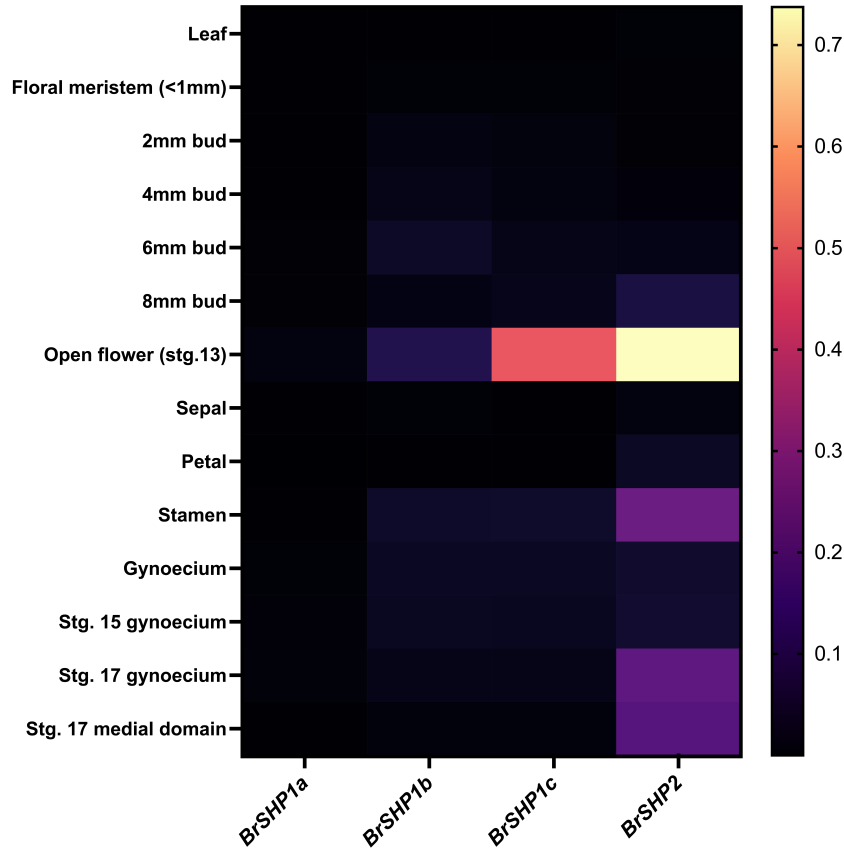


Figure 4.11: Heat map of expression of *BrSHP* mRNAs relative to *EF1α*.

The expression data shown in **Figure 4.10** were colour-coded as indicated by the colour bar on the right. Yellow and red represent high expression levels, black and blue no or low expression.

to determine the expression levels of the *BrSHP* genes. The results of this experiment showed highest level of expression in stamen filaments of 10 mm floral buds (**Figure 4.15**), which is equivalent to approximately stage 12 in *Arabidopsis* flowers (Smyth et al., 1990). This stage of flower development in *B. rapa* is depicted in **Figure 4.16**, for reference.

Taken together, the results of the analyses described above suggest that the *BrSHP* genes are expressed in the same tissues as their homologs in *Arabidopsis*. However, their expression patterns appears to be broader with high expression also detected in stamens - the filaments, primarily - at more advanced stages of flower development. Thus, these genes may have functions in stamens that are not shared with the *SHP* genes from *Arabidopsis*.

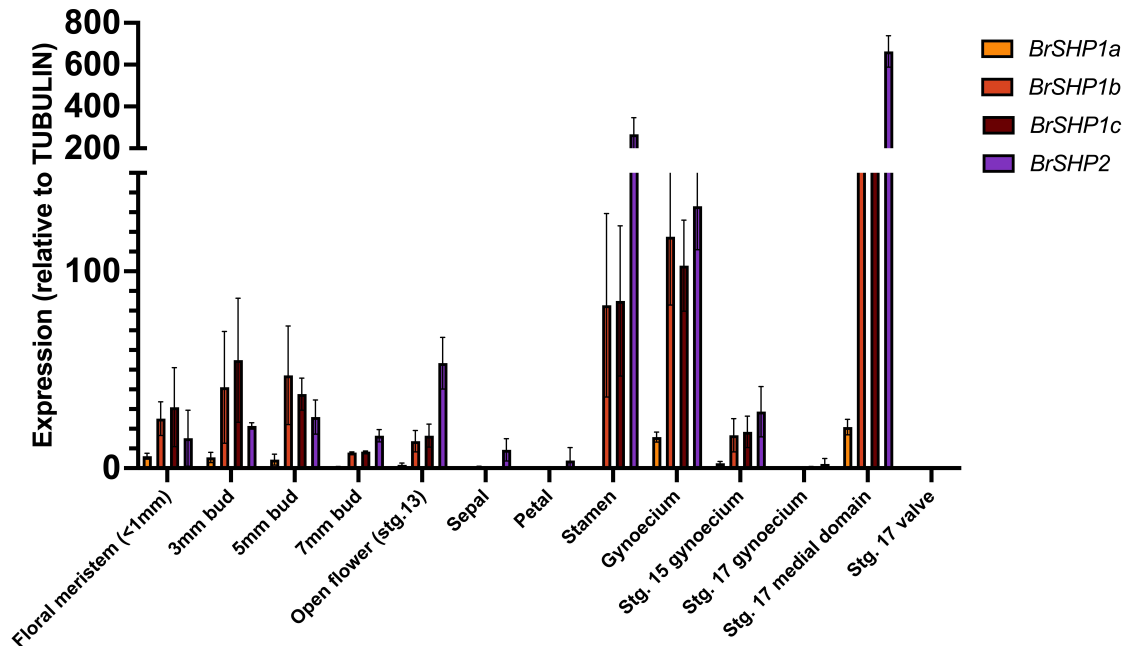


Figure 4.12: Expression of *BrSHP* mRNAs relative to *TUB*.

RT-qPCR results measuring the level of *BrSHP* mRNA in various tissue types (labelled) of the R-o-18 wild-type accession of *Brassica rapa*. Values are relative to the reference gene *TUBULIN* (*TUB*). Values shown are the mean of three biological replicates for each tissue type. Two technical replicates were performed for each sample. Error bars represent the standard deviation of the replicates.

4.2.4 Complementation of SHATTERPROOF activity in *Arabidopsis* using both native and *B. rapa* coding sequences

To further characterise the *BrSHP* genes, I tested whether they could complement the loss of *SHP1/2* gene function in *Arabidopsis*. To this end, I focused on *BrSHP2* as, according to my data, it is the most highly expressed *SHP* gene in *B. rapa*, as described in the section above. For these experiments, I generated a set of constructs (see Section 2.7 for details) containing the CDS of either *BrSHP2* or *AtSHP2* under the control of the constitutive CaMV 35S promoter. I also made constructs that drive the *BrSHP2* or *AtSHP2* under their endogenous promoters. Finally, I made another set of constructs in which the *BrSHP2* or *AtSHP2* promoters had been switched so that *BrSHP2* would be driven from the *AtSHP2* promoter, and *vice versa*. These constructs were transformed into both wild-type

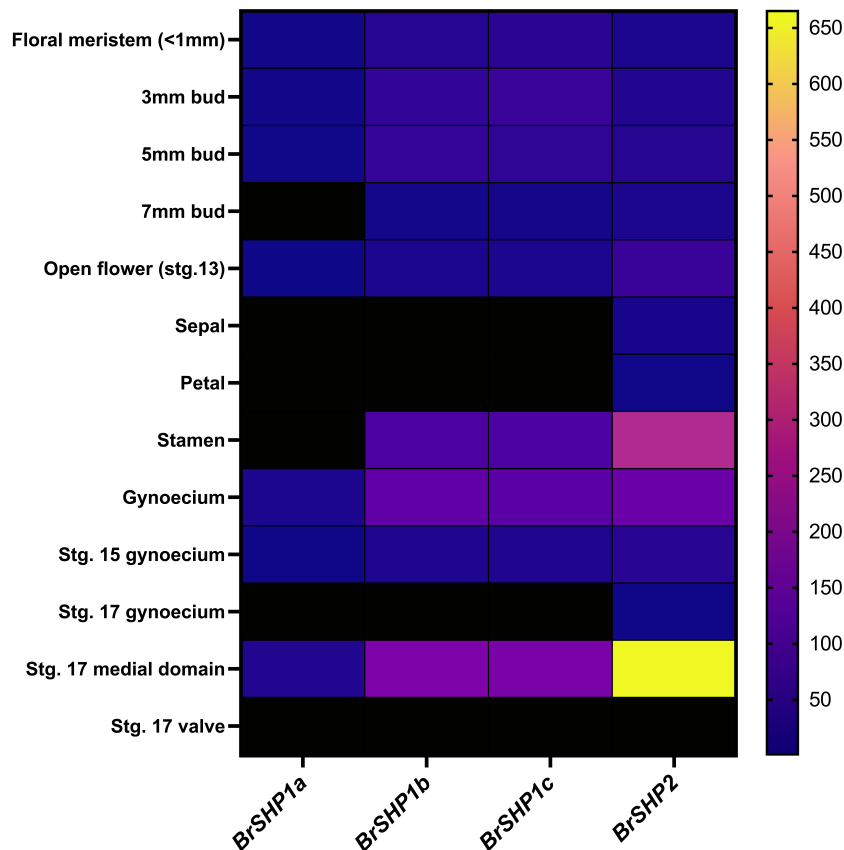


Figure 4.13: Heat map of expression of *BrSHP* mRNAs relative to *TUB*.

The expression data shown in **Figure 4.12** were colour-coded as indicated by the colour bar on the right. Yellow and red represent high expression levels, black and blue no or low expression.

(accession: L-*er*) and *shp1/2* double-mutant plants.

Flowers of primary transformants carrying the constructs in a wild-type background appeared phenotypically normal. However, whole plant architecture in a wild-type background was sometimes affected, in that the plants had increased height and formed numerous lateral inflorescences (**Figures 4.17 – 4.19**). This phenotype appeared to be especially strong in lines containing the *BrSHP2* promoter, possibly indicating that its activity in *Arabidopsis* is not restricted to just the flowers. In a *shp1 shp2* double-mutant background, introduction of all constructs led to a rescue of the mutant phenotype in all of the (5 to 10) primary transformants that were analysed per line, so that the siliques readily opened after a mechanical treatment (**Figure 4.20**). Thus, it appears that *BrSHP2*

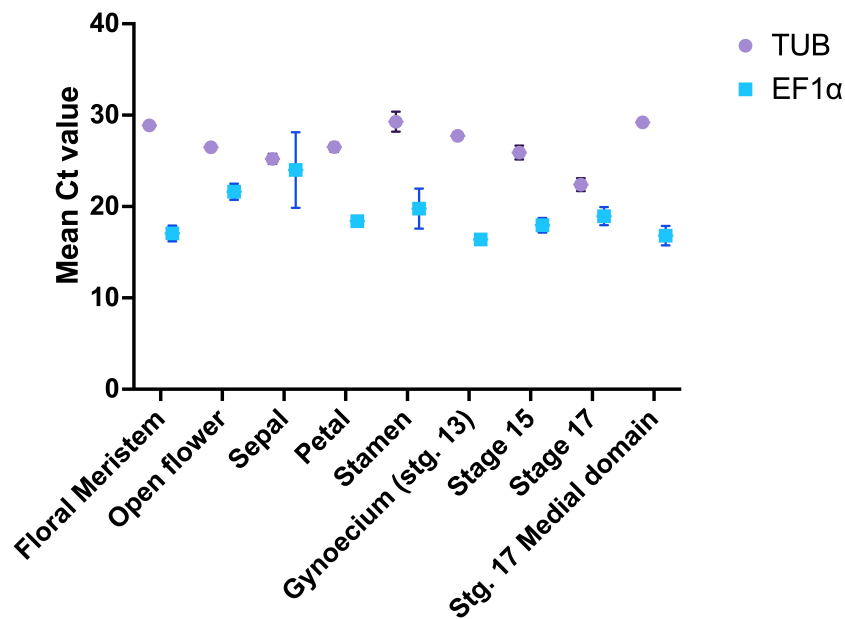


Figure 4.14: Scatter plot of the mean Ct values across various tissues of the reference genes tested, TUB and EF1 α .

Values shown are the mean of three biological replicates, with two technical replicates completed for each sample. Error bars represent the standard deviation.

can functionally complement a loss of *AtSHP* function and that the *BrSHP2* promoter is active in the *Arabidopsis* gynoecium.

As stated above, when introduced to a *shp1 shp2* background, all of the constructs described were capable of rescuing the shatterproof phenotype of this background. No other phenotype was observed in most of these lines. The only genotype in which this differed was the p35S::*BrSHP2* CDS construct expressed in a *shp1 shp2* background. An example phenotype is shown in **Figure 4.21**. Not only does this phenotype demonstrate the lateral growth which is discussed above in reference to the transgenic lines transformed in to *L-er* plants, but this line also showed severely stunted growth.

It should be noted that this phenotype was examined in the T₁ generation, so these plants were subjected to BASTATM spraying as seedlings and this may cause some level of stress. This was due to there not be sufficient time to carry

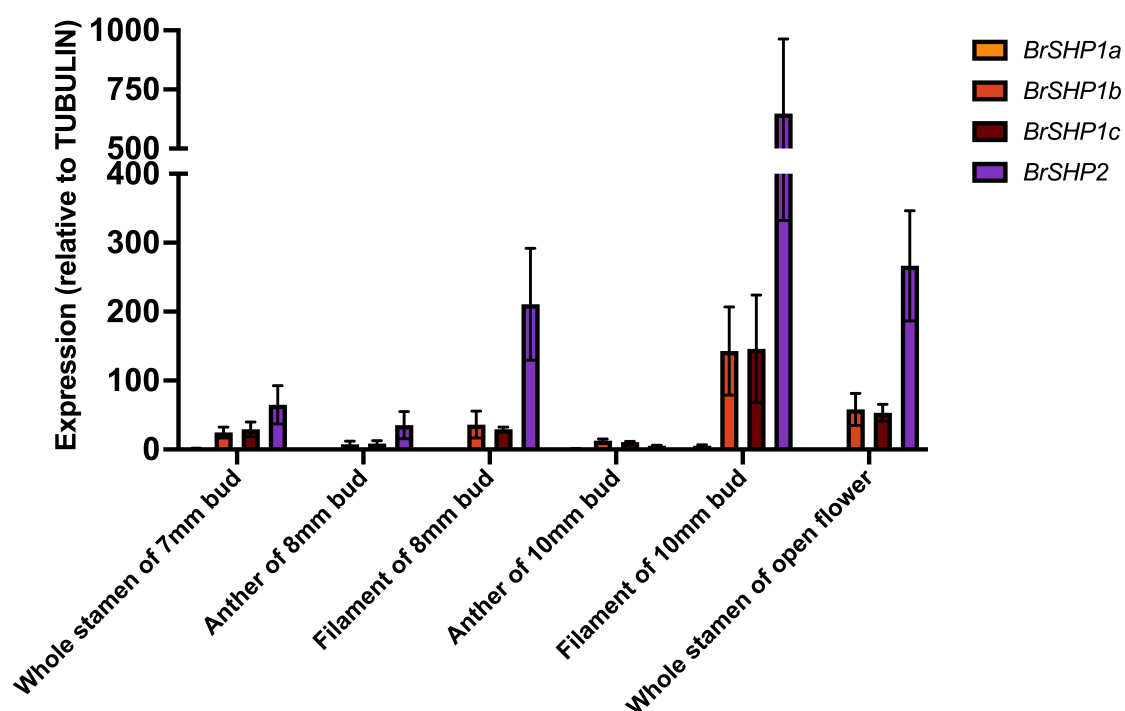


Figure 4.15: Expression of *BrSHP* genes in stamens relative to *TUB*.

RT-qPCR results measuring the level of *BrSHP* mRNA in various tissue types (labelled) of the R-o-18 wild-type accession of *B. rapa*. Values are relative to the reference gene *TUB*. Values shown are the mean of three biological replicates for each tissue type. Two technical replicates were performed for each sample. Error bars represent standard deviations.

the line through to T₂ for phenotypic evaluation. However, this same phenotype was observed in 7 independent lines of this genotype of a total of 10 transformants, and such a phenotype had not been observed in any other lines in the T₁, which were subjected to identical BASTATM treatment. This phenotype would need to be verified in subsequent generations to be deemed heritable and as an effect of the transgene.

4.2.5 The identification of the 3' UTRs of the *BrSHP* homologs

An additional step I undertook in the characterisation of these identified homologs was to amplify and sequence their 3' untranslated region (UTR). This could prove advantageous for future experimental design for designing gene-specific probes or primers, as the sequences of all 4 *BrSHP* genes are highly sim-



Figure 4.16: An example of a 10 mm *B. rapa* flower.

(A) A closed 10 mm floral bud. (B) A 10 mm bud with a sepal removed. (C) A 10 mm bud with a sepal and a petal removed. (D) An intact open flower (approx. 11-12 mm) (E) An open flower with a sepal removed to visualise the floral organs. Scale bar = 1 mm. Photographs kindly provided by Dr Christina Fitzsimons.

ilar and the 3' UTR tends to be less conserved. This could potentially be a target site of interest if undertaking *in situ* hybridisation or other similar methods, and these UTRs have not previously been described. To this end, I employed a 3' rapid amplification of cDNA ends (RACE) protocol detailed in Scotto-Lavino et al., 2007. The principle of this method is based on the use of three nested gene-specific primers as well as a specialised oligo d(T), in multiple rounds of amplification. Once successfully amplified, the product of the 3' RACE PCR was sent for Sanger sequencing. Then, each of the 3' RACE products were aligned with the known 3' UTRs of *AtSHP1* and *AtSHP2* (Figures 4.22 & 4.23).

According to the alignment of these sequences, the *SHP1* 3' UTRs share a 68% pairwise identity, accounting for indels which can be seen in the alignment (Figure 4.22). While they are quite similar, each has unique segments which fulfil the purpose of isolating unique sequences to be used as targets. The known 3' UTR of *AtSHP1* is 175 bp, while the lengths of the 3'UTR sequences identified

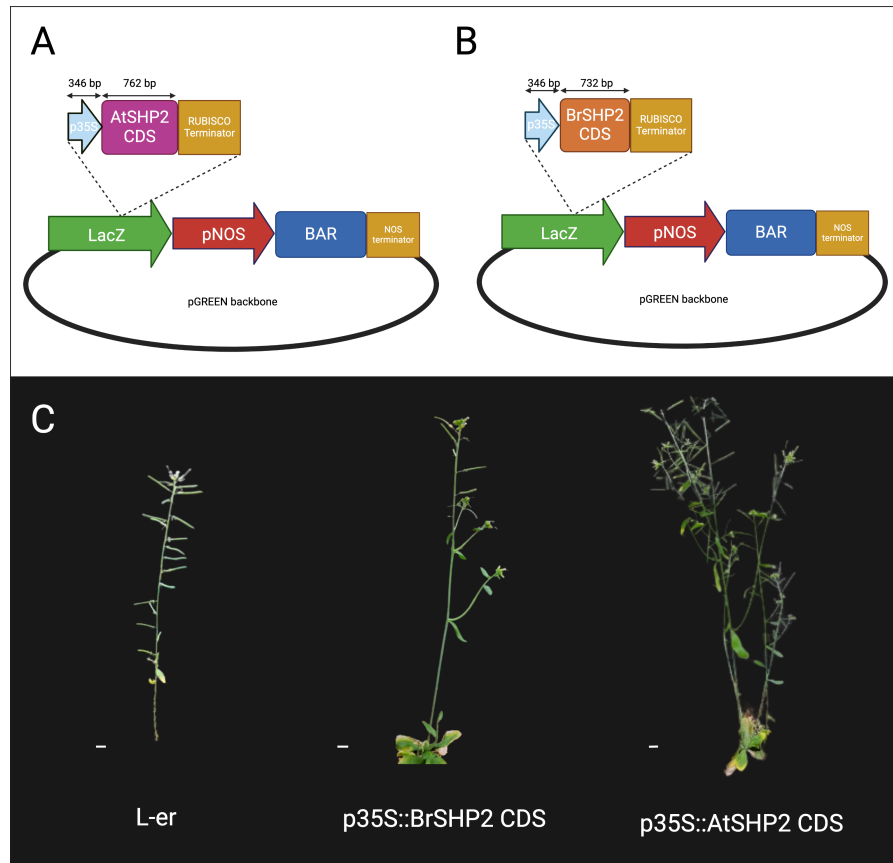


Figure 4.17: Overexpression of the *Arabidopsis* and *B. rapa* copies of *SHP2*. (A) Map of construct for the *Arabidopsis* overexpression line (B) Map of construct for the *B. rapa* overexpression line. (C) Plant architecture of L-*er* and both over-expression lines (labelled). Scale bar: 1 cm. Figure created with Biorender.com

in *BrSHP1a/b/c* were 127 bp, 132 bp and 139 bp, respectively.

