THE PRODUCTION OF FEMALE-STERILE FLOWERS IN *ARABIDOPSIS THALIANA* (L.) HEYNH. AND *BRASSICA NAPUS* L.

A Thesis Submitted to the College
of Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Biology
University of Saskatchewan
Saskatoon

By
Jeffrey Dennis Pylatuik
Fall 2001

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries at this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or in part should be addressed to:

Head of the Department of Biology
University of Saskatchewan
112 Science Place
Saskatoon, Saskatchewan, S7N 5E2

ABSTRACT

The objectives of this study were to examine the feasibility of producing female-sterile flowers in *Arabidopsis thaliana* and *Brassica napus*, while concurrently identifying the contribution of each of those genes involved in reproductive organ development and testing the integrity of the ABC model for flower development in *B. napus*.

The production and viability of pollen produced by an *Arabidopsis* mutant, *flo10-1*, was examined. *flo10-1* mutants are female-sterile due to the proliferation of additional stamens in place of the gynoecium. Although the quantity and viability of pollen produced by the median and lateral stamens of *flo10-1* did not differ from wild type flowers, the additional stamens of *flo10-1* produced significantly less pollen of lower viability than the stamens of wild-type flowers. However, the latter still effectively fertilized ovules, producing normal, viable seed. To create a floral phenotype similar to *flo10-1*, it was decided to alter the floral morphology using homeotic genes involved in the ABC model.

The *Arabidopsis* class C homeotic gene *AGAMOUS* (*AG*) is expressed primarily in the reproductive organs and is required for their proper development. Expression by *Arabidopsis AG* regulatory elements was tested in *B. napus*. Spatially and temporally, the expression pattern of *AG* in *B. napus* was very similar to that in *Arabidopsis*, with the exception that no expression was observed in the ovules of *B. napus*.

A cDNA isolated and cloned from *Brassica napus*, *BnAP3*, was found to be homologous to the B class homeotic gene *APETALA3* of *Arabidopsis*. In order to convert a wild-type gynoecium to stamens, *BnAP3* was targeted to the reproductive organs of *Arabidopsis* and *B. napus* flowers under the control of the *AG* regulatory elements. In both species, sepals, petals, and stamens of transformed plants were identical to those of wild-type plants, while the gynoecium of transformed plants was replaced with four organs that were partially converted to stamens. The conversion was only partial because L2 and L3 derived tissues of these organs were converted to those

of stamens while L1 derived tissues retained their carpeloid properties. The number, arrangement, and vascular supply of organs produced in the fourth whorl however, indicated that organ number and patterning were not random, but were possibly governed by the patterning of the vestigial gynoecium of the transformed flowers.

Finally, a gene orthologous to *SHATTERPROOF1* (*SHP1*) of *Arabidopsis*, *BnSHP1* was isolated and characterized from *B. napus*. *SHP1* is required for the proper development of dehiscence zones in the gynoecium of *Arabidopsis*. Sequence analysis of the large first intron of *BnSHP1*, as well as the regulatory regions of both *SHP1* and *SHP2* of *Arabidopsis*, identified a conserved LEAFY binding site within these genes, suggesting that they may be regulated, in part, by the LEAFY protein.

These studies were successful in that they demonstrated that (1) the approach taken to generate female sterility in flowers can be successful, (2) homologous genes controlling flower development were identified in *B. napus*, (3) aspects of the ABC model of flower development hold true in *B. napus*, and (4) when the identity of the gynoecium of *Arabidopsis* is altered, the number and patterning of organs that are produced are not random, suggesting that the patterning is governed by the vestigial gynoecium.

PERMISSION TO REPRODUCE

Chapter 2 is a revised version of:

Pylatuik, J.D., Bonham-Smith, P.C. and Davis, A.R. (1998) The additional stamens of *flo10-1* mutants of *Arabidopsis thaliana* are compromised in production and viability of pollen. *Can. J. Bot.*, **76,** 1733-1742.

and is reprinted with the kind permission of National Research Council of Canada, NRC Press, Ottawa, ON, K1A 0R6, Canada (Permission granted August 14, 2001).

ACKNOWLEDGEMENTS

I am indebted to my supervisors Dr. Peta Bonham-Smith and Dr. Arthur Davis who have made possible this opportunity of a lifetime. In addition to providing guidance through the labyrinth of science, they have taught by example, how to be master teachers. Their devoted time, patience and unlimited resourcefulness are qualities I can only hope to reciprocate to others in the future.