The *SHP2* 3' UTR sequences share a 67.9% pairwise identity, accounting for the indels present across them (**Figure 4.23**). The known 3' UTR of *AtSHP2* is 207 bp in length, while the *BrSHP2* sequence identified is 161 bp long. Additionally, the identified *BrSHP2* 3' UTR was aligned to the 3' UTRs of *BrSHP1a/b/c* and was found to only share a 60% pairwise identity (data not shown). So, there are also unique regions within this sequence which can be exploited for the purpose of generating gene-specific probes and primers.

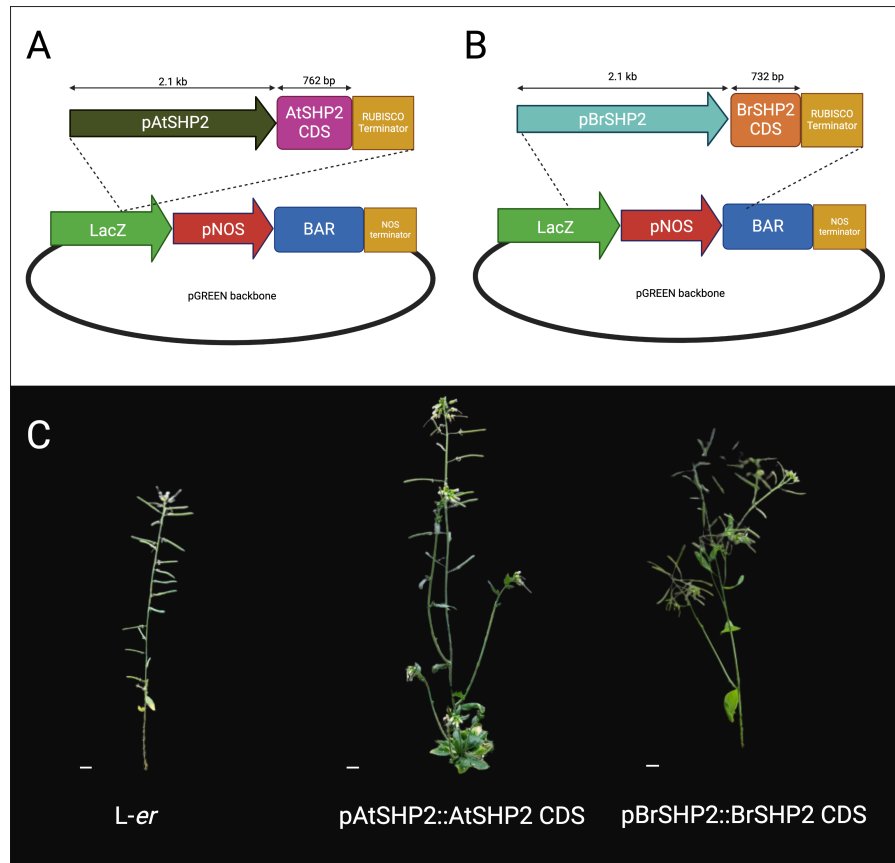


Figure 4.18: The expression of the *Arabidopsis* and *B. rapa* copies of *SHP2* driven under their native promoters.

(A) Map of construct for the *Arabidopsis* SHP2 endogenous expression line (B) Map of construct for the *B. rapa* SHP2 endogenous expression line. (C) Phenotype and plant architecture of the wild-type and both native promoter lines (labelled). Scale bar: 1 cm. Figure created with Biorender.com

4.2.6 Selection of mutant *brshp1/2* lines from TILLING screen by RevGen UK

In order to characterise the function of the *BrSHP* genes in *B. rapa*, I aimed at using a mutant approach. To this end, I screened a collection of mutant lines distributed by RevGen UK. RevGen UK is a company operating from the John Innes Centre in Norwich offering Targeting Induced Local Lesions in Genomes (TILLING) mutant lines of *B. rapa subsp. trilocularis*. To generate these lines, the plants of a recombinant inbred line called R-o-18 were mutagenised with ethyl methanesulfonate (EMS) before undergoing rigorous screening and exome

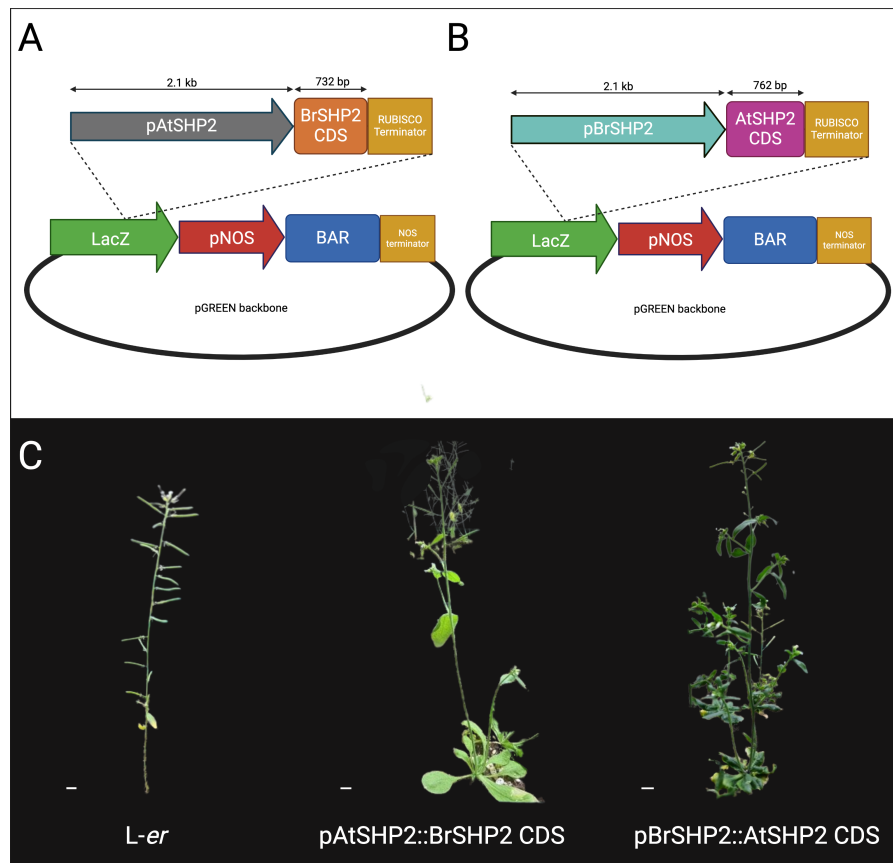


Figure 4.19: The expression of the *Arabidopsis* and *B. rapa* copies of SHP2 with the promoter of the converse.

(A) Map of construct for the *B. rapa* SHP2 driven by the *Arabidopsis* SHP2 promoter (B) Map of construct for the *Arabidopsis* SHP2 driven by the *B. rapa* SHP2 promoter. (C) Phenotype and plant architecture of the wild-type and both promoter swap lines (labelled). Scale bar: 1 cm. Figure created with Biorender.com

sequencing to identify mutations in gene sequences on a global scale. Under current law in the EU, common mutagenesis techniques such as CRISPR cannot legally be employed in plants which are intended for human consumption, but TILLING lines are legal to use. This, accompanied by the fact that *B. rapa* is quite technically difficult to transform in order to use an approach such as CRISPR, was why TILLING lines were used for this work.

To identify mutations in the *BrSHP* genes, I screened the exome sequencing information provided by RevGen UK. While premature stop codons can be identified for many genes in this mutant collection, in the case of all four *BrSHP* genes this was not the case. The only exception was *BrSHP2*, but in that



Figure 4.20: The rescue of the *shp1/2* phenotype with complementation constructs.

(A) A stage 18 (Smyth et al., 1990) *L-er* silique, fused and split open. (B) A stage 18 (Smyth et al., 1990) *shp1-1 shp2-1* silique, fused and partially split. (C) A stage 18 (Smyth et al., 1990) silique from the *AtSHP2* rescue line, fused and split open. Scale bars: 1 mm. All split siliques subjected to the same force by a No 4. jeweller's forceps, to demonstrate the difficulty to open a *shp1 shp2* silique and that this is rescued by the transgene.

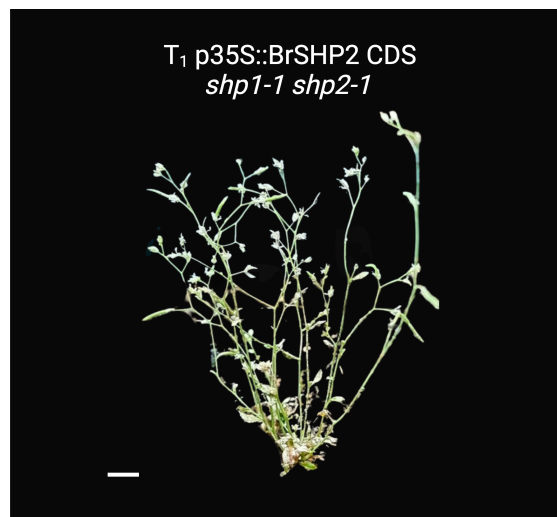


Figure 4.21: The phenotype of the overexpression of the *BrSHP2* CDS in a *shp1 shp2* background.

Scale bar: 1 cm.

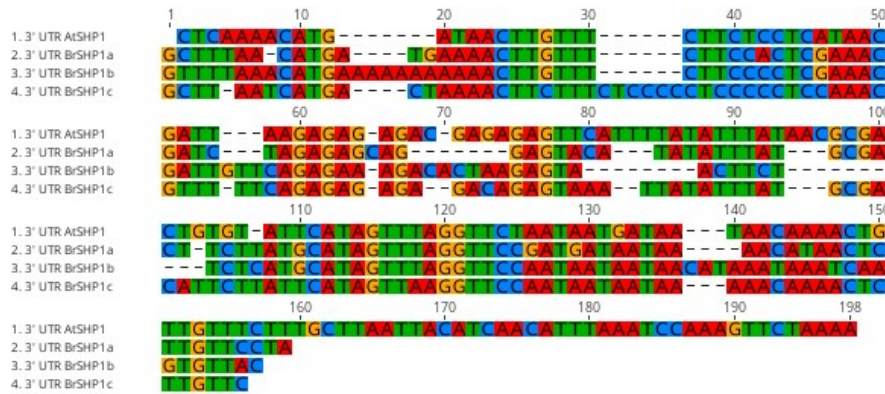


Figure 4.22: Alignment of the 3' UTRs of *AtSHP1* and the three copies of *BrSHP1*.

Analysis completed using the *Geneious Prime* software. Each base (A, T, G, C) is colour-coded for easier visualisation (red, green, yellow and blue, respectively). When the nucleotides are shaded in colour, this indicates that they are identical across both sequences at this position.

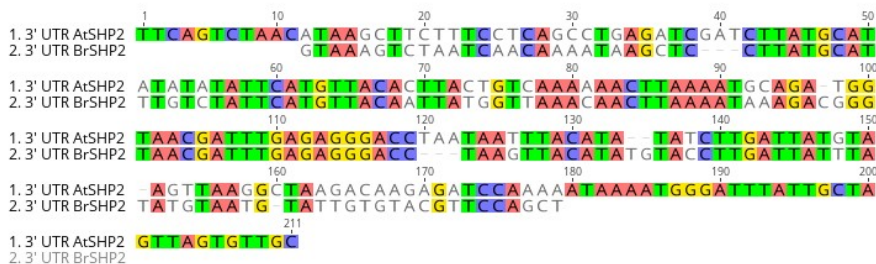


Figure 4.23: Alignment of the 3' UTRs of *AtSHP2* and *BrSHP2* homolog.

Analysis completed using the *Geneious Prime* software. Each base (A, T, G, C) is colour-coded for easier visualisation (red, green, yellow and blue, respectively). When the nucleotides are shaded in colour, this indicates that they are identical across both sequences at this position.

particular case the premature stop codons were located in the last exon of the gene and were deemed not likely to alter protein function. A second screen of additional lines of the TILLING collection for premature stop codons in the *BrSHP* genes was kindly undertaken by RevGen UK but unfortunately, it did not yield any new alleles of interest. Therefore, for all four *BrSHP* genes, missense or splice sites mutation alleles were selected. In the case of missense mutations, non-conservative amino acid changes (i.e. those that would have altered the po-

larity or charge of an amino acid) in highly conserved regions of the proteins, especially the MADS domain, were desirable. The mutant alleles selected for this work and the effects of the mutations on the corresponding proteins are summarised in **Table 4.6** and **Figure 4.24**.

Table 4.6: TILLING alleles selected for this work.

Gene names, RevGen line identifiers, and given allele names are listed. The nucleotide position of the mutation in a each line and its effect on the corresponding amino acid sequence are shown as well.

Gene	RevGen ID	Shorthand	Mutation	Remarks
<i>BrSHP1a</i>	J130010-B	<i>brshp1a-5</i>	missense: 175G>A:59A>T	Targets well-conserved Arg in Beta sheet of MADS domain
<i>BrSHP1b</i>	J130720-A	<i>brshp1b-2</i>	missense 2241C>T:82L>F	Targets conserved leucine in alpha helix
<i>BrSHP1c</i>	J130055-A	<i>brshp1c-2</i>	missense 191C>T:64S>F	Targets extremely well conserved ($\simeq 100\%$) residue in Beta sheet
<i>BrSHP2</i>	J131501-B	<i>brshp2-8</i>	missense: 64G>A:22E>K	K sometimes found at this position

While all of the alleles I chose have amino acid substitutions in the MADS domain, not all positions affected in these mutants are as highly conserved as others. Therefore, it is possible or even likely that some of these alleles are not full loss-of-function alleles. However, this was not necessarily problematic for the purposes of this work. As discussed in Section 4.1.4, one aim of this work was to generate *B. rapa* lines that are more shatter resistant than wild-type plants. Because siliques of plants that are fully shatterproof due to a complete loss of *SHP* function can only be opened through a relatively strong mechanical force, it may be advantageous to work with lines that are based on partial loss-of-function alleles for the purposes of agriculture. Thus, the selection of the alleles for this work may be ideal to test the potential of this approach.

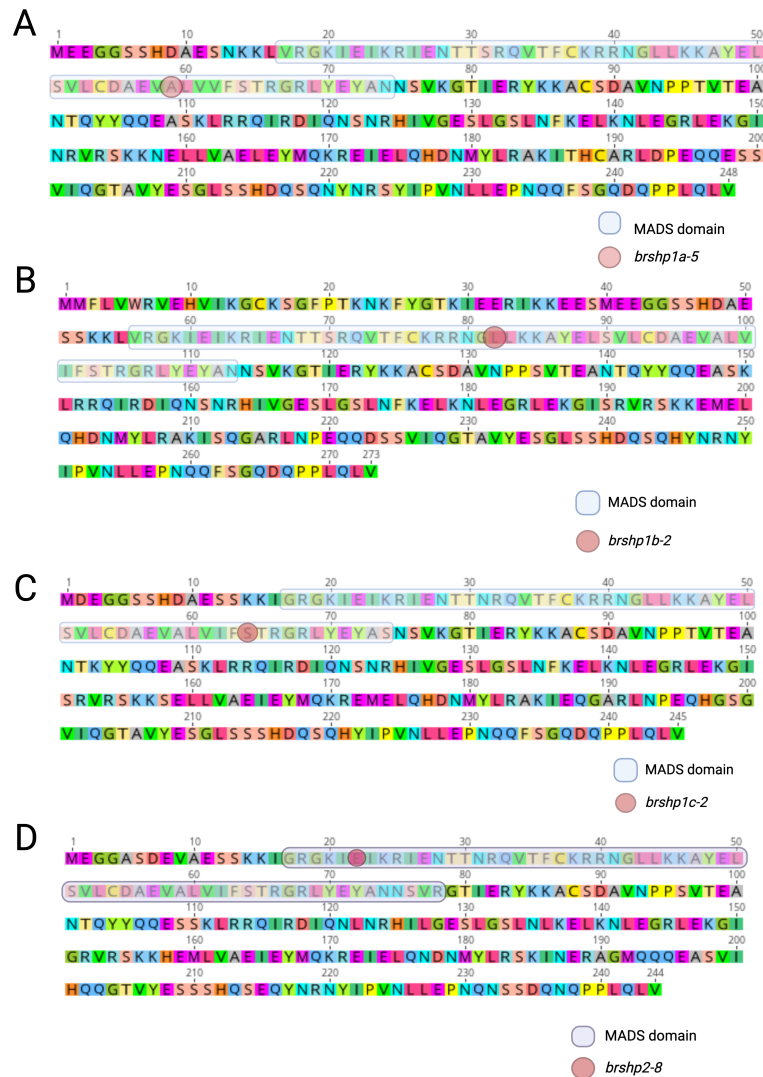


Figure 4.24: The position of the SNPs of the TILLING alleles selected.
The amino acid positions affected in the different BrSHP TILLING lines are indicated. The position of MADS domain is highlighted in each case. Figure created with Biorender.com.

4.2.7 Identification of mutant plants through Double-mismatch allele-specific (DMAS) PCR

In order to be able to deal with high sample volume required to allow for homozygous mutants to be isolated from segregating populations, as well as genotyping the progeny of crosses made, a suitable high throughput genotyping assay needed to be established. Sanger sequencing was utilised in the first gen-

eration population received from RevGen UK to ensure the alleles were as expected. However, this would be an expensive and time-consuming method for downstream use when up to 200 plants may be being dealt with at a time.

An assay which could be carried out in 96-well plate format seemed the most efficient for experimental set-up. Additionally, this was suitable for our laboratory as the Roche LightCycler 480 could be used for this format. The DMAS PCR assay is relatively cheap per reaction, as well as being technically simple to perform and suitable for a high throughput experiment. The principle of the assay is to perform two parallel qPCRs on the same sample simultaneously (Lefever et al., 2019). Both reactions use one common primer and each has an allele-specific primer, either for the wild-type (WT) or mutant (mut) nucleotide. This assay was optimised for the identification of SNPs. By performing a qPCR with these primers, the efficiency of the PCR can be ascertained by its C_p value. The genotype of the sample is determined by simply calculating $x = C_{p_{WT}} - C_{p_{mut}}$, where $x > 1$ is assigned a homozygous mutant, $x < -1$ is assigned homozygous wild type and $-1 < x < 1$ is heterozygous.

4.2.8 Test via RT-PCR on a splice mutant line

The nucleotides immediately surrounding a splice site are well conserved across all organisms, but this motif varies slightly across kingdoms (Shang et al., 2017). The position weight distribution of a typical splice donor site is shown in **Figure 4.25 (A)**. The conservation of this sequence is essential to ensure splicing is regulated during transcription. When the splicing process is impaired, a different mRNA will be produced and therefore a different protein product.

When selecting mutations to a gene sequence, SNPs which result in splice acceptor/donor mutations are tentatively predicted to have stronger phenotypes. Although this may not result in the strongest phenotype possible (such as in an early stop codon) it is hypothesised that this will have a more severe effect on the formation and folding of the protein due to incorrect transcript splicing.

One of the TILLING alleles available for *BrSHP2*, with the RevGen ID JI31461, altered a splice donor site. This was of interest to acquire as it altered the splice site between exon 1 and 2. If this SNP effectively hindered the splicing process, it could have a dramatic effect on the resulting amino acid sequence and therefore greatly alter protein function. This particular TILLING allele was a G > A mutation in the position equivalent to “+5” in **Figure 4.25 (A)**. The splice donor site mutation was promising as a disruption to protein function, but it needed to be established whether splicing of the intron was actually affected. A wild-type plant and a plant that was homozygous for the splice mutation were both subjected to reverse transcription PCR (RT-PCR) to test if there was a difference in the length of the cDNA product. Initially, the RT-PCR was run on an agarose gel and the bands of the splice mutant and wild-type plants appeared to be the same size.

So, to further investigate the nature of the mutation, the RT-PCR product was sent for Sanger sequencing to screen for any small indels which may have resulted in a frameshift mutation that would have an impact on protein formation and folding. By referring to the diagram from Shang et al., 2017 this guanine base is well conserved in human splice donor sites. However, while it is the most commonly found base at this position in *Arabidopsis*, guanine is not as well-conserved as it is in humans. Therefore, it was important to test whether splicing was altered in this mutant line before continuing to work with it and using it to generate higher-order mutant lines. The sequencing data showed that splicing was in fact not altered and therefore the mRNA and amino acid sequences were identical to the wild-type. Thus, this line was shown to not affect the protein sequence of *BrSHP2*, so it was not used in this work.

4.2.9 Generating higher-order mutants of the *BrSHP* genes

Due to the substantially longer generation time of *B. rapa* when compared with *Arabidopsis*, a plan had to be implemented to obtain high-order mutants in as

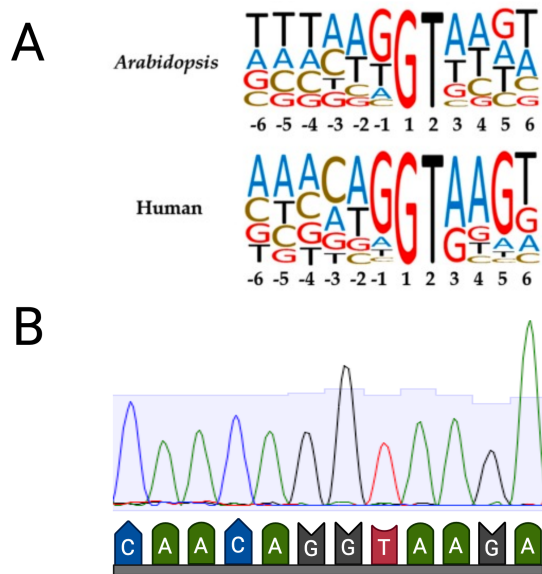


Figure 4.25: Splice donor site in *BrSHP2*.

(A) Frequency weighted outline of the sequence of splice donor sites in *Arabidopsis* and humans compared. Adapted from Shang et al., 2017 (B) The sequence of the corresponding region in *BrSHP 2*, situated in the first intron, between exon 1 and 2. Created with BioRender.com

efficient a manner as possible. While *Arabidopsis* will typically cycle from seed to seed in approximately 8 weeks, this will take up to 4 months in *B. rapa*. Implementing an efficient method of generating high-order mutants was especially critical because of additional generations having to be grown, as we found the TILLING lines first needed to be back-crossed in to the wild-type R-o-18 background due to the health issues that were observed in the heavily mutagenised TILLING plants. Then, the mutant allele would have to be identified from the segregating population once again. Additionally, in this initial back-cross, the poor health of the parent TILLING plant would often lead to few seeds being produced, so in some cases only a heterozygote would be isolated and yet another generation would need to be screened.

Through planning for a cross of two double mutants, rather than making every possible crossing combination, the quadruple mutant should be quicker to obtain. Additionally, Mendelian segregation laws would suggest that in the

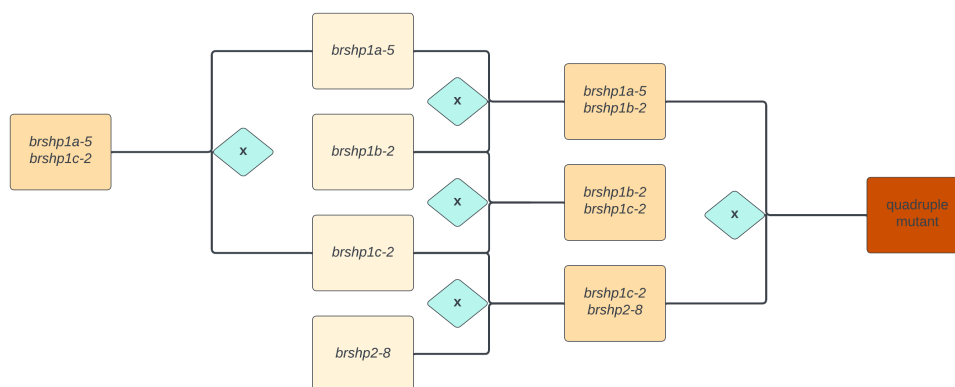


Figure 4.26: Crossing plan for *B. rapa* TILLING mutant lines.
Figure generated using *Lucid Chart* software.

course of screening the F_2 of this cross to isolate quadruple mutants, it should also be possible to isolate every other possible mutant combination as well (**Figure 4.26**). However, due to unforeseen circumstances, the double mutant stage was what was achieved. Crosses for the generation of triple and quadruple mutants were carried out with the plants of the F_1 generation growing at the time of writing. The quadruple mutant will be isolated, but unfortunately no analyses were possible for this thesis.

4.2.10 Phenotypic observations via Random Impact Test

Single or double *brshp* mutants exhibited no abnormal phenotypes upon first inspection. This was not unexpected as in *Arabidopsis*, only a full loss of both *SHP1* and *SHP2* function leads to a mutant phenotype. However, I sought to test whether the disruption of some of the *BrSHP* genes would lead to some effect on lignification and seed dispersal. To this end, I made use of a Random Impact Test (RIT), first described over two decades ago (Morgan et al., 1998). In this test, dry siliques are subjected to random impacts by ball bearings over a time series and the time required for siliques to open is recorded (**Figure 4.27**). The test results in RIT_{50} values, which is the pod shatter ‘half-life’, i.e. the time required for half of the siliques of a given genotype to shatter (see Section 2.3

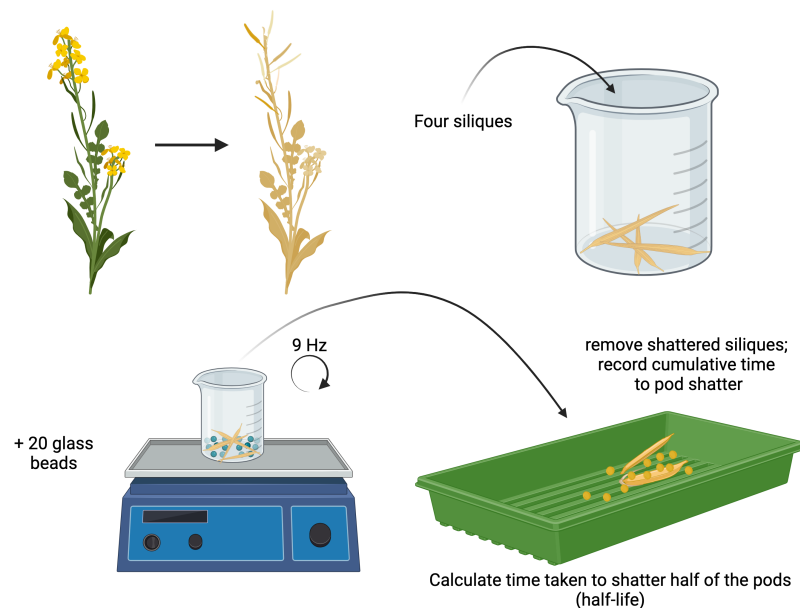


Figure 4.27: Schematic of the workflow of a Random Impact Test (RIT).

See text for details. Figure created with BioRender.com

for how the RIT_{50} is calculated). The RIT has been optimised over the years for use in various species (Lenser and Theissen, 2014; Stephenson et al., 2019). I used an adapted version of the protocol to suit materials available to me in the laboratory (Lenser and Theissen, 2014).

Table 4.7: Results of Random Impact Test of *brshp* mutants.

Cultivar background of the mutant alleles given in parentheses. C-634 and *brshp2-8* in a C-634 background included to test if cultivar background had an impact on pod shatter.

Genotype	RIT_{50}
R-o-18	5 s
C-634	6.08 s
<i>brshp1a-5</i> (R-o-18)	5 s
<i>brshp1b-2</i> (R-o-18)	5 s
<i>brshp1c-2</i> (R-o-18)	5 s
<i>brshp2-8</i> (R-o-18)	5 s
<i>brshp2-8</i> (C-634)	6.08 s
<i>brshp1a-5 brshp1b-2</i>	16.78 s
<i>brshp1a-5 brshp1c-2</i>	5 s
<i>brshp1b-2 brshp1c-2</i>	6.94 s
<i>brshp1c-2 brshp2-8</i>	8.02 s

The results of the RIT show that the single mutants do not display any increase in shatter resistance. This was unsurprising to observe as the same is true in *Arabidopsis*, due to the high level of functional redundancy between the *SHP* genes. Surprisingly, however, it appears that even a double mutant is sufficient to substantially increase shatter resistance, compared to the wild type or a single mutant. The particularly surprising result was the *brshp1a brshp1b* double mutant, which increased in shatter resistance almost three-fold. The data in **Figures 4.10-4.13** suggest that these two homologs are the least expressed in the medial domain. However, these RIT₅₀ measurements would ideally need to be compared alongside a quadruple mutant to accurately indicate the extent to which the double mutants are shatter-resistant.

4.3 Discussion

4.3.1 The identification and characterisation of *SHP* homologs in *B. rapa*

The aim of this Chapter was to identify and characterise homologs of *SHP1/2* in *B. rapa*. This was successfully undertaken in a thorough manner using a combination of techniques. Firstly, *in silico* analyses were undertaken to identify genes sharing high similarity with *Arabidopsis SHP1/2*, align the sequences, examine the genes in the surrounding regions of the chromosome and also identify the loci and structure of the identified genes. In all, four genes were identified as being homologous to *AtSHP1/2*, with the BRAD gene IDs Bra014552, Bra003356, Bra007419 and Bra004716. They were given the aliases *BrSHP1a*, *BrSHP1b*, *BrSHP1c* and *BrSHP2*, respectively. Each of these genes are situated on different chromosomes in *B. rapa*, which means they are not linked. As described in this Chapter, this posed an advantage to my work as I would be able to cross mutant lines for each of the genes with each other without having to consider recombination, or alleles on the same chromosome being inherited together.

When I had successfully identified the *BrSHP* genes, as they were termed, I then began characterising them by examining their expression profiles via RT-qPCR. I examined samples across floral tissues of multiple stages. This analysis revealed that, indeed, the *BrSHPs* are expressed in the gynoeceium. Additionally, as the fruit matures they are localised to the medial domain and not present in the valves, much like *AtSHP1/2*. While *BrSHP1a* expression was detected in most tissues, it was globally expressed at extremely low levels in comparison to the other *BrSHP* homologs. This indicated that, for the purpose of isolating mutant lines producing a shatter resistant phenotype, that a *brshp1a* mutant would not likely produce shatter resistance or contribute to the phenotype in higher order mutants in a significant way. However, the results of the RIT suggested that

this may not be the case and that mutating *BrSHP1a* may indeed have an effect on shatter resistance. Perhaps this is due to a dosage effect that arose following gene duplication.

The expression analysis of the *BrSHPs* also revealed that in addition to the gynoecium, they are expressed in the stamens. This expression level of the *BrSHPs* steadily increases in the developing stamens and is at its highest at anthesis. This was an interesting result as this differs from the expression pattern of *AtSHP1/2*. This bore similarity to *AtAG*, which is a regulator of the reproductive organs (Section 1.2). However, *AtAG* acts in the very early stages of flower development, which isn't reflected in the *BrSHP* expression profiles (Mizukami and Ma, 1997). The relationship of the *BrSHP* genes to *AtSHP2* and *AtAG* needs to be investigated further, which will be addressed in Chapter 5. In *AtAG* the large intron is known to have many regulatory functions and drive normal *AG* expression (Lenhard et al., 2001). Thus, it would be expected that this sequence would be conserved. So, in terms of *BrSHP* genes sharing functional similarities with *AtAG*, it would be expected that the large introns of these genes may also share similarities. However, upon alignment of the genomic sequences, the intron of *AtSHP2* and *BrSHP2* shared 63.6% identity, while the large introns of *AtAG* and *BrSHP2* shared only 52% identity (**Appendix A1.4**).

It is important to note that in examining the literature, it seems that *Arabidopsis* is in fact an exceptional species in having such a highly restricted domain of *SHP* expression. Many other species of agricultural interest, ranging from peach to cucumber, have been found to display *SHP* expression within stamen tissue when characterising the MADS-box TFs of these species. This will be expanded upon in Chapter 5. Because *Arabidopsis* has been adopted as the model plant species, bias can be introduced wherein we assume that because another species displays functional differences to *Arabidopsis* that the other species has independently evolved this alternative function, while *Arabidopsis* characteristics are generally considered the “default”. So we must examine many different

species in order to address this potential source of bias, and the function of the *BrSHPs* required further examination.

As part of characterising these genes, I also identified the 3' UTRs of the *BrSHP* genes. This was done via 3' RACE and sequencing. I found that the 3' UTR sequences across the four *BrSHPs* are not well conserved and do not share a very high level of similarity. This allows for the identification of unique regions within genes which share such a high sequence similarity, if the genes need to be differentiated from each other. This could be potentially useful for future experiments seeking to compare the *BrSHP* genes, such as the use of *in situ* hybridisation to further characterise and confirm the expression domains of each *BrSHP* gene.

To further characterise the *BrSHP* genes, complementation of *SHP2* in *Arabidopsis* using both its native copy as well as the *B. rapa* copy, *BrSHP2*, was undertaken in order to demonstrate that *BrSHP2* is capable of functionally replacing *AtSHP2*. This also provides an opportunity to examine how *BrSHP2* functions and what controls the altered expression profile when compared to *AtSHP2*. These experiments demonstrated that *BrSHP2* complementation does indeed rescue the phenotype of a *shp1 shp2* double mutant in *Arabidopsis*. Additionally, the phenotype of *BrSHP2* being expressed in the wild-type *L-er* accession of *Arabidopsis* demonstrated that *BrSHP2* may in fact share functional similarity with *AtAG*. This could potentially provide an explanation for the stamen expression seen in the *BrSHP* homologs, as this is a known expression domain of *AG*. As discussed in Section 4.2.4, the phenotype observed in *L-er* plants with transgenic constructs introduced were reminiscent of a weak overexpression of *AG*. The lateral growths are reminiscent of a weak *ap1* mutation (Irish and Sussex, 1990). These phenotypes, in accompaniment with the expression data, led to the investigation of the similarity of *BrSHP* function to *AtAG* function, as well as the redundancy of *AtAG* and *AtSHP1/2*. This is the main focus of Chapter 5.

4.3.2 Isolating and examining *brshp* mutant lines

The final aim of this Chapter was to isolate mutant lines of the *BrSHP* genes and to examine the phenotypes of these mutants. As discussed in Section 4.2.6, the strength of the alleles obtained from RevGen UK could not be accurately predicted. For this reason, it must be considered that while these alleles were chosen due to their induction of changes in the most conserved portion of the gene, it is also possible that the alleles simply were not strong enough to obtain the desired phenotype. Every effort was made in selecting mutant alleles for analysis to ensure that they would be likely to have some impact on protein charge and folding, but it won't be fully understood what the effect of these alleles are until they are observed together in a quadruple *brshp* mutant. By referring to **Figure 4.24** to identify the residue of the MADS domain affected in each mutant line, **Figure 4.28** can then be employed in order to predict the impact to the protein sequence of the mutation. For example, in *brshp1a-5* the altered residue corresponds to residue 43 in **Figure 4.28**. The residue here is almost always a non-polar Alanine, which suggests that it being changed may have functional implications. In the mutant allele there is a polar Threonine at this position, which may impact protein tertiary structure. In *brshp1b-2*, the affected residue corresponds to position 27 in **Figure 4.28**. This is a highly conserved non-polar Leucine, and the Phenylalanine residue it is replaced with in the mutant allele is not found at this position. The polar Serine residue corresponding to position 48 in **Figure 4.28** is replaced by a non-polar Phenylalanine residue in the *brshp1c-2* line. While Serine isn't the most commonly found residue at this position in the MADS domain, it is the third highest occurrence and Phenylalanine is not normally found at this position. Finally, *brshp2-8* sees a negatively charged Glutamine residue in position 6 (**Figure 4.28**) replaced by a positively charged Lysine. Similarly to *brshp1c-2*, Glutamine is not the most conserved residue at this position in MADS domains broadly, but at the same time, Lysine is not nor-

demonstrated that there is in fact an increase in the shatter resistance within double mutants, which is encouraging for the purposes of this work and the outlook going forward. This suggests that an intermediate phenotype may be attainable in *B. rapa*. This would be incredibly advantageous to the agriculture sector. Given the additional copies of *SHP* genes due to polyploidy (see Section 4.1.3), the *BrSHP* genes may not be fully redundant or may be subjected to gene dosage effects and thus an intermediate phenotype may be possible. The most unexpected result of the RIT was in the *brshp1a-5 brshp1b-2* double mutant. Due to the low levels of expression of *BrSHP1a* (**Figures 4.10-4.13**) it would not have been predicted that a *brshp1a* mutant would have a drastic impact on the DZ. However, the *brshp1a brshp1b* double mutant was the most shatter resistant according to the RIT. The small sample sizes of this experiment must be taken into account, where $4 < n < 8$, as well as the fact that this test was not conducted over multiple generations to evaluate this phenotype in as many independent replicates as possible. Based on the expression data (**Figures 4.10-4.13**) the double mutant which would be expected to have the largest effect on pod shatter of all the possible combinations of double mutants is *brshp1c-2 brshp2-8*, however these alleles may be weaker than the *brshp1a* and *brshp1b* alleles, as discussed above.

4.4 Future Work

For the purpose of the project undertaken, the main aim of this Chapter was to identify homologs of *SHP1* and *SHP2* in *B. rapa* and to characterise them through a variety of techniques. From this work, it has been observed that there is further study required of the *BrSHPs* given their apparent functional differences to their *Arabidopsis* counterparts. Continuing to generate higher order mutants would be paramount to establish exactly what combination of mutations is required both for a fully fused phenotype and also for the desired phenotype for agricultural purposes, with an increased shatter resistance but still suitable for harvesting seeds.

In addition, further experiments could be conducted to elucidate the relationship between *SHP* and *AG* in *B. rapa*. Given their possible redundancy, it would be important to employ mutant lines generated previously in the lab by Dr Christina Fitzsimons, who identified homologs of *AG* in *B. rapa* and isolated a double *BrAG* mutant. It would be a valuable experiment to evaluate the phenotype of *brag brshp* sextuple mutants. It is possible, based on the findings of this work, that the resulting phenotype would be reminiscent of an *ag* mutant in *Arabidopsis*. Further investigation is also required of the large intron of the *BrSHP* genes. This intron is not known to have any promoter activity in *Arabidopsis*. However, the large intron of *AtAG* is sufficient to drive normal expression of *AG* (Lenhard et al., 2001). From the complementation experiments I conducted, it appears that this could also be the case in *BrSHP2*. This will be investigated and discussed in Chapter 5.

Further characterisation of these complementation lines would also be valuable in elucidating the genetic interactions that both *AtSHP2* and *BrSHP2* are involved in to compare them. For example, in Section 4.2.4, I mentioned that the branching phenotype in transgenic plants of the wild-type *L-er* background could possibly be due to a down regulation of *AP1* activity, due to the similarity

of the SHP2 and AG proteins. Further analyses could be conducted on these lines to assess *AP1* activity as well as other floral meristem determinators to verify this suggestion.

Another step toward fully characterising the *BrSHPs* and also evaluating their level of redundancy by examining the extent to which *BrSHP* function needs to be perturbed in order to obtain a fully shatterproof phenotype, is to obtain null mutant alleles. As discussed in Section 4.2.6, there were no null alleles available from RevGen UK at the time this work was undertaken. An additional TILLING screen or employing CRISPR/Cas9 would both be options to fully knock out the *BrSHP* genes. As discussed in Section 4.2.6, CRISPR has been shown in the literature to not work efficiently in *B. rapa* which is why it was not employed during this work. Another reason for avoiding the use of CRISPR in this work was the aim of translating these mutant lines to agriculture. Currently in the EU, CRISPR-mutagenised plants are considered transgenic and cannot be grown for food legally. However, TILLING lines are suitable for use in the field. Despite this, I believe it would be valuable to employ as a proof of concept experiment to analyse null allele phenotypes.

5 Examining the roles of *AG* and *SHP1/2* in inhibiting trichome initiation on the gynoecium

5.1 Introduction

5.1.1 The role of *AG* in gynoecium development

As discussed in Section 1.2, the role of *AG* as a regulator of gynoecium development was initially uncovered upon observation of the *ag-1* allele, where the 3rd and 4th whorls are replaced by supernumerary petals and sepals in what is known as a homeotic transformation (Bowman et al., 1989). Given that a strong *ag* mutant allele is incapable of producing any reproductive organs, microarray analyses as well as genome-wide localisation studies of *AG* were completed to further elucidate its role in the development of reproductive organs. These studies revealed that *AG* regulates hundreds of genes involved in these processes (Wellmer et al., 2004; Ó Maoiléidigh et al., 2013). For this reason, *AG* is considered the master regulator of reproductive floral organ development.

It has been shown that *AG* is expressed throughout gynoecium formation until fertilisation occurs and fruit development commences (**Figure 5.1**)(Drews et al., 1991; Klepikova et al., 2016). While the entire regulatory network directed by *AG* is incredibly complex and not fully understood, as discussed in Section

1.4, AG works in conjunction with SEP proteins in order to form quartets which can activate downstream effectors.