I am grateful to Dr. Vipen Sawhney for his valuable insight and thought-provoking discussions about my research as well as Drs. William Crosby and Isobel Parkin for their help and encouragement throughout the project. A special thanks to Professor Emeritus Taylor Steeves for his insightful discussions and valuable comments on my work. Many thanks to Dennis Dyck, Jeaniene Smith, Yukio Yano and Dr. Susan Gilmer for technical assistance.

I am grateful for the support, encouragement, and friendship of my colleagues; Kerri McIntosh, Lester Young, Rebecca Cross, Ashley Byun, Kathy Lockhart, Laureen Blahut-Beatty, Jacquie Hulm, Donna Lindsay-Postnikoff, Song Wang, and Zhuan Chen who, over the years, have been a constant resource of information. I would also like to express my sincere appreciation to the summer students who helped with aspects of my work, Erin Gunderson and Stephanie Dotchin.

I am forever indebted to my parents for their unconditional love, support and encouragement. From my father Dennis, I learned the ability of abstract thought, reason and determination. From my mother Sonia, I learned the benefit that attention to detail brings. Thank you both.

Finally, my beautiful wife Jacqueline, you are my greatest discovery. My inspiration, confidence, and strength of mind I owe to you. Above all else, this work is a product of your love and understanding.

PGS-A and PGS-B Natural Sciences and Engineering Research Council Awards and a Dean's Allocation University of Saskatchewan Graduate Scholarship are gratefully acknowledged. This research was supported by grants from the Natural

Sciences and Engineering Research Council awarded to Dr. P. C. Bonham-Smith and Dr. A. R. Davis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX FIGURES

Chapter 1. GENERAL INTRODUCTION

1.1 The importance of *Brassica napus*

The Brassicaceae is the largest family of the Brassicales, an order characterized by the presence of glucosinolates and consisting of more than 4100 species in over 400 genera (Judd *et al.*, 1999). Although the vegetation among members of this family can take the form of trees, shrubs, or herbs (Judd *et al.*, 1999), the floral architecture is remarkably stable (Endress, 1992), predominantly containing bilaterally symmetrical flowers of four sepals, four petals, six stamens, and a bi-loculate gynoecium. Both *Brassica napus* L. and *Arabidopsis thaliana* (L.) Heynh., are important members of this family.

History suggests that *B. napus* was cultivated for its seed oil as early as 22,000 years ago in India, and although it was primarily used in cooking, its demand as a lubricant led to its introduction into Canada in the 1940's (Canola Council of Canada, 1999). Varieties of *B. napus*, bred to contain less than 2% erucic acid and less than 30 μmoles/g glucosinolates in the seed, are now referred to by the registered trademark 'canola', of which Canada is the world's second largest producer (Canola Council of Canada, 1999). *B. napus* therefore, plays a very important role in Canada's agricultural economy.

1.2 The benefits and shortcomings of hybrid *B. napus*

Hybrid plants, derived from heterozygous seed from crosses between two distinct genotypes, often produce higher yields and/or higher seed quality. In *B. napus*, heterosis for seed yield can average as high as 50%, with a range of 20% to 80% (Lefort-Buson and Dattee, 1982). These high levels of heterosis are sufficient to ensure widespread

adoption of hybrid *B. napus* in the future, however at present, low efficiency of hybrid seed production is one factor deterring a large-scale adoption (McVetty, 1995).

The inherent obstacle to promoting outcrossing (and thus heterosis) in *B. napus* is the species' self-pollinating, bisexual flower. Current production techniques for *B. napus* hybrid seed have commonly overcome this problem by generating male-sterility in the acceptor or A-line of the system. However, pollen donor lines, which contain one or more dominant restorer gene(s) for male sterility (R-line), are invariably self-pollinating and are usually destroyed when flowering is complete. Therefore physical separation between A and R-lines is required to facilitate mechanized hybrid seed production.

To facilitate the movement of pollen from R-line rows to A-line rows, an insect pollen vector is required. Both honey bees (*Apis mellifera* L.) and leafcutter bees (*Megachile rotundata* F.) have been tested and successfully used in hybrid seed production (McVetty *et al.*, 1995; Pinnisch and McVetty, 1990; Hogarth *et al.*, 1995). However, cross-pollination decreases linearly with increased distance between A and R-lines (Zasshi