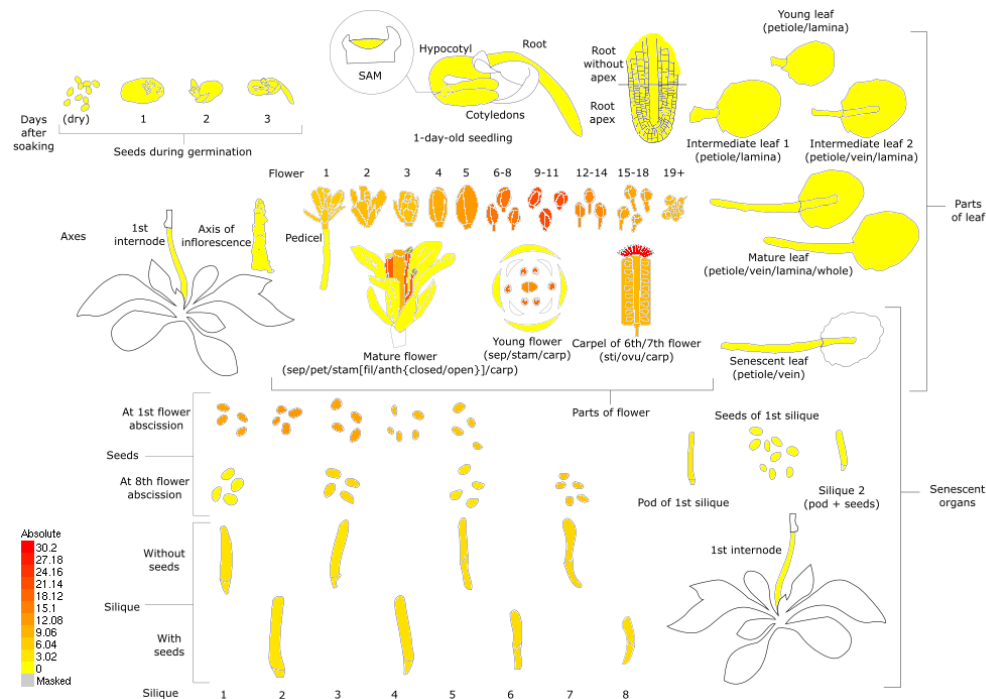


Figure 5.1: The expression domain of AG in *Arabidopsis*.

Expression heat map for AG, generated using the *Arabidopsis* eFP Browser, based on available RNA-seq data (Klepikova et al., 2016).

5.1.2 The role of AG in floral trichome suppression

As previously outlined in Section 1.7, AG acts as an upstream regulator in suppressing trichome initiation on floral organs. This is demonstrated when we observe the phenotype in an AG knockdown background, which is exacerbated by knocking out the trichome repressors *TRY* and *CPC* (Figure 1.8). While the precise mechanism by which this is regulated is not fully understood, work in our lab effectively showed that AG interacts in some manner with trichome repressors *TRY* and *CPC* to prevent the formation of trichomes on the gynoecium (Figure 1.10).

Much in the way that the leaf trichome phenotype of *try* single mutant plants shows some clustering of trichomes, which is then exacerbated in the

try cpc double mutant (**Figure 3.2**), the work previously conducted in our lab demonstrated that this additive phenotype is present on the gynoecium also. In an *AG* amiRNA knockdown, when there is a sufficiently low level of *AG* expression, some trichomes may be observed on the surface of the gynoecium (**Figure 1.8 (A, B)**). When *AG* is knocked down in a *try* mutant background, there is a significant increase in the number of trichomes on the gynoecium (**Figure 1.8 (D)**). Furthermore, the most drastic phenotype is seen in an *AG* knockdown introduced to the *try cpc* double mutant, with trichomes present on both the abaxial and adaxial sides of the carpels (**Figure 1.8 (E)**).

While this work was pivotal in understanding the role *AG* plays in floral trichome repression, using an amiRNA knockdown line would not be suitable in an effort to generate a stable mutant which displayed carpel trichomes. For this reason, crosses were also made into the *ag-10* background, which is a weak *ag* allele (Ji et al., 2011). When examining these lines, it can be seen that an *ag try* combination is sufficient to produce numerous carpel trichomes (**Figure 5.2 (B)**).

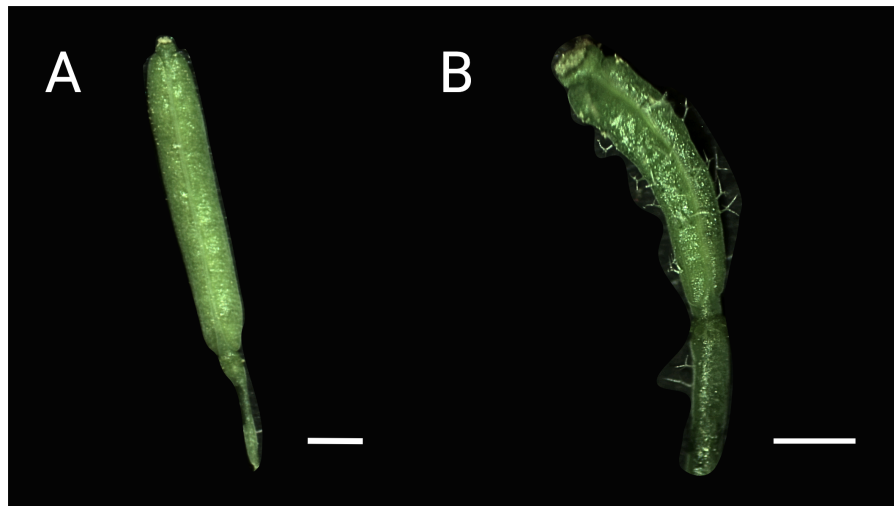


Figure 5.2: The phenotype of an *ag-10 try-29760* gynoecium.
(A) A stage 17 *L-er* silique. (B) A stage 17 *ag-10 try-29760* silique (Smyth et al., 1990). Scale bars: 1 mm.

5.1.3 The role of *SHP1/2* in *Arabidopsis* and *B. rapa*

As outlined in Chapter 4, based on experiments I conducted in *B. rapa* while characterising the *SHP* homologs, the expression domain of the *BrSHPs* differs from that in *AtSHP1/2*. The identification of expression of the *BrSHPs* in stamen tissue was the initial indication that these genes may be functioning in a different manner to *AtSHPs*.

As discussed in Chapter 1, *AG* and *SHP1/2* are closely related, and in fact, if *SHP1/2* expression is induced earlier than in the wild type, they are capable of functionally replacing *AG* (Colombo et al., 2010; Ó Maoiléidigh et al., 2018). However, *AG* activates *SHP1/2* specifically in the gynoecium of *Arabidopsis* only at later stages - when floral organ specification has already occurred - which prevents *SHP1/2* being functionally redundant with *AG* in the wild type (Savidge et al., 1995).

This motivated further studies to be conducted to assess the redundancy of *AtAG* and *AtSHP1/2* further. More specifically, in this research, I focussed on the redundancy in their roles suppressing the initiation of trichomes on gynoecium tissue. Obtaining a “hairy silique” phenotype in *B. rapa* was the primary aim of the grant funding my research, so it was a mechanism we sought to understand more deeply in *Arabidopsis* for use in translational research. Then, as discussed previously, I also sought to gain similar insights in *B. rapa*, as the *BrSHPs* have shown evidence of sharing functional similarities to *AtAG*. For the purposes of this work, the analyses were focussed on *BrSHP2* due to the fact that it displayed much higher expression levels (**Figures 4.10 - 4.13**). This would act as a model for the function of the *BrSHPs*.

5.1.4 The role of *SHP* genes in other species

One active area of research currently in plant biology is the translation of flower development genetics already characterised in *Arabidopsis* to additional species

which are of agronomic interest and are less well characterised than the model organism *Arabidopsis*. This was discussed in Section 1.8. This is useful research to inform plant breeding programs. When analysing the literature, through characterisation of *SHP* genes in species more distantly related to *Arabidopsis* than *B. rapa* – such as apple, cucumber, peach and tomato – expression of the *SHPs* is commonly observed in stamen tissue (van der Linden et al., 2002; Cheng et al., 2020; Tani et al., 2007; Vrebalov2009FleshyiTAGL1/i). In fact, these expression analyses show very similar patterns to those shown in Chapter 4. Moreover, in addition to my own work in *B. rapa*, similar characterisation studies were carried out in our lab by Joseph Beegan in the white mustard crop *Sinapis alba*. *S. alba* is also a member of the family *Brassicaceae*, like *B. rapa* and *Arabidopsis*, so is closely related to the two species. The characterisation of common MADS-domain TFs in *S. alba* through RNA-sequencing experiments revealed that there is also *SHP* expression observed in stamen tissue (**Figure 5.3**). Based on these data together, it would therefore be plausible that *Arabidopsis* is in fact the exceptional species, as mentioned in Section 4.3.

5.1.5 Aims of the chapter

The primary aim of this Chapter was to compare the functions of *AtAG* and *AtSHP1/2* in suppressing floral trichome formation. This was based on previous work from our lab which demonstrated that an overexpression of *SHP2* was capable of partially rescuing the phenotype of *ag try cpc* (Ó Maoiléidigh et al., 2018). Given the objectives of the SFI grant which funded my work (to generate *Brassica* crops with supernumerary trichomes to mitigate herbivory), my aim in this chapter was to establish if it was possible to produce the phenotype of trichomes on siliques by using a *shp1 shp2* double mutant instead of an *ag* mutant. This is briefly mentioned in Section 4.1.4, as it could be extremely beneficial to obtain this phenotype without the need to perturb *AG* function, which could result in defects in floral development and fertility. To this end, I aimed to gen-

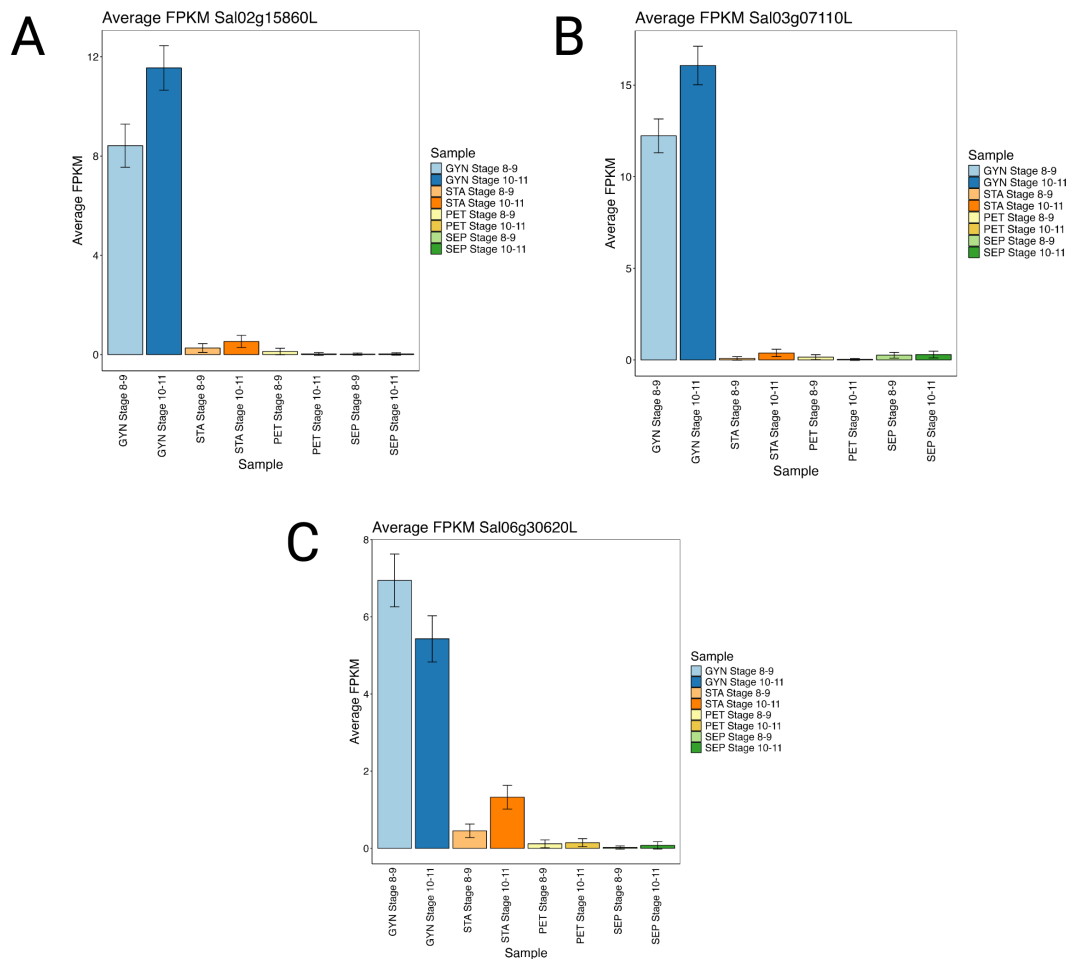


Figure 5.3: The expression of *SH1/2* homologs in the floral organs of *S. alba*. Expression of *SH1* homologs identified in *S. alba*, from RNA-seq experiments performed by Joseph Beegan. **(A)** Sal02g15860L, a homolog of *AtSH1*. **(B)** Sal03g07110L, a homolog of *AtSH1*. **(C)** Sal06g30620L, a homolog of *AtSH2*. Error bars indicate standard errors of three biological replicates. Y-axis shows average Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values, which normalises read counts of a given gene to the gene length and total number of reads. X-axis shows the tissue type, denoted by "GYN" (gynoecium), "STA" (stamen), "PET" (petal) and "SEP" (sepal), and developmental stage, based on Smyth et al. (1990). Figure and data kindly provided by Joseph Beegan.

erate higher-order mutants involving *ag-10*, *shp1-1/shp2-1*, *try-29760* and *cpc-1*. I would then examine their trichome phenotype and complete BAP treatments of selected lines, as cytokinin is a positive regulator of trichome formation (Section 3.1).

The next aim of this chapter was to follow on from the characterisation of the *BrSH1* homologs described in Chapter 4. The data presented in Chapter 4

led us to question whether the *SHP* homologs identified in *B. rapa* could share an increased level of redundancy with the *BrAG* homologs, when compared to the level of redundancy between *AtAG* and *AtSHP1/2*, which is described in the section above. To this end, I endeavoured to analyse the promoter of *BrSHP2* through complementation in *Arabidopsis*. To add to this, I would complete *in silico* analyses to identify binding motifs within the genomic sequences of *AG* and *SHP2* in both *Arabidopsis* and *B. rapa*. This can be done to provide some insight into the similarities and differences of these MADS-box genes across these two species.

5.2 Results

5.2.1 Characterisation of the *ag-10 shp1-1 shp2-1* triple mutant

As previously described (Ó Maoiléidigh et al., 2018), a perturbation of AG through expression of an AG-amiRNA leads to the formation of trichomes on the gynoecium. This phenotype can be partially rescued by SHP2 overexpression, suggesting that AG and the redundant *SHP1/SHP2* genes act together in the suppression of trichome initiation. In agreement with this idea, a perturbation of AG through expression of an AG-amiRNA in a *shp1-1 shp2-1* double-mutant background leads to a slight increase in trichome numbers relative to an AG perturbation in a wild-type background alone (Ó Maoiléidigh et al., 2018). To further assess the level of redundancy between AG and *SHP1/2* in this process, I generated an *ag-10 shp1-1 shp2-1* triple mutant. Given that *ag-10* is a weak allele that does not form trichomes on the gynoecium, I wondered whether a mild perturbation of AG, when combined with a full loss of *SHP1/2* function, would lead to floral trichomes (Ji et al., 2011). The characterisation of the *ag-10 shp1-1 shp2-1* triple mutant did not reveal any evidence of trichome formation on carpel valves. Instead, the triple mutant displayed an additive phenotype with the shorter, bulged gynoecia of *ag-10* and the eponymous shatterproof phenotype of the *shp1/2* double mutant (**Figure 5.4**). Thus, it appears that in *ag-10*, the level of AG activity remaining is sufficient to suppress floral trichome formation without a contribution of the *SHP* genes.

5.2.2 Interaction of SHP1/SHP2 with TRY and cytokinin

To further assess the contribution of *SHP1/2* to the suppression of trichome initiation on the gynoecium, I asked whether disruption of their activities can lead to trichome formation in a background where *TRY* is mutated. As described in Chapter 1, *TRY* is one of the key suppressors of trichome initiation in the gy-

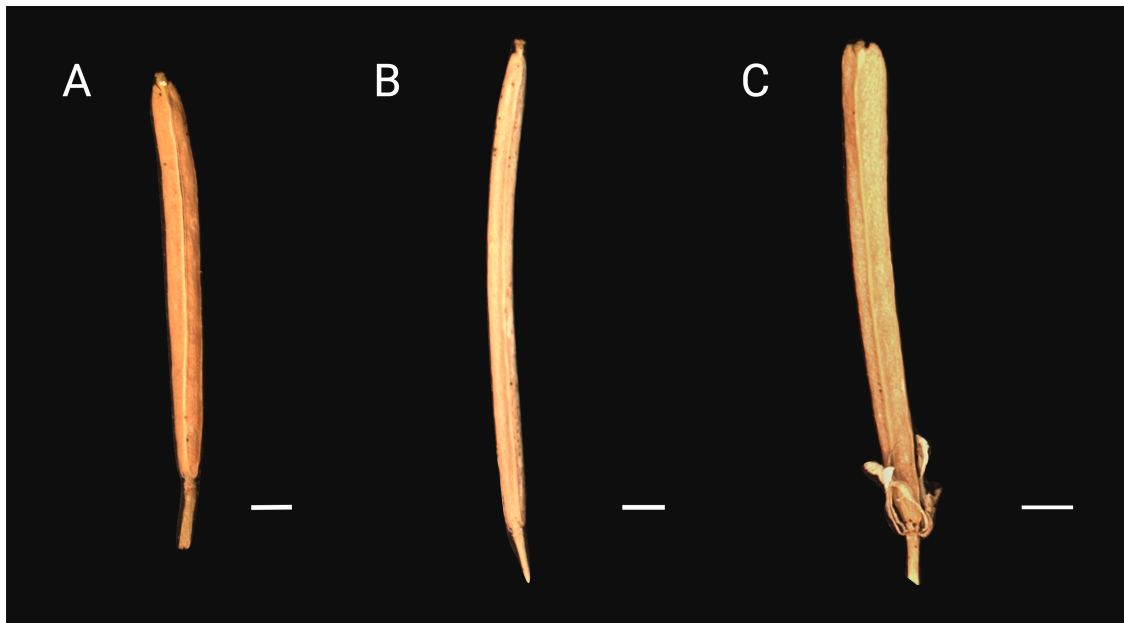


Figure 5.4: The phenotype of the *ag-10 shp1-1 shp2-1* triple mutant. Siliques of stage 18 wild-type (*L-er*) (A); *shp1-1 shp2-1* (B); and *ag-10 shp1-1 shp2-1* (C) flowers are shown. Scale bars: 1 mm.

noecium. However, in *try* mutants, gynoecia are glabrous as long as AG activity is normal in this background (Ó Maoiléidigh et al., 2013; Ó Maoiléidigh et al., 2018).

When I isolated and characterised a *shp1-1 shp2-1 try-29760* triple mutant, I did not observe any trichomes on the gynoecium. Thus, it appears that in this genetic background, sufficient activity for the suppression of trichomes remains. To further test this, I treated plants with BAP, as cytokinin has been shown to have a strong positive effect on trichome initiation on the gynoecium (Figure 1.9). BAP-treated wild-type and *shp1-1 shp2-1* double mutant plants did not form any floral trichomes (Figure 5.5 (A, B)). Although it had been previously reported that BAP treatment of *try-29760* leads occasionally to trichomes on gynoecia (Ó Maoiléidigh et al., 2018), in my experiments, I did not observe this phenotype (Figure 5.5 (C)) perhaps due to slight difference in the treatment conditions. In contrast, BAP treatment of *shp1-1 shp2-1 try-29760* triple-mutant plants led to the formation of a small number of trichomes on the gynoecium

(Figure 5.5 (D)). This suggests that the *shp1 shp2* double-mutant background does indeed de-repress trichome inhibition to some extent.

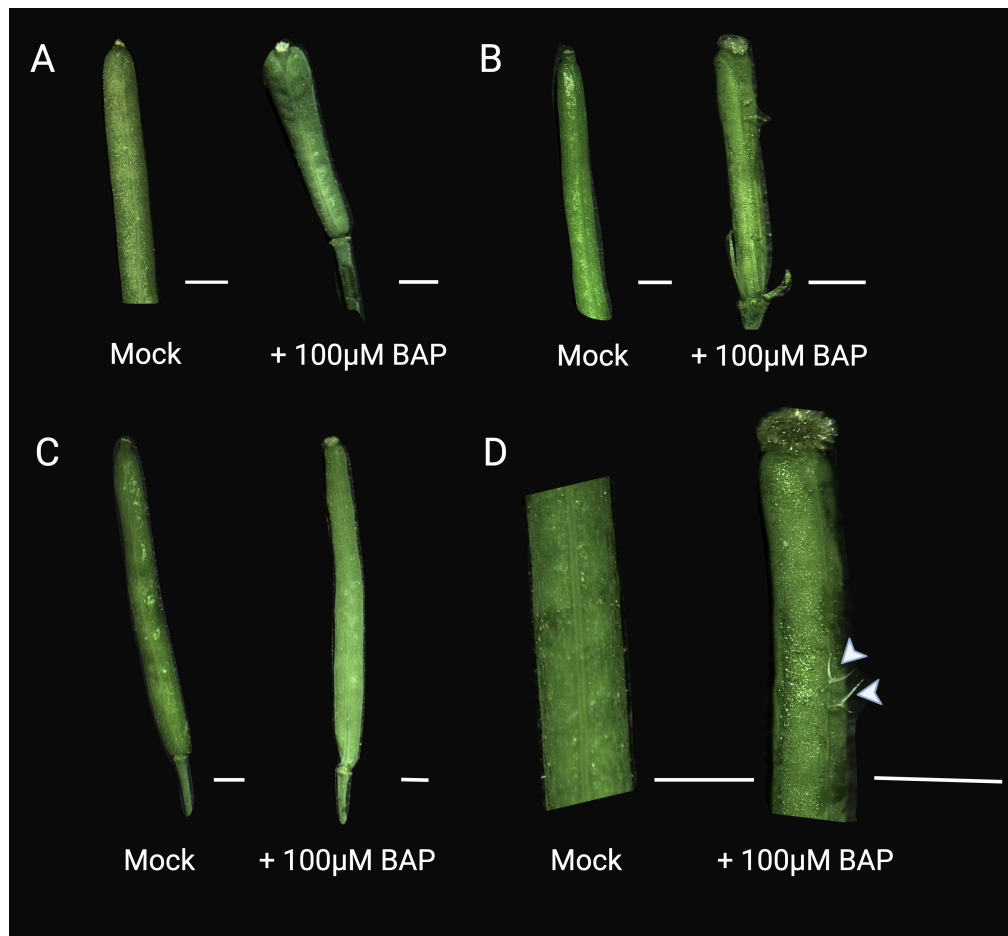


Figure 5.5: The effect of CK on trichome formation on siliques of *shp1-1 shp2-1 try-29760* triple mutant plants.
(A-D) Siliques of plants after mock and BAP treatments. **(A)** Wild type (*L-er*); **(B)** *shp1-1 shp2-1*; **(C)** *try-29760*; **(D)** *shp1-1 shp2-1 try-29760*. Arrow heads indicate trichomes. Scale bars: 1 mm.

5.2.3 Sequence comparison of *BrSHP2* and *AtAG*

The first step toward establishing a potential redundancy between *BrSHP2* and *AtAG* was to compare their coding and protein sequences. This would assist in establishing that the *B. rapa* homologs were appropriately identified in Chapter 4, and whether the genes were in fact homologs of *AtAG*. As seen in **Figure 5.6**, there is a region near the beginning of the *BrSHP2* CDS that shares a high

degree of similarity with *AtAG*. However, it can be seen from the amino acid sequence (Figure 5.7) that this region is in fact the MADS domain, which is highly conserved.

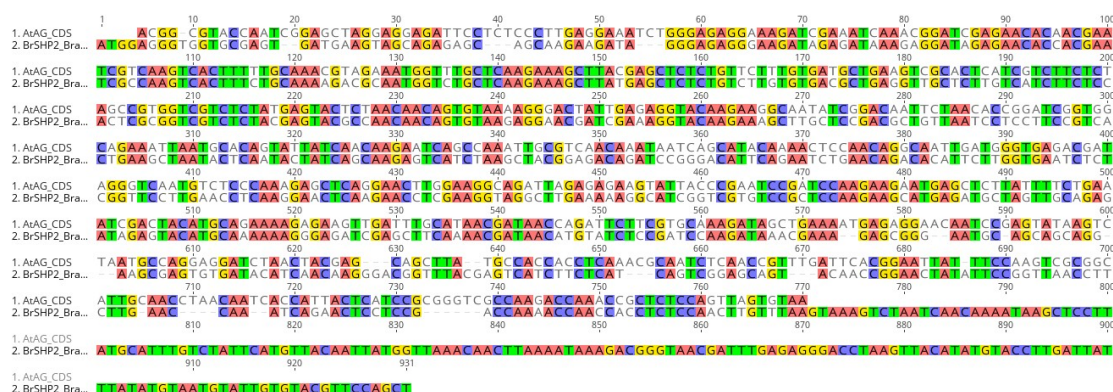


Figure 5.6: CDS alignment of *AtAG* and *BrSHP2*.

Analysis completed using the *Geneious Prime* software. Each base (A, T, G, C) is colour-coded for easier visualisation (red, green, yellow and blue, respectively). When the nucleotides are shaded in colour, this indicates that they are identical across both sequences at this position.

From these analyses, I established that the pairwise identity of *AtAG* CDS and *AtSHP2* CDS is 63.7% (Appendix A1.4), while for *AtAG* and *BrSHP2* it is 64.4% (Figure 5.6). Thus, *BrSHP2* would not be deemed a homolog of *AtAG* as the percentage identity was below 70% (Quiros et al., 2001). Therefore, we were able to proceed with this work using the nomenclature outlined in Chapter 4.

Additionally, the protein sequences of *AtAG* and *AtSHP2* share a 58.3% pairwise identity (Appendix A1.4), while the *AtAG* and *BrSHP2* protein sequences share a 59.4% identity. This alignment also does not suggest that these proteins are in fact orthologs of each other (Figure 5.7) (Quiros et al., 2001). Thus, the assignment of *BrSHP2* as a *SHP* homolog and not a homolog of *AG* in Chapter 4 can be deemed accurate.

Table 5.1: Predicted MADS-domain transcription factor binding sites.

The region which is being analysed is indicated in the top row; "upstream" denotes the 2.1 kb upstream of the start codon; "intron" denotes the largest intron in the genomic sequence. The TFs and their corresponding JASPAR Matrix IDs are also listed. The cut-off for accepting a predicted site was a weighted relative score $\geq 90\%$.

Gene binding site	JASPAR Matrix ID	<i>BrSHP2</i> upstream	<i>BrSHP2</i> intron	<i>AtSHP2</i> upstream	<i>AtSHP2</i> intron	<i>AtAG</i> upstream	<i>AtAG</i> intron
AG	MA0005.1 MA0005.2 MA0005.3	2	1	6	2	5	7
AGL15	MA0548.1 MA0548.2	1	0	3	0	0	3
AP1	MA0005.1 MA0005.2 MA0005.3	2	1	6	2	5	7
AP3	MA0005.1 MA0005.2 MA0005.3	2	1	6	2	5	7
FLC	MA0005.1 MA0005.2 MA0005.3	2	1	6	2	5	7
PI	MA0005.1 MA0005.2 MA0005.3	2	1	6	2	5	7
SEP1	MA0005.1 MA0005.2 MA0005.3	2	1	6	2	5	7
SEP3	MA0005.1 MA0005.2 MA0005.3	2	1	6	2	5	7
SEP4	MA0005.1 MA0005.2 MA0005.3	2	1	6	2	5	7
SOC1	MA0005.1 MA0005.2 MA0005.3	2	1	6	2	5	7
	Total	12	17	29	26	19	38

5.2.5 Complementation of *SHP* expression in *Arabidopsis* using the promoter from *B. rapa*

Due to the findings presented in Chapter 4 that the expression pattern of the *Br-SHP* genes is highly similar to that of *AtAG* (Figures 4.10-4.13), it was important

to establish what may be responsible for this: the *BrSHP* promoters driving their expression, or the BrSHP proteins themselves. For this reason, the *Arabidopsis* and *B. rapa* copies of *SHP2* were driven under their native promoters and their promoters were swapped with each other in additional constructs generated.

In all, these complementation experiments suggested that the promoter of *BrSHP2* (upstream of the start codon) increased the number of lateral shoots in transgenic plants, when compared to constructs where the promoter of *AtSHP2* was used (**Figures 4.18 & 4.19**). In the *AtSHP2* rescue line where the native *Arabidopsis* promoter was used, there was some additional branching compared to the wild type (**Figure 4.18**). However, in the transgenic lines where *BrSHP2* was driven by its native promoter and when *AtSHP2* was driven by the promoter of *BrSHP2*, this phenotype was much more exaggerated (**Figures 4.18 & 4.19**). In some individuals, the phenotype was even more pronounced than in the 35S promoter overexpression lines (**Figure 4.17**). This was another indication that it is possible the *B. rapa* promoter is driving an expression pattern which is similar to *AtAG*. In these lines, a 2.1 kb region upstream of the start codon of either *AtSHP2* or *BrSHP2* was used as a promoter, as this was shown to be sufficient to drive normal expression in *AtSHP2* (Savidge et al., 1995).

To investigate further if the *BrSHP* homologs may function more similarly to *AtAG* than *AtSHP1/2*, I endeavoured to further characterise the promoter activity of *BrSHP2*. Given that it has been previously shown that the large second intron of *AtAG* is sufficient to drive *AG* expression in its expected domains (Lenhard et al., 2001), it was of interest to investigate whether this may hold true in *BrSHP2*. It has been previously shown via *AG* genome localisation studies that the putative promoter sequence of *AtSHP2* is upstream of the start codon, and binding to the large intron does not occur (Ó Maoiléidigh et al., 2013). It has long been accepted in the literature that a region approximately 2.1 kb upstream of the start codon acts as a promoter of *AtSHP2* (Savidge et al., 1995).

As a test of whether there was any difference between using the 5' se-

quence or the intron as a promoter, as in *AG*, *BrSHP2* was driven by the large intron found in the *BrSHP2* genomic sequence (**Figure 5.8**). This investigation was prompted by the findings of the RT-qPCR experiments in Chapter 4, which found that the expression domain of *BrSHP2* is in keeping with that of *AtAG*. Not only did this line rescue the phenotype of a *shp1 shp2* mutant in 6 independent lines, but it also altered the plant architecture in *L-er* plants transformed with this construct, much like the other transgenic lines driven by the upstream promoter of *BrSHP2* (**Figures 5.8 & 4.18, 4.19**). This phenotype was observed in four independent transgenic lines.

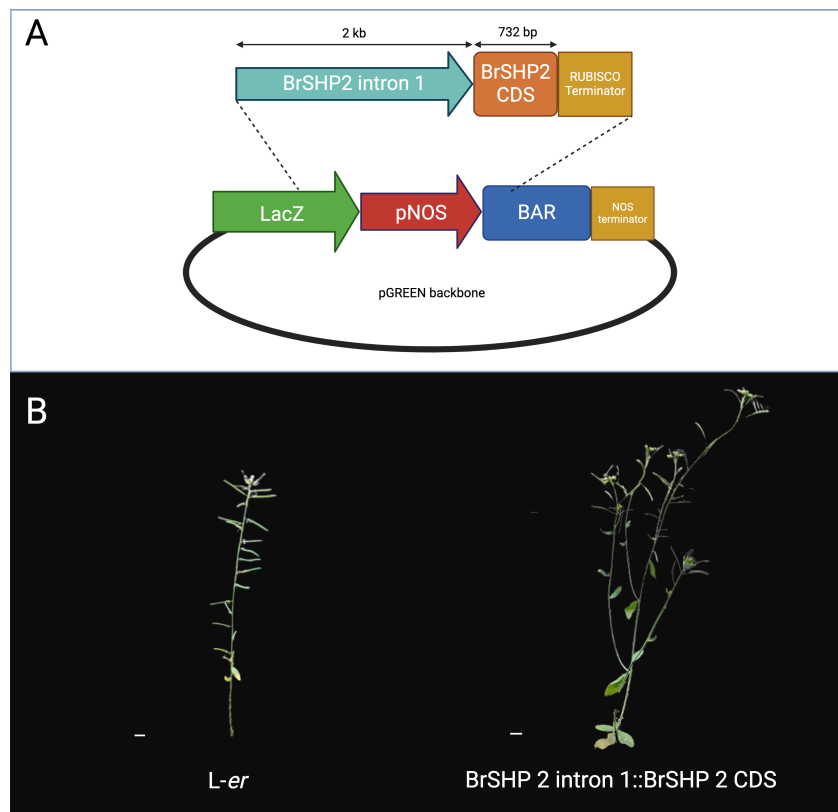


Figure 5.8: Expression of *BrSHP2* driven by its large intron. (A) Map of the construct carrying the first intron of *BrSHP2* as a promoter. Panel created with Biorender.com. (B) Phenotype and plant architecture of the wild type and intron promoter line transformed into *L-er* (labelled). Scale bars: 1 cm.

All together, these data are in keeping with *BrSHP2* having promoter activity similar to *AtAG*, but perhaps not expressed as strongly. This is due to the fact that even when driven by its large intron (**Figure 5.8**), *BrSHP2* introduced

into wild-type *Arabidopsis* does not display secondary flowers growing from the primary floral axis, as observed in *ap1* mutants (Irish and Sussex, 1990). This is the phenotype that would be expected by introducing additional gene copies driven by a promoter that is identical in activity to the *AtAG* promoter.

5.3 Discussion

The aims of this Chapter were to examine the redundancy of *AtAG* and *At-SHP1/2* in their roles as suppressors of trichome formation on floral organs, with a specific focus on the gynoecium due to the objectives of the work which I was funded to complete. As outlined in Section 5.1.1, it had previously been established that *SHP2* overexpression is capable of rescuing the *ag try* carpel trichome phenotype (Ó Maoiléidigh et al., 2018). In this Chapter, I sought to explore whether it was necessary to perturb *AG* function in order to obtain a carpel trichome phenotype.

Through this work, I determined that there is a certain level of redundancy shared between *AG* and *SHP1/2*. However, as demonstrated in Ó Maoiléidigh et al. (2018), *AG* knockdown alone can be sufficient to induce some trichomes on carpels. In a *shp1 shp2* mutant background, additional factors need to be introduced for the trichome inhibition mechanism to be dysregulated enough that trichomes will begin to form on the gynoecium. Specifically in my work, I found that a *shp1 shp2 try* triple mutant, together with exogenous BAP application, is sufficient in *Arabidopsis* for trichomes to form on the gynoecium (**Figure 5.5**).

A secondary aim of this Chapter, motivated by the data presented in Chapter 4, was to examine the function of *BrSHP2* by comparing it to *AtAG* and *At-SHP1/2* in order to establish whether it may behave more similarly to the former, rather than the latter. In previous work conducted under the same grant in our laboratory, Dr Christina Fitzsimons identified and characterised two putative *AG* homologs in *B. rapa* - Bra012564 and Bra013364 – which were then termed *BrAG1* and *BrAG2*, respectively. Upon isolating a double *brag1 brag2* mutant, as it was termed, it was found that flowers did not display homeotic transformations as observed in an *Arabidopsis ag-1* mutant (**Figure 5.9**). A possible reason for this lack of homeotic transformation could be due to additional functional redundancy between the *BrAG* and *BrSHP* genes. As seen in **Figure 5.9**, the

stamens and carpels are impaired but they are still present, unlike in the *ag-1* mutant where stamens and carpels are replaced by additional sepals and petals (Bowman et al., 1989). A sextuple *brag brshp* mutant would need to be observed in order to test this hypothesis.

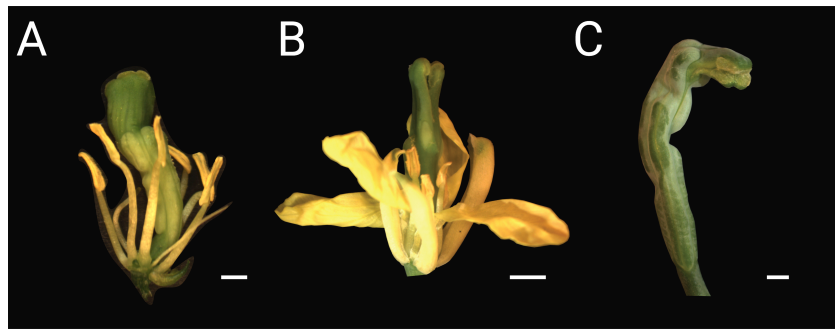


Figure 5.9: The phenotype of *brag1-1 brag2-1* mutant flowers.

Note the impaired, but present, stamens and carpels. **(A)** Open flower with sepals and petals removed to show the reproductive organs. Note the six stamens and two filamentous structures that lack anthers. **(B)** Flower of approximately stage 13-14 (Smyth et al., 1990). All organs are phenotypically normal except the gynoecium, which is partially unfused. **(C)** The bent, shortened silique produced by one of the first flowers of a *brag1-1 brag2-1* plant. Scale bars: 1 mm. Photos provided by Dr Christina Fitzsimons.

As a method of testing whether *BrSHP2* shared more functional similarity with *AtAG* than *AtSHP2* does, I generated a transgenic *Arabidopsis* line where *BrSHP2* was driven by its own large intron. This can be done in *AtAG* to achieve normal expression patterns (Lenhard et al., 2001). As discussed in Chapter 4, transgenic lines expressing additional copies of *SHP2* resemble a weak *AG* over-expression phenotype, due to slight downregulation of *AP1* (Wigge et al., 2005). This was also true for the *BrSHP2* intron 1::*BrSHP2* CDS line generated (**Figure 5.8**). This, accompanied by the rescue of the *shp1 shp2* phenotype by this construct, demonstrates that the large intron of *BrSHP2* is indeed capable of driving its expression. Additionally, I went on to conduct *in silico* analyses to identify binding motifs within the promoters and introns of *AtAG*, *AtSHP2* and *BrSHP2* to assess which of these sequences are likely to act as promoters (**Table 5.1**). This analysis demonstrated that the large intron of *BrSHP2*, like *AtAG*, contains more

binding sites than the region 2.1 kb upstream of the start codon. The converse is true in *AtSHP2*. However, the difference in the number of binding sites across both regions in *AtSHP2* and *BrSHP2* was not as great as it is in *AtAG*.

5.4 Future Work

Obtaining a *shp1 shp2 try cpc* quadruple mutant, as well as an *ag shp1 shp2 try cpc* quintuple mutant and assessing their respective phenotypes would be a key development in ascertaining the redundancy of *AG* and *SHP1/2* in their roles as trichome repressors in *Arabidopsis*. Unfortunately, the quadruple mutant has not yet been isolated and thus could not be examined. The trichome phenotype of the BAP-treated silique of the *shp1 shp2 try* triple mutant was encouraging and led me to question whether the *shp1 shp2 try cpc* quadruple mutant may be sufficient to enable trichomes to form on the gynoecium. One potential issue that I identified during this work is the fact that, in *Arabidopsis*, *SHP2* and *CPC* are both located on the second chromosome. This could potentially lead to linkage of the two genes, due to higher rates of recombination, making it less likely to isolate an individual homozygous for both *shp2-1* and *cpc-1*. These two genes are located at a distance of 5 cM from each other (Delseny et al., 1996), however, which would not normally be expected to pose an issue for linkage.

Another avenue of further research could be in the form of further complementation experiments. As outlined above, the large intron of *BrSHP2* appears to be functional as a promoter, as is the case in *AtAG*. The constructs generated throughout the course of this work were introduced only in to a *shp1 shp2* double mutant background due to their foremost purpose being in the effort to characterise the *BrSHP* homologs as part of the work of Chapter 4. However, given the data presented in Chapter 4 and also in this Chapter, I believe it would be highly informative if these constructs were to also be transformed into an *ag-1* background. If the *BrSHP2* protein is capable of functionally replacing *AG*, or indeed if either of the *BrSHP2* promoter sequences cloned could drive *SHP2* expression such that the *ag-1* phenotype was rescued, this would support the hypothesis presented that *BrSHPs* are functionally more similar to *AtAG* than *AtSHP1/2*.

A1 Appendix

A1.1 Buffers used in molecular biology

A1.1.1 0.5 M ethylenediamine tetraacetic acid (EDTA), pH 8.0

Dissolve 186.1 g $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ in 700 mL H_2O

Adjust pH to 8.0 with 10 M NaOH (≈ 50 mL)

Add H_2O to 1 litre

A1.1.2 DNA extraction buffer for "Edward's preparations"

200 mM Tris-HCl (pH 7.5-8.0)

250 mM NaCl

25 mM EDTA

0.5% (w/v) SDS

A1.1.3 2% Cetyltrimethyl Ammonium Bromide (CTAB) DNA extraction buffer

100 mM Tris-HCl (pH 8.0)

20 mM EDTA

1.4 M NaCl

2% (w/v) cetyltrimethyl ammonium bromide (CTAB)

1% (w/v) polyvinyl pyrrolidone (PVP)

A1.1.4 *Agrobacterium* pH8.0 cell lysis buffer

10 mM Tris-HCl (pH 8)

0.1% Triton X-100

A1.1.5 Tris/EDTA (TE) pH8.0 buffer for resuspending DNA

10 mM Tris (pH 8)

1 mM EDTA

A1.1.6 Tris/acetate/EDTA (TAE) buffer for electrophoresis

50× stock solution:

242 g Tris base

57.1 mL glacial acetic acid

37.2 g Na₂EDTA.2H₂O

ddH₂O to 1 litre

Dilute fifty-fold to 1x working concentration for use in gels and gel tanks

A1.1.7 Agarose gel loading dye

100x stock solution:

0.04 g Bromophenol blue

0.04 g Xylene cyanol FF

0.1 g Orange G

5 mL 100% glycerol

5 mL H₂O

Dilute twenty-fold in 50% glycerol to make a 10x working solution

A1.2 Solutions used for microbiology

A1.2.1 1M CaCl₂ stock solution

Used in the preparation of chemically competent bacterial cells

147 g CaCl₂·2H₂O

Make up with ddH₂O to 1 litre

Dilute 100-fold for a working solution of 10 mM

A1.2.2 Super Optimal Broth (SOB)

Used to culture *E. coli* when preparing chemically competent cells

5g bactotryptone

1.25 g Yeast extract

0.125 g NaCl

Add 250 mL of ddH₂O and transfer solution into a 2 L flask; autoclave. Store at room temperature. Add 2.5 mL of sterile 1 M MgSO₄ and 2.5 mL of sterile 1 M MgCl₂ before use.

A1.2.3 Transformation buffer (TB)

Used to resuspend chemically competent *E. coli* cells

To make 250 mL:

0.76 g PIPES/KOH pH 6.7

0.55 g CaCl₂

4.66 g KCl

2.72 g MnCl₂

Make sure to use PIPES free acid (MW = 302.3). Weigh out substances

except for MnCl₂ and dissolve in water. Adjust pH to 6.7 with KOH. Add MnCl₂ and stir until dissolved. Filter solution (0.45 μ m) and store at 4 °C.

A1.3 Oligonucleotides used in this work

Identifier	Use	Sequence (5' to 3')
DM-682	Genotyping	CACATCATCTCATTGATGCTTGGT
DM-683	Genotyping	GTATAAACTCGTTGGCGACAG
DM-684	Genotyping	AGTAATTCAAGGACAGGTAC
DM-685	Genotyping	TGATAAACTCCCATTTCGATACTGCT
DM-686	Genotyping	AACATCGTTGAACTTGCATCTG
oDS128	Genotyping	TGGTTCACGTAGTGGGCCATCG
DM-733	Genotyping	GTCTACTGATGAGTTGTCACTAGG
DM-734	Genotyping	GTGACGGAAGGAGGGTTGACG
DM-735	Genotyping	GATGCACTCGAAATCAGCCAATTTTAGAC
DM-736	Genotyping	GAGGATAGAGAACACTACGAATCGTC
DM-737	Genotyping	CAGGTCAAGTCAATAGATTCCCTAC
DM-242	RT-qPCR	AAGCGGTTGTGGAGAACATGATACG
DM-243	RT-qPCR	TGGAGAGCTTGATTTGCGAAATACCG
CB_266	RT-qPCR	AACAAAGACGTAATCAACCAGGAAGTG
CB_267	RT-qPCR	CTCCCACTGCCCTTTCGATC
CB_268	RT-qPCR	TTCCGGCTTGAGGTACGAGTG
CB_269	RT-qPCR	AGCTCCTCGAACATCCCTGAC
CB_270	RT-qPCR	CAGTGCAGCAGGTTTCATCAGC
CB_271	RT-qPCR	AACGAGAAGCTAACACAGAGAGAGAC
BT_436	Colony PCR	TGTCCCAGGATTAGAATGATTAGGC
BT_437	Colony PCR	CCCCAGAAATTGAACGCCGAAGAAC
CB_61	RT-qPCR	TATTAACCTTCTTGGAGCGGACACG

CB_62	RT-qPCR	AGTACCCTTCACGCTGTTGTTGG
CB_65	RT-qPCR	ACGCCAGCAACAGTGTGAAGG
CB_66	RT-qPCR	CCCGAATCTGCCTCCGAAGC
CB_69	RT-qPCR	TGAGTACGCTAACAACAGTGTGAAG
CB_70	RT-qPCR	TTCGGTCACAGTAGGAGGGTTG
CB_73	RT-qPCR	TCTTGGTGAATCTCTCGGTTCCCTTG
CB_74	RT-qPCR	CATCTCATGCTTCTTGGAGCGGAC
CB_187	RT-qPCR	ATGGTGATGCTGGTATGG
CB_188	RT-qPCR	TCCTTCTTGTCAACACTCTT
oCF98	RT-qPCR	ACGGAGGGAGCTGAACTGATTG
oCF99	RT-qPCR	AGAACCAGTTCCTCCACCCAATG
CB_118	3' RACE	AGAGCGGGAATGCAGCAGCAGG
CB_119	3' RACE	ACACACTGCGCTAGGCTGGATC
CB_120	3' RACE	AACAAGGCGCGAGATTGAATCC
CB_121	3' RACE	CCGGAGCAGCAGGATTCGAG
CB_122	3' RACE	GGCATCGGTCGTGTCC
CB_123	3' RACE	CGCGTCCGATCCAAGAAG
CB_124	3' RACE	CCGCGTCCGATCCAAG
CB_125	3' RACE	TCAGCCGTGTCCGATC
CB_126	3' RACE	CAAGGGACGGTTTACGAG
CB_127	3' RACE	CTGCGGTTTATGAATCTGGTC
CB_128	3' RACE	AGGGACGGCGGTTTATG
CB_129	3' RACE	CCGGTCTATCTTCCCATG
oCF205	3' RACE	CCAGTGAGCAGAGTGACGAG GACTCGAGCTCAAGC(T) ₁₇
oCF206	3' RACE	CCAGTGAGCAGAGTGACG
oCF207	3' RACE	GAGGACTCGAGCTCAAGC

CB_205	Cloning	AACAGGTCTCAACCTGGGTAA TTTACTTGTCTAATCTGAGTC
CB_206	Cloning	AACAGGTCTCATGTTTTATA TCAACCCTATAGCTTAAAGATA
CB_207	Cloning	AACAGGTCTCAACCTAACC TCTACGAGTACGCCAACAACAG
CB_208	Cloning	AACAGGTCTCATGTTTT AACAGCGTCCGAGCAAG
CB_215	Cloning	AACAGGTCTCAGGCTcc ATGGAGGGTGGTGCGAG
CB_216	Cloning	AACAGGTCTCACTGAAAC AAGTTGGAGAGGTGGTTGG
CB_217	Cloning	AACAGGTCTCAGGCTcc ATGGAGGGTGGTGCGAG
CB_218	Cloning	AACAGGTCTCACTGAAA CAAGTTGCAGAGGTGGTT
CB_219	Cloning	AACAGGTCTCAACCTAT CTCCAACGCATTGTTACG
CB_220	Cloning	AACAGGTCTCATGTTTTC TATAAGCCCTAGCTGAAGT
CB_245	DMAS	TGTCTTGTGTGATGCGGAAaTTG
CB_246	DMAS	TGTCTTGTGTGATGCGGAAaTTA
CB_137	DMAS	ACCCTAATCTTGGCTCTAAACCTCTC
CB_257	DMAS	ACCCTCCTACTGTGACCtAAG
CB_258	DMAS	ACCCTCCTACTGTGACCtAAA
CB_92	DMAS	TGAATGTCCCGAATCTGCCTCC
CB_247	DMAS	TTCTGCAAACGACGCAATaGTC
CB_248	DMAS	TTCTGCAAACGACGCAATaGTT
CB_98	DMAS	GCAAAGATCAAAGGTGCAGGAGAAG

CB_250	DMAS	TGAAGTTGCCCTCGTTATCTcCTC
CB_251	DMAS	TGAAGTTGCCCTCGTTATCTcCTT
CB_259	DMAS	AGATCAAGGTGTAGGTAGGAGAAGC
CB_253	DMAS	GGGTTTTGAGTTTTGCCATAAACCATaTAG
CB_254	DMAS	GGGTTTTGAGTTTTGCCATAAACCATaTAA
CB_104	DMAS	TCGGTTAGAGGATGAAGACGTGAC
CB_237	DMAS	CAAGAAGATAGGGAGAGGGAAGgTAG
CB_238	DMAS	CAAGAAGATAGGGAGAGGGAAGgTAA
CB_239	DMAS	TGGCGATTCGTGGTGTTCCTC

A1.4 Sequence alignments

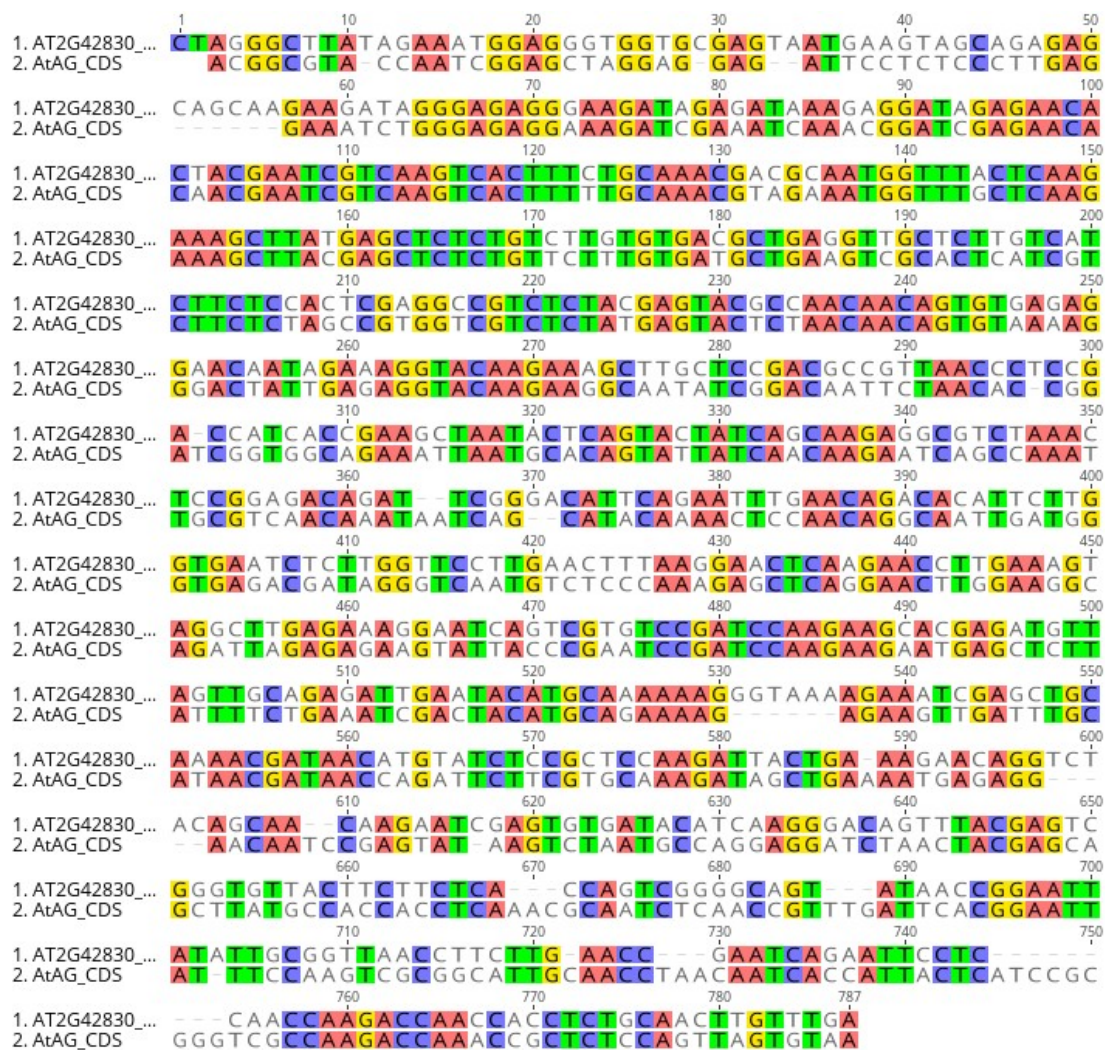


Figure A1.1: Alignment of the *AtSHP2* and *AtAG* CDS.

Analysis completed using the *Geneious Prime* software. Each base (A, T, G, C) is colour-coded for easier visualisation (red, green, yellow and blue, respectively). When the nucleotides are shaded in colour, this indicates that they are identical across both sequences at this position.



Figure A1.2: Alignment of the *AtSHP2* and *AtAG* protein sequences.
Analysis completed using the *Geneious Prime* software.



Figure A1.3: Alignment of the *AtSHP2* and *BrSHP2* genomic sequences. Analysis completed using the *Geneious Prime* software. Each base (A, T, G, C) is colour-coded for easier visualisation (red, green, yellow and blue, respectively). When the nucleotides are shaded in colour, this indicates that they are identical across both sequences at this position. Pairwise identity: 63.5%



Figure A1.4: Alignment of the *AtSHP2* and *BrSHP2* large introns.

Analysis completed using the *Geneious Prime* software. Each base (A, T, G, C) is colour-coded for easier visualisation (red, green, yellow and blue, respectively). When the nucleotides are shaded in colour, this indicates that they are identical across both sequences at this position. Pairwise identity: 63.6%



Figure A1.5: Alignment of the *AtSHP2* and *BrSHP2* genomic sequences. Analysis completed using the *Geneious Prime* software. Each base (A, T, G, C) is colour-coded for easier visualisation (red, green, yellow and blue, respectively). When the nucleotides are shaded in colour, this indicates that they are identical across both sequences at this position. Pairwise identity: 52%



Figure A1.6: Alignment of the *AtAG* and *BrSHP2* large introns. Analysis completed using the *Geneious Prime* software. Each base (A, T, G, C) is colour-coded for easier visualisation (red, green, yellow and blue, respectively). When the nucleotides are shaded in colour, this indicates that they are identical across both sequences at this position. Pairwise identity: 52%

References

- Abbott, R. J., & Gomes, M. F. (1989). Population genetic structure and outcrossing rate of *Arabidopsis thaliana* (L.) Heynh. *Heredity*, 62(3), 411–418. <https://doi.org/10.1038/hdy.1989.56>
- Alonso-Cantabrana, H., Ripoll, J. J., Ochando, I., Vera, A., Ferrándiz, C., & Martínez-Laborda, A. (2007). Common regulatory networks in leaf and fruit patterning revealed by mutations in the *Arabidopsis* ASYMMETRIC LEAVES1 gene. *Development*, 134(14), 2663–2671. <https://doi.org/10.1242/DEV.02864>
- Alvarez-Buylla, E. R., Benítez, M., Corvera-Poiré, A., Chaos Cador, Á., de Folter, S., Gamboa de Buen, A., Garay-Arroyo, A., García-Ponce, B., Jaimes-Miranda, F., Pérez-Ruiz, R. V., Piñeyro-Nelson, A., & Sánchez-Corrales, Y. E. (2010). Flower Development. In *The arabidopsis book* (e0127). BioOne. <https://doi.org/10.1199/tab.0127>
- Alves-Ferreira, M., Wellmer, F., Banhara, A., Kumar, V., Riechmann, J. L., & Meyerowitz, E. M. (2007). Global expression profiling applied to the analysis of *Arabidopsis* stamen development. *Plant Physiology*, 145(3), 747–762. <https://doi.org/10.1104/pp.107.104422>
- Antonelli, A., Fry, C., Smith, R., Eden, J., Govaerts, R., Kersey, P., Nic Lughadha, E., Onstein, R., Simmonds, M., Zizka, A., & Ackerman, J. (2023). *State of the World's Plants and Fungi 2023* (tech. rep.). Royal Botanic Gardens, Kew.

- Bar-On, Y. M., Phillips, R., & Milo, R. (2018). The biomass distribution on Earth. *Proceedings of the National Academy of Sciences*, 115(25), 6506–6511. <https://doi.org/10.1073/pnas.1711842115>
- Bateson, W., Saunders, E., & Punnett, R. (1904). *Experimental Studies in the Physiology of Heredity* (tech. rep.). The Evolution Committee of the Royal Society.
- Bernhardt, C., Lee, M. M., Gonzalez, A., Zhang, F., Lloyd, A., & Schiefelbein, J. (2003). The bHLH genes GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) specify epidermal cell fate in the Arabidopsis root. *Development (Cambridge, England)*, 130(26), 6431–9. <https://doi.org/10.1242/dev.00880>
- Birchler, J. A., & Yang, H. (2022). The multiple fates of gene duplications: Deletion, hypofunctionalization, subfunctionalization, neofunctionalization, dosage balance constraints, and neutral variation. *The Plant Cell*, 34(7), 2466–2474. <https://doi.org/10.1093/plcell/koac076>
- Blanc, G., & Wolfe, K. H. (2004). Functional divergence of duplicated genes formed by polyploidy during Arabidopsis evolution. *The Plant Cell*, 16(7), 1679–1691. <https://doi.org/10.1105/tpc.021410>
- Boettcher, M., & McManus, M. T. (2015). Choosing the right tool for the job: RNAi, TALEN, or CRISPR. *Molecular Cell*, 58(4), 575–585. <https://doi.org/10.1016/j.molcel.2015.04.028>
- Bouyer, D., Geier, F., Kragler, F., Schnittger, A., Pesch, M., Wester, K., Balkunde, R., Timmer, J., Fleck, C., & Hülkamp, M. (2008). Two-dimensional patterning by a trapping/depletion mechanism: The role of TTG1 and GL3 in Arabidopsis trichome formation. *PLoS Biology*, 6(6), e141. <https://doi.org/10.1371/journal.pbio.0060141>
- Bowman, J. L., Smyth, D. R., & Meyerowitz, E. M. (1989). Genes directing flower development in Arabidopsis. *The Plant Cell*, 1(1), 37–52. <https://doi.org/10.1105/tpc.1.1.37>

- Bowman, J. L., Smyth, D. R., & Meyerowitz, E. M. (2012). The ABC model of flower development: then and now. *Development*, 139(22), 4095–4098. <https://doi.org/10.1242/dev.083972>
- Brouns, S. J. J., Jore, M. M., Lundgren, M., Westra, E. R., Slijkhuis, R. J. H., Snijders, A. P. L., Dickman, M. J., Makarova, K. S., Koonin, E. V., & van der Oost, J. (2008). Small CRISPR RNAs Guide Antiviral Defense in Prokaryotes. *Science*, 321(5891), 960–964. <https://doi.org/10.1126/science.1159689>
- Causier, B., Schwarz-Sommer, Z., & Davies, B. (2010). Floral organ identity: 20 years of ABCs. *Seminars in Cell & Developmental Biology*, 21(1), 73–9. <https://doi.org/10.1016/j.semcdb.2009.10.005>
- Charpentier, E., & Doudna, J. A. (2013). Rewriting a genome. *Nature*, 495(7439), 50–51. <https://doi.org/10.1038/495050a>
- Charpentier, E., & Marraffini, L. A. (2014). Harnessing CRISPR-Cas9 immunity for genetic engineering. *Current Opinion in Microbiology*, 19(0), 114–119. <https://doi.org/10.1016/j.mib.2014.07.001>
- Chen, H., Choi, J., & Bailey, S. (2014). Cut Site Selection by the Two Nuclease Domains of the Cas9 RNA-guided Endonuclease. *Journal of Biological Chemistry*, 289(19), 13284–13294. <https://doi.org/10.1074/jbc.M113.539726>
- Cheng, Z., Zhuo, S., Liu, X., Che, G., Wang, Z., Gu, R., Shen, J., Song, W., Zhou, Z., Han, D., & Zhang, X. (2020). The MADS-Box Gene CsSHP Participates in Fruit Maturation and Floral Organ Development in Cucumber. *Frontiers in Plant Science*, 10, 485382. <https://doi.org/10.3389/fpls.2019.01781>
- Clark, J. W., & Donoghue, P. C. J. (2018). Whole-Genome Duplication and Plant Macroevolution. *Trends in plant science*, 23(10), 933–945. <https://doi.org/10.1016/j.tplants.2018.07.006>
- Coen, E. S., & Meyerowitz, E. M. (1991). The war of the whorls: genetic interactions controlling flower development. *Nature*, 353(6339), 31–37. <https://doi.org/10.1038/353031a0>

- Colombo, M., Brambilla, V., Marcheselli, R., Caporali, E., Kater, M. M., & Colombo, L. (2010). A new role for the SHATTERPROOF genes during Arabidopsis gynoecium development. *Developmental Biology*. <https://doi.org/10.1016/j.ydbio.2009.10.043>
- Cui, W., Chen, Z., Shangguan, X., Li, T., Wang, L., Xue, X., & Cao, J. (2022). TRY intron2 determined its expression in inflorescence activated by SPL9 and MADS-box genes in Arabidopsis. *Plant Science*, 321. <https://doi.org/10.1016/J.PLANTSCI.2022.111311>
- Custers, R., & Dima, O. (2022). *Genome-edited crops and 21st century food system challenges* (tech. rep.). Scientific Foresight Unit, European Parliamentary Research Service.
- Dalin, P., Ågren, J., Björkman, C., Huttunen, P., & Kärkkäinen, K. (2008). Leaf Trichome Formation and Plant Resistance to Herbivory. In *Induced plant resistance to herbivory* (pp. 89–105). Springer Netherlands. https://doi.org/10.1007/978-1-4020-8182-8{_}4
- Delseny, M., Raynal, M., Laudié, M., Varoquaux, F., Comella, P., Wu, H. J., Cooke, R., & Grellet, F. (1996). Sequencing and mapping the Arabidopsis genome: a weed model for real crops. *Symposia of the Society for Experimental Biology*, 50, 5–9.
- Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., Eckert, M. R., Vogel, J., & Charpentier, E. (2011). CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature*, 471(7340), 602–607. <https://doi.org/10.1038/nature09886>
- Ditta, G., Pinyopich, A., Robles, P., Pelaz, S., & Yanofsky, M. F. (2004). The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. *Current Biology*, 14(21), 1935–40. <https://doi.org/10.1016/j.cub.2004.10.028>

- Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science*, 346(6213). <https://doi.org/10.1126/science.1258096>
- Drews, G. N., Bowman, J. L., & Meyerowitz, E. M. (1991). Negative regulation of the Arabidopsis homeotic gene AGAMOUS by the APETALA2 product. *Cell*, 65(6), 991–1002. [https://doi.org/10.1016/0092-8674\(91\)90551-9](https://doi.org/10.1016/0092-8674(91)90551-9)
- Duan, W., Song, X., Liu, T., Huang, Z., Ren, J., Hou, X., & Li, Y. (2015). Genome-wide analysis of the MADS-box gene family in Brassica rapa (Chinese cabbage). *Molecular Genetics and Genomics*, 290(1), 239–255. <https://doi.org/10.1007/s00438-014-0912-7>
- Edwards, K., Johnstone, C., & Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic acids research*, 19(6), 1349. <https://doi.org/10.1093/nar/19.6.1349>
- EEA. (2023). Pesticides: what are the risks to our health and to the environment? <https://www.eea.europa.eu/en/newsroom/editorial/pesticides-what-are-the-risks>
- Eliazer Nelson, A. R. L., Ravichandran, K., & Antony, U. (2019). The impact of the Green Revolution on indigenous crops of India. *Journal of Ethnic Foods*, 6(1), 8. <https://doi.org/10.1186/s42779-019-0011-9>
- Espinosa-Soto, C., Immink, R. G., Angenent, G. C., Alvarez-Buylla, E. R., & Folter, S. (2014). Tetramer formation in Arabidopsis MADS domain proteins: analysis of a protein-protein interaction network. *BMC Systems Biology*, 8(1), 9. <https://doi.org/10.1186/1752-0509-8-9>
- FAO. (2015). *Climate change and food systems: global assessments and implications for food security and trade*. (tech. rep.). Food Agriculture Organization of the United Nations.
- Ferrándiz, C., Liljegren, S. J., & Yanofsky, M. F. (2000). Negative regulation of the SHATTERPROOF genes by FRUITFULL during Arabidopsis fruit development. *Science*. <https://doi.org/10.1126/science.289.5478.436>

- Flanagan, C. A., Hu, Y., & Ma, H. (1996). Specific expression of the AGL1 MADS-box gene suggests regulatory functions in Arabidopsis gynoecium and ovule development. *The Plant journal : for cell and molecular biology*, 10(2), 343–53. <https://doi.org/10.1046/j.1365-313x.1996.10020343.x>
- Galway, M. E., Masucci, J. D., Lloyd, A. M., Walbot, V., Davis, R. W., & Schiefelbein, J. W. (1994). The TTG gene is required to specify epidermal cell fate and cell patterning in the Arabidopsis root. *Developmental Biology*, 166(2), 740–754. <https://doi.org/10.1006/dbio.1994.1352>
- Garneau, J. E., Dupuis, M.-È., Villion, M., Romero, D. A., Barrangou, R., Boyaval, P., Fremaux, C., Horvath, P., Magadán, A. H., & Moineau, S. (2010). The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature*, 468(7320), 67–71. <https://doi.org/10.1038/nature09523>
- German, D. A., Hendriks, K. P., Koch, M. A., Lens, F., Lysak, M. A., Bailey, C. D., Mummenhoff, K., & Al-Shehbaz, I. A. (2023). An updated classification of the Brassicaceae (Cruciferae). *PhytoKeys*, 220, 127–144. <https://doi.org/10.3897/phytokeys.220.97724>
- Girin, T., Sorefan, K., & Østergaard, L. (2009). Meristematic sculpting in fruit development. *Journal of Experimental Botany*, 60(5), 1493–1502. <https://doi.org/10.1093/jxb/erp031>
- Goethe, J. W. V. (1790). *The Metamorphosis of Plants* (1st). Gotha, C. W. Ettinger. <https://www.loc.gov/item/agr08000317/>
- Gómez-Felipe, A., Marconi, M., Branchini, E., Wang, B., Bertrand-Rakusova, H., Stan, T., Burkiewicz, J., Folter, S. d., Routier-Kierzkowska, A.-L., Wabnik, K., & Kierzkowski, D. (2023). Competing differentiation gradients coordinate fruit morphogenesis. *bioRxiv*, 2023.01.19.524793. <https://doi.org/10.1101/2023.01.19.524793>
- González-Reig, S., Ripoll, J. J., Vera, A., Yanofsky, M. F., & Martínez-Laborda, A. (2012). Antagonistic Gene Activities Determine the Formation of Pat-

- tern Elements along the Mediolateral Axis of the Arabidopsis Fruit. *PLoS Genet*, 8(11), 1003020. <https://doi.org/10.1371/journal.pgen.1003020>
- Goslin, K., Zheng, B., Serrano-Mislata, A., Rae, L., Ryan, P. T., Kwa, K., Thomson, B., Ó'maoiléidigh, D. S., Madueño, F., Wellmer, F., & Graciet, E. (2017). Transcription Factor Interplay between LEAFY and APETALA1/CAULIFLOWER during Floral Initiation. *Plant Physiology*, 174, 1097–1109. <https://doi.org/10.1104/pp.17.00098>
- Goto, K., Kyoizuka, J., & Bowman, J. L. (2001). Turning floral organs into leaves, leaves into floral organs. *Current opinion in genetics & development*, 11(4), 449–56. [https://doi.org/10.1016/s0959-437x\(00\)00216-1](https://doi.org/10.1016/s0959-437x(00)00216-1)
- Gramzow, L., & Theissen, G. (2010). A hitchhiker's guide to the MADS world of plants. *Genome Biology*, 11(6), 214. <https://doi.org/10.1186/gb-2010-11-6-214>
- Gu, Q., Ferrándiz, C., Yanofsky, M. F., & Martienssen, R. (1998). The FRUITFULL MADS-box gene mediates cell differentiation during Arabidopsis fruit development. *Development (Cambridge, England)*.
- Hedenus, F., Wirsenius, S., & Johansson, D. J. A. (2014). The importance of reduced meat and dairy consumption for meeting stringent climate change targets. *Climatic Change*, 124(1-2), 79–91. <https://doi.org/10.1007/s10584-014-1104-5>
- Hendriks, K. P., Kiefer, C., Al-Shehbaz, I. A., Bailey, C. D., Hooft van Huysduy-
nen, A., Nikolov, L. A., Nauheimer, L., Zuntini, A. R., German, D. A.,
Franzke, A., Koch, M. A., Lysak, M. A., Toro-Núñez, Ó., Özüdoğru, B.,
Invernón, V. R., Walden, N., Maurin, O., Hay, N. M., Shushkov, P., ...
Lens, F. (2023). Global Brassicaceae phylogeny based on filtering of 1,000-
gene dataset. *Current Biology*, 33(19), 4052–4068. <https://doi.org/10.1016/j.cub.2023.08.026>

- Honma, T., & Goto, K. (2001). Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature*, 409(6819), 525–529. <https://doi.org/10.1038/35054083>
- Hugouvieux, V., Silva, C. S., Jourdain, A., Stigliani, A., Charras, Q., Conn, V., Conn, S. J., Carles, C. C., Parcy, F., & Zubieta, C. (2018). Tetramerization of MADS family transcription factors SEPALLATA3 and AGAMOUS is required for floral meristem determinacy in Arabidopsis. *Nucleic Acids Research*, 46(10), 4966–4977. <https://doi.org/10.1093/nar/gky205>
- Hülkamp, M., Miséra, S., & Jürgens, G. (1994). Genetic dissection of trichome cell development in Arabidopsis. *Cell*, 76(3), 555–566. [https://doi.org/10.1016/0092-8674\(94\)90118-X](https://doi.org/10.1016/0092-8674(94)90118-X)
- Inoue, H., Nojima, H., & Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. *Gene*, 96(1), 23–28. [https://doi.org/10.1016/0378-1119\(90\)90336-P](https://doi.org/10.1016/0378-1119(90)90336-P)
- IPNI. (2024). International Plant Names Index. <http://www.ipni.org>
- Irish, V. F., & Sussex, I. M. (1990). Function of the *apetala-1* gene during Arabidopsis floral development. *The Plant Cell*, 2(8), 741–53. <https://doi.org/10.1105/tpc.2.8.741>
- Ito, T., Ng, K.-H., Lim, T.-S., Yu, H., & Meyerowitz, E. M. (2007). The homeotic protein AGAMOUS controls late stamen development by regulating a jasmonate biosynthetic gene in Arabidopsis. *The Plant cell*, 19(11), 3516–29. <https://doi.org/10.1105/tpc.107.055467>
- Ito, T., Wellmer, F., Yu, H., Das, P., Ito, N., Alves-Ferreira, M., Riechmann, J. L., & Meyerowitz, E. M. (2004). The homeotic protein AGAMOUS controls microsporogenesis by regulation of SPOROCTELESS. *Nature*, 430(6997), 356–360. <https://doi.org/10.1038/nature02733>
- Ji, L., Liu, X., Yan, J., Wang, W., Yumul, R. E., Kim, Y. J., Dinh, T. T., Liu, J., Cui, X., Zheng, B., Agarwal, M., Liu, C., Cao, X., Tang, G., & Chen, X. (2011). ARGONAUTE10 and ARGONAUTE1 Regulate the Termination of Floral

- Stem Cells through Two MicroRNAs in Arabidopsis (L.-J. Qu, Ed.). *PLoS Genetics*, 7(3), e1001358. <https://doi.org/10.1371/journal.pgen.1001358>
- Jiang, W.-k., Liu, Y.-l., Xia, E.-h., & Gao, L.-z. (2013). Prevalent Role of Gene Features in Determining Evolutionary Fates of Whole-Genome Duplication Duplicated Genes in Flowering Plants. *Plant Physiology*, 161(4), 1844–1861. <https://doi.org/10.1104/pp.112.200147>
- Jiao, Y., & Meyerowitz, E. M. (2010). Cell-type specific analysis of translating RNAs in developing flowers reveals new levels of control. *Molecular systems biology*, 6, 419. <https://doi.org/10.1038/msb.2010.76>
- Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science*, 337(6096), 816–821. <https://doi.org/10.1126/science.1225829>
- Kaufmann, K., Wellmer, F., Muino, J. M., Ferrier, T., Wuest, S. E., Kumar, V., Serrano-Mislata, A., Madueno, F., Krajewski, P., Meyerowitz, E. M., Angenent, G. C., & Riechmann, J. L. (2010). Orchestration of Floral Initiation by APETALA1. *Science*, 328(5974), 85–89. <https://doi.org/10.1126/science.1185244>
- Kaufmann, K., Muiño, J. M., Jauregui, R., Airoidi, C. A., Smaczniak, C., Krajewski, P., & Angenent, G. C. (2009). Target Genes of the MADS Transcription Factor SEPALLATA3: Integration of Developmental and Hormonal Pathways in the Arabidopsis Flower (D. Weigel, Ed.). *PLoS Biology*, 7(4), e1000090. <https://doi.org/10.1371/journal.pbio.1000090>
- Klepikova, A. V., Kasianov, A. S., Gerasimov, E. S., Logacheva, M. D., & Penin, A. A. (2016). A high resolution map of the *Arabidopsis thaliana* developmental transcriptome based on RNA-seq profiling. *The Plant Journal*, 88(6), 1058–1070. <https://doi.org/10.1111/tpj.13312>

- Koornneef, M., & Meinke, D. (2010). The development of Arabidopsis as a model plant. *The Plant journal : for cell and molecular biology*, 61(6), 909–21. <https://doi.org/10.1111/j.1365-313X.2009.04086.x>
- Lampropoulos, A., Sutikovic, Z., Wenzl, C., Maegele, I., Lohmann, J. U., & Forner, J. (2013). GreenGate - A Novel, Versatile, and Efficient Cloning System for Plant Transgenesis (P. J. Janssen, Ed.). *PLoS ONE*, 8(12), e83043. <https://doi.org/10.1371/journal.pone.0083043>
- Lefever, S., Rihani, A., Van der Meulen, J., Pattyn, F., Van Maerken, T., Van Dorpe, J., Hellemans, J., & Vandesompele, J. (2019). Cost-effective and robust genotyping using double-mismatch allele-specific quantitative PCR. *Scientific Reports*, 9(1), 2150. <https://doi.org/10.1038/s41598-019-38581-z>
- Lenhard, M., Bohnert, A., Jürgens, G., & Laux, T. (2001). Termination of stem cell maintenance in Arabidopsis floral meristems by interactions between WUSCHEL and AGAMOUS. *Cell*, 105(6), 805–14. [https://doi.org/10.1016/s0092-8674\(01\)00390-7](https://doi.org/10.1016/s0092-8674(01)00390-7)
- Lenser, T., & Theissen, G. (2014). Quantifying Fruit Dehiscence Using the Random Impact Test (RIT). *BIO-PROTOCOL*, 4(15). <https://doi.org/10.21769/BioProtoc.1200>
- Li, F., Kitashiba, H., Inaba, K., & Nishio, T. (2009). A Brassica rapa linkage map of EST-based SNP markers for identification of candidate genes controlling flowering time and leaf morphological traits. *DNA research : an international journal for rapid publication of reports on genes and genomes*, 16(6). <https://doi.org/10.1093/DNARES/DSP020>
- Lieber, M. R. (2010). The Mechanism of Double-Strand DNA Break Repair by the Nonhomologous DNA End-Joining Pathway. *Annual Review of Biochemistry*, 79(1), 181–211. <https://doi.org/10.1146/annurev.biochem.052308.093131>

- Liljegren, S. J., Ditta, G. S., Eshed, Y., Savidge, B., Bowmant, J. L., & Yanofsky, M. F. (2000). SHATTERPROOF MADS-box genes control dispersal in *Arabidopsis*. *Nature*. <https://doi.org/10.1038/35008089>
- Ma, H., Yanofsky, M. F., & Meyerowitz, E. M. (1991). AGL1-AGL6, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. *Genes & development*, 5(3), 484–95. <https://doi.org/10.1101/gad.5.3.484>
- Marsch-Martínez, N., Ramos-Cruz, D., Irepan Reyes-Olalde, J., Lozano-Sotomayor, P., Zúñiga-Mayo, V. M., & de Folter, S. (2012). The role of cytokinin during *Arabidopsis* gynoecia and fruit morphogenesis and patterning. *The Plant Journal*, 72(2), 222–234. <https://doi.org/10.1111/j.1365-313X.2012.05062.x>
- Marsch-Martínez, N., Zúñiga-Mayo, V. M., Herrera-Ubaldo, H., Ouwerkerk, P. B. F., Pablo-Villa, J., Lozano-Sotomayor, P., Greco, R., Ballester, P., Balanzá, V., Kuijt, S. J. H., Meijer, A. H., Pereira, A., Ferrándiz, C., & de Folter, S. (2014). The NTT transcription factor promotes replum development in *Arabidopsis* fruits. *The Plant Journal*, 80(1), 69–81. <https://doi.org/10.1111/tpj.12617>
- Masucci, J. D., Rerie, W. G., Foreman, D. R., Zhang, M., Galway, M. E., Marks, M. D., & Schiefelbein, J. W. (1996). The homeobox gene GLABRA2 is required for position-dependent cell differentiation in the root epidermis of *Arabidopsis thaliana*. *Development (Cambridge, England)*, 122(4), 1253–60. <https://doi.org/10.1242/dev.122.4.1253>
- McBride, K. E., & Summerfelt, K. R. (1990). Improved binary vectors for *Agrobacterium*-mediated plant transformation. *Plant Molecular Biology*, 14(2), 269–276. <https://doi.org/10.1007/BF00018567>
- McLaughlin, H. M., Lü, T.-F., Natarajan, B., Østergaard, L., & Dong, Y. (2024). Conserved auxin signalling synergism through the canonical and ETT-mediated pathways promotes gynoecium development in members of

- the Brassicaceae. *bioRxiv*, 2023.03.02.530771. <https://doi.org/10.1101/2023.03.02.530771>
- Melzer, R., & Theissen, G. (2009). Reconstitution of 'floral quartets' in vitro involving class B and class E floral homeotic proteins. *Nucleic Acids Research*. <https://doi.org/10.1093/nar/gkp129>
- Meyerowitz, E. M. (2001). Prehistory and History of Arabidopsis Research. *Plant Physiology*, 125(1), 15–19. <https://doi.org/10.1104/pp.125.1.15>
- Michaels, S. D., & Amasino, R. M. (2008). Memories of winter: vernalization and the competence to flower. *Plant, Cell & Environment*, 23(11), 1145–1153. <https://doi.org/10.1046/j.1365-3040.2000.00643.x>
- Mizukami, Y., & Ma, H. (1997). Determination of Arabidopsis floral meristem identity by AGAMOUS. *The Plant cell*, 9(3), 393–408. <https://doi.org/10.1105/tpc.9.3.393>
- Mizukami, Y., & Ma, H. (1992). Ectopic expression of the floral homeotic gene AGAMOUS in transgenic Arabidopsis plants alters floral organ identity. *Cell*, 71(1), 119–131. [https://doi.org/10.1016/0092-8674\(92\)90271-D](https://doi.org/10.1016/0092-8674(92)90271-D)
- Montgomery, K., Walden-Schreiner, C., Saffer, A., Jones, C., Seliger, B. J., Worm, T., Tateosian, L., Shukunobe, M., Kumar, S., & Meentemeyer, R. K. (2023). Forecasting global spread of invasive pests and pathogens through international trade. *Ecosphere*, 14(12). <https://doi.org/10.1002/ecs2.4740>
- Morgan, C., Bruce, D., Child, R., Ladbroke, Z., & Arthur, A. (1998). Genetic variation for pod shatter resistance among lines of oilseed rape developed from synthetic *B. napus*. *Field Crops Research*, 58(2), 153–165. [https://doi.org/10.1016/S0378-4290\(98\)00099-9](https://doi.org/10.1016/S0378-4290(98)00099-9)
- Mulugeta Habtewold, T., & Heshmati, A. (2023). Impacts of improved agricultural technologies on food security and child nutrition in rural Ethiopia. *Cogent Food & Agriculture*, 9(2). <https://doi.org/10.1080/23311932.2023.2276565>

- Nagaharu, U. (1935). Genome analysis in Brassica with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Japanese Journal of Botany*, 7(7), 389–452.
- Nutter, F., Teng, P., & Royer, M. (1993). Terms and Concepts for Yield, Crop Loss, and Disease Thresholds. *Plant Disease*, 77(2). <https://doi.org/10.1094/PD-77-211>
- Ó Maoiléidigh, D. S., Graciet, E., & Wellmer, F. (2014). Genetic Control of *Arabidopsis* Flower Development. In *Advances in botanical research* (pp. 159–190). <https://doi.org/10.1016/B978-0-12-417162-6.00006-7>
- Ó Maoiléidigh, D. S., Stewart, D., Zheng, B., Coupland, G., & Wellmer, F. (2018). Floral homeotic proteins modulate the genetic program for leaf development to suppress trichome formation in flowers. *Development*, 145(3). <https://doi.org/10.1242/dev.157784>
- Ó Maoiléidigh, D. S., Wuest, S. E., Rae, L., Raganelli, A., Ryan, P. T., Kwaśniewska, K., Das, P., Lohan, A. J., Loftus, B., Graciet, E., & Wellmer, F. (2013). Control of Reproductive Floral Organ Identity Specification in *Arabidopsis* by the C Function Regulator AGAMOUS. *The Plant Cell*, 25(7), 2482–2503. <https://doi.org/10.1105/tpc.113.113209>
- Oerke, E. C. (2006). Crop losses to pests. *The Journal of Agricultural Science*, 144(1), 31–43. <https://doi.org/10.1017/S0021859605005708>
- Oerke, E. C., Dehne, H., Schönbeck, F., & Weber, A. (1994). *Crop Production and Crop Protection*. Elsevier. <https://doi.org/10.1016/C2009-0-00683-7>
- Oppenheimer, D., Herman, P., Sivakumaran, S., Esch, J., & Marks, M. (1991). A myb gene required for leaf trichome differentiation in *Arabidopsis* is expressed in stipules. *Cell*, 67(3). [https://doi.org/10.1016/0092-8674\(91\)90523-2](https://doi.org/10.1016/0092-8674(91)90523-2)
- Ori, N., Eshed, Y., Chuck, G., Bowman, J. L., & Hake, S. (2000). Mechanisms that control knox gene expression in the *Arabidopsis* shoot. *Development*

- (Cambridge, England), 127(24), 5523–32. <https://doi.org/10.1242/dev.127.24.5523>
- Pajoro, A., Madrigal, P., Muiño, J. M., Matus, J. T., Jin, J., Mecchia, M. A., Debernardi, J. M., Palatnik, J. F., Balazadeh, S., Arif, M., Ó'Maoiléidigh, D. S., Wellmer, F., Krajewski, P., Riechmann, J. L., Angenent, G. C., & Kaufmann, K. (2014). Dynamics of chromatin accessibility and gene regulation by MADS-domain transcription factors in flower development. *Genome Biology*, 15(3). <https://doi.org/10.1186/gb-2014-15-3-r41>
- Panchy, N., Lehti-Shiu, M., & Shiu, S.-H. (2016). Evolution of Gene Duplication in Plants. *Plant Physiology*, 171(4), 2294–316. <https://doi.org/10.1104/pp.16.00523>
- Parcy, F. (2005). Flowering: a time for integration. *The International Journal of Developmental Biology*, 49(5-6), 585–93. <https://doi.org/10.1387/ijdb.041930fp>
- Parcy, F., Nilsson, O., Busch, M. A., Lee, I., & Weigel, D. (1998). A genetic framework for floral patterning. *Nature*, 395(6702), 561–566. <https://doi.org/10.1038/26903>
- Pattanaik, S., Patra, B., Singh, S. K., & Yuan, L. (2014). An overview of the gene regulatory network controlling trichome development in the model plant, Arabidopsis. *Frontiers in Plant Science*, 5(259). <https://doi.org/10.3389/fpls.2014.00259>
- Payne, C. T., Zhang, F., & Lloyd, A. M. (2000). GL3 encodes a bHLH protein that regulates trichome development in arabidopsis through interaction with GL1 and TTG1. *Genetics*, 156(3), 1349–62. <https://doi.org/10.1093/genetics/156.3.1349>
- Pelaz, S., Tapia-López, R., Alvarez-Buylla, E. R., & Yanofsky, M. F. (2001). Conversion of leaves into petals in Arabidopsis. *Current Biology*, 11(3), 182–4. [https://doi.org/10.1016/s0960-9822\(01\)00024-0](https://doi.org/10.1016/s0960-9822(01)00024-0)

- Pelaz, S., Ditta, G. S., Baumann, E., Wisman, E., & Yanofsky, M. F. (2000). B and C floral organ identity functions require SEPALLATA MADS-box genes. *Nature*, 405(6783), 200–203. <https://doi.org/10.1038/35012103>
- Pesch, M., & Hülskamp, M. (2009). One, two, three...models for trichome patterning in Arabidopsis? *Current Opinion in Plant Biology*, 12(5), 587–92. <https://doi.org/10.1016/j.pbi.2009.07.015>
- Pesch, M., & Hülskamp, M. (2004). Creating a two-dimensional pattern de novo during Arabidopsis trichome and root hair initiation. *Current Opinion in Genetics & Development*, 14(4), 422–7. <https://doi.org/10.1016/j.gde.2004.06.007>
- Pinyopich, A., Ditta, G. S., Savidge, B., Liljegren, S. J., Baumann, E., Wisman, E., & Yanofsky, M. F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature*, 424(6944), 85–88. <https://doi.org/10.1038/nature01741>
- Price, J., Hobson, R., Neale, M., & Bruce, D. (1996). Seed Losses in Commercial Harvesting of Oilseed Rape. *Journal of Agricultural Engineering Research*, 65(3), 183–191. <https://doi.org/10.1006/jaer.1996.0091>
- Provart, N. J., Alonso, J., Assmann, S. M., Bergmann, D., Brady, S. M., Brkljacic, J., Browse, J., Chapple, C., Colot, V., Cutler, S., Dangl, J., Ehrhardt, D., Friesner, J. D., Frommer, W. B., Grotewold, E., Meyerowitz, E., Nemhauser, J., Nordborg, M., Pikaard, C., ... McCourt, P. (2016). 50 years of Arabidopsis research: highlights and future directions. *The New Phytologist*, 209(3), 921–44. <https://doi.org/10.1111/nph.13687>
- Purugganan, M. D. (2019). Evolutionary Insights into the Nature of Plant Domestication. *Current Biology*, 29(14), R705–R714. <https://doi.org/10.1016/j.cub.2019.05.053>
- Quiros, C. F., Grellet, F., Sadowski, J., Suzuki, T., Li, G., & Wroblewski, T. (2001). Arabidopsis and Brassica comparative genomics: sequence, structure and gene content in the ABI-Rps2-Ck1 chromosomal segment and related re-

- gions. *Genetics*, 157(3), 1321–30. <https://doi.org/10.1093/genetics/157.3.1321>
- Rajani, S., & Sundaresan, V. (2001). The Arabidopsis myc/bHLH gene ALCA-TRAZ enables cell separation in fruit dehiscence. *Current Biology*, 11(24), 1914–1922. [https://doi.org/10.1016/S0960-9822\(01\)00593-0](https://doi.org/10.1016/S0960-9822(01)00593-0)
- Raven, P. H. (2021). Plants make our existence possible. *PLANTS, PEOPLE, PLANET*, 3(1), 2–6. <https://doi.org/10.1002/ppp3.10173>
- Rerie, W., Feldmann, K., & Marks, M. (1994). The GLABRA2 gene encodes a homeo domain protein required for normal trichome development in Arabidopsis. *Genes & Development*, 8(12). <https://doi.org/10.1101/GAD.8.12.1388>
- Roeder, A. H., Ferrándiz, C., & Yanofsky, M. F. (2003). The Role of the REPLUM-LESS Homeodomain Protein in Patterning the Arabidopsis Fruit. *Current Biology*, 13(18), 1630–1635. <https://doi.org/10.1016/J.CUB.2003.08.027>
- Ryan, P. T., Ó'Maoiléidigh, D. S., Drost, H. G., Kwaśniewska, K., Gabel, A., Grosse, I., Graciet, E., Quint, M., & Wellmer, F. (2015). Patterns of gene expression during Arabidopsis flower development from the time of initiation to maturation. *BMC Genomics*, 16(1). <https://doi.org/10.1186/s12864-015-1699-6>
- Saha, G., Park, J.-I., Jung, H.-J., Ahmed, N. U., Kayum, M. A., Chung, M.-Y., Hur, Y., Cho, Y.-G., Watanabe, M., & Nou, I.-S. (2015). Genome-wide identification and characterization of MADS-box family genes related to organ development and stress resistance in Brassica rapa. *BMC genomics*, 16(1), 178. <https://doi.org/10.1186/s12864-015-1349-z>
- Savary, S., Mille, B., Rolland, B., & Lucas, P. (2006a). Patterns and management of crop multiple pathosystems. In *Plant disease epidemiology: Facing challenges of the 21st century* (pp. 123–138). Springer Netherlands. https://doi.org/10.1007/1-4020-5020-8_9

- Savary, S., Teng, P. S., Willocquet, L., & Nutter, F. W. (2006b). Quantification and Modeling of Crop Losses: A Review of Purposes. *Annual Review of Phytopathology*, 44(1), 89–112. <https://doi.org/10.1146/annurev.phyto.44.070505.143342>
- Savidge, B., Rounsley, S., & Yanofsky, M. (1995). Temporal relationship between the transcription of two Arabidopsis MADS box genes and the floral organ identity genes. *The Plant Cell*, 7(6). <https://doi.org/10.1105/TPC.7.6.721>
- Schellmann, S., Schnittger, A., Kirik, V., Wada, T., Okada, K., Beerman, A., Thumfahrt, J., Jürgens, G., & Hülskamp, M. (2002). TRIPTYCHON and CAPRICE mediate lateral inhibition during trichome and root hair patterning in Arabidopsis. *EMBO Journal*, 21(19), 5036–5046. <https://doi.org/10.1093/emboj/cdf524>
- Schmülling, T., Werner, T., Riefler, M., Krupková, E., & Bartrina y Manns, I. (2003). Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis and other species. *Journal of Plant Research*, 116(3), 241–252. <https://doi.org/10.1007/s10265-003-0096-4>
- Schwab, R., Ossowski, S., Riester, M., Warthmann, N., & Weigel, D. (2006). Highly specific gene silencing by artificial microRNAs in Arabidopsis. *Plant Cell*, 18(5), 1121–1133. <https://doi.org/10.1105/tpc.105.039834>
- Scotto-Lavino, E., Du, G., & Frohman, M. A. (2007). 3' End cDNA amplification using classic RACE. *Nature Protocols*, 1(6), 2742–2745. <https://doi.org/10.1038/nprot.2006.481>
- Shang, X., Cao, Y., & Ma, L. (2017). Alternative splicing in plant genes: A means of regulating the environmental fitness of plants. *International Journal of Molecular Sciences*, 18(2). <https://doi.org/10.3390/ijms18020432>
- Simpson, G. G., & Dean, C. (2002). Arabidopsis, the Rosetta stone of flowering time? *Science*, 296(5566), 285–9. <https://doi.org/10.1126/science.296.5566.285>

- Smyth, D. R., Bowman, J. L., & Meyerowitz, E. M. (1990). Early flower development in *Arabidopsis*. *Plant Cell*, 2(8), 755–767. <https://doi.org/10.1105/tpc.2.8.755>
- Song, X.-M., Wang, J.-P., Sun, P.-C., Ma, X., Yang, Q.-H., Hu, J.-J., Sun, S.-R., Li, Y.-X., Yu, J.-G., Feng, S.-Y., Pei, Q.-Y., Yu, T., Yang, N.-S., Liu, Y.-Z., Li, X.-Q., Paterson, A. H., & Wang, X.-Y. (2020). Preferential gene retention increases the robustness of cold regulation in Brassicaceae and other plants after polyploidization. *Horticulture Research*, 7(1), 20. <https://doi.org/10.1038/s41438-020-0253-0>
- Soroka, J. J., Holowachuk, J. M., Gruber, M. Y., & Grenkow, L. F. (2011). Feeding by flea beetles (Coleoptera: Chrysomelidae; *Phyllotreta* spp.) is decreased on canola (*Brassica napus*) seedlings with increased trichome density. *Journal of Economic Entomology*, 104(1), 125–36. <https://doi.org/10.1603/ec10151>
- Stephenson, P., Baker, D., Girin, T., Perez, A., Amoah, S., King, G. J., & Østergaard, L. (2010). A rich TILLING resource for studying gene function in *Brassica rapa*. *BMC Plant Biology*, 10(62). <https://doi.org/10.1186/1471-2229-10-62>
- Stephenson, P., Stacey, N., Brüser, M., Pullen, N., Ilyas, M., O'Neill, C., Wells, R., & Østergaard, L. (2019). The power of model-to-crop translation illustrated by reducing seed loss from pod shatter in oilseed rape. *Plant Reproduction*. <https://doi.org/10.1007/s00497-019-00374-9>
- Stokstad, E. (2018). European Union expands ban of three neonicotinoid pesticides. *Science*. <https://doi.org/10.1126/science.aau0152>
- Szymanski, D. B., & Marks, M. D. (1998). GLABROUS1 overexpression and TRIPTYCHON alter the cell cycle and trichome cell fate in *Arabidopsis*. *The Plant Cell*, 10(12), 2047–62. <https://doi.org/10.1105/tpc.10.12.2047>
- Tani, E., Polidoros, A. N., & Tsaftaris, A. S. (2007). Characterization and expression analysis of FRUITFULL- and SHATTERPROOF-like genes from

- peach (*Prunus persica*) and their role in split-pit formation. *Tree Physiology*, 27(5), 649–659. <https://doi.org/10.1093/treephys/27.5.649>
- Theissen, G., & Saedler, H. (2001). Plant biology. Floral quartets. *Nature*, 409(6819), 469–71. <https://doi.org/10.1038/35054172>
- Theissen, G., Melzer, R., & Rümpler, F. (2016). MADS-domain transcription factors and the floral quartet model of flower development: linking plant development and evolution. *Development*, 143(18). <https://doi.org/10.1242/dev.134080>
- Tominaga, R., & Wada, T. (2017). Triptychon (TRY) Protein Accumulation in the Roots of Mutant Auxinresistant 1 (axr1) *Arabidopsis thaliana*. *Journal of Plant Biochemistry & Physiology*, 05(03). <https://doi.org/10.4172/2329-9029.1000192>
- Ulijaszek, S. J. (1991). Human dietary change. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 334(1270), 271–279. <https://doi.org/10.1098/rstb.1991.0116>
- van der Linden, C., Vosman, B., & Smulders, M. (2002). Cloning and characterization of four apple MADS box genes isolated from vegetative tissue. *Journal of Experimental Botany*, 53(371), 1025–1036. <https://doi.org/10.1093/jexbot/53.371.1025>
- Wada, T., Tachibana, T., Shimura, Y., & Okada, K. (1997). Epidermal cell differentiation in *Arabidopsis* determined by a Myb homolog, CPC. *Science*, 277(5329), 1113–1116. <https://doi.org/10.1126/science.277.5329.1113>
- Walker, A. R., Davison, P. A., Bolognesi-Winfield, A. C., James, C. M., Srinivasan, N., Blundell, T. L., Esch, J. J., Marks, M. D., & Gray, J. C. (1999). The TRANSPARENT TESTA GLABRA1 Locus, Which Regulates Trichome Differentiation and Anthocyanin Biosynthesis in *Arabidopsis*, Encodes a WD40 Repeat Protein. *The Plant Cell*, 11(7), 1337–1350. <https://doi.org/10.1105/tpc.11.7.1337>

- Wang, L., Yu, P., Lyu, J., Hu, Y., Han, C., Bai, M.-Y., & Fan, M. (2021). BZR1 Physically Interacts with SPL9 to Regulate the Vegetative Phase Change and Cell Elongation in Arabidopsis. *International Journal of Molecular Sciences*, 22(19), 10415. <https://doi.org/10.3390/ijms221910415>
- Wang, S., & Chen, J.-G. (2014). Regulation of cell fate determination by single-repeat R3 MYB transcription factors in Arabidopsis. *Frontiers in Plant Science*, 5, 87297. <https://doi.org/10.3389/fpls.2014.00133>
- Wang, Z.-P., Xing, H.-L., Dong, L., Zhang, H.-Y., Han, C.-Y., Wang, X.-C., & Chen, Q.-J. (2015). Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in Arabidopsis in a single generation. *Genome Biology*, 16(1), 144. <https://doi.org/10.1186/s13059-015-0715-0>
- Wellmer, F., Alves-Ferreira, M., Dubois, A., Riechmann, J. L., & Meyerowitz, E. M. (2006). Genome-Wide Analysis of Gene Expression during Early Arabidopsis Flower Development. *PLoS Genetics*, preprint(2006), e117. <https://doi.org/10.1371/journal.pgen.0020117.eor>
- Wellmer, F., Graciet, E., & Riechmann, J. L. (2014). Specification of floral organs in Arabidopsis. *Journal of Experimental Botany*, 65(1), 1–9. <https://doi.org/10.1093/jxb/ert385>
- Wellmer, F., Riechmann, J. L., Alves-Ferreira, M., & Meyerowitz, E. M. (2004). Genome-Wide Analysis of Spatial Gene Expression in Arabidopsis Flowers. *The Plant Cell*, 16(5), 1314–1326. <https://doi.org/10.1105/tpc.021741>
- Wendel, J. F. (2000). Genome evolution in polyploids. *Plant Molecular Biology*, 42(1), 225–49.
- Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., & Schmülling, T. (2003). Cytokinin-Deficient Transgenic Arabidopsis Plants Show Multiple Developmental Alterations Indicating Opposite Functions of Cytokinins in the Regulation of Shoot and Root Meristem Activity. *The Plant Cell*, 15(11), 2532–2550. <https://doi.org/10.1105/tpc.014928>

- Werner, T., Motyka, V., Strnad, M., & Schmülling, T. (2001). Regulation of plant growth by cytokinin. *Proceedings of the National Academy of Sciences*, 98(18), 10487–10492. <https://doi.org/10.1073/pnas.171304098>
- Wigge, P. A., Kim, M. C., Jaeger, K. E., Busch, W., Schmid, M., Lohmann, J. U., & Weigel, D. (2005). Integration of spatial and temporal information during floral induction in *Arabidopsis*. *Science*, 309(5737), 1056–9. <https://doi.org/10.1126/science.1114358>
- Winter, C. M., Austin, R. S., Blanvillain-Baufumé, S., Reback, M. A., Monniaux, M., Wu, M.-F., Sang, Y., Yamaguchi, A., Yamaguchi, N., Parker, J. E., Parcy, F., Jensen, S. T., Li, H., & Wagner, D. (2011). LEAFY target genes reveal floral regulatory logic, cis motifs, and a link to biotic stimulus response. *Developmental Cell*, 20(4), 430–43. <https://doi.org/10.1016/j.devcel.2011.03.019>
- Winter, C. M., Yamaguchi, N., Wu, M.-F., & Wagner, D. (2015). Transcriptional programs regulated by both LEAFY and APETALA1 at the time of flower formation. *Physiologia Plantarum*, 155(1), 55–73. <https://doi.org/10.1111/ppl.12357>
- Winter, K.-U., Saedler, H., & Theissen, G. (2002). On the origin of class B floral homeotic genes: functional substitution and dominant inhibition in *Arabidopsis* by expression of an orthologue from the gymnosperm *Gnetum*. *The Plant Journal*, 31(4), 457–75. <https://doi.org/10.1046/j.1365-313x.2002.01375.x>
- Wolfe, K. H. (2001). Yesterday's polyploids and the mystery of diploidization. *Nature Reviews Genetics*, 2(5), 333–341. <https://doi.org/10.1038/35072009>
- Wu, W., Huang, X., Cheng, J., Li, Z., de Folter, S., Huang, Z., Jiang, X., Pang, H., & Tao, S. (2011). Conservation and Evolution in and among SRF- and MEF2-Type MADS Domains and Their Binding Sites. *Molecular Biology and Evolution*, 28(1), 501–511. <https://doi.org/10.1093/molbev/msq214>

- Wuest, S. E., O'Maoileidigh, D. S., Rae, L., Kwasniewska, K., Raganelli, A., Hanczaryk, K., Lohan, A. J., Loftus, B., Graciet, E., & Wellmer, F. (2012). Molecular basis for the specification of floral organs by APETALA3 and PISTILLATA. *Proceedings of the National Academy of Sciences of the United States of America*, 109(33), 13452–7. <https://doi.org/10.1073/pnas.1207075109>
- Yu, N., Cai, W.-J., Wang, S., Shan, C.-M., Wang, L.-J., & Chen, X.-Y. (2010). Temporal control of trichome distribution by microRNA156-targeted SPL genes in *Arabidopsis thaliana*. *The Plant Cell*, 22(7), 2322–35. <https://doi.org/10.1105/tpc.109.072579>
- Zhang, X., Henriques, R., Lin, S.-S., Niu, Q.-W., & Chua, N.-H. (2006). Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nature Protocols*, 1(2), 641–646. <https://doi.org/10.1038/nprot.2006.97>
- Zúñiga-Mayo, V. M., Reyes-Olalde, J. I., Marsch-Martinez, N., & de Folter, S. (2014). Cytokinin treatments affect the apical-basal patterning of the *Arabidopsis* gynoecium and resemble the effects of polar auxin transport inhibition. *Frontiers in Plant Science*, 5(191), 191. <https://doi.org/10.3389/fpls.2014.00191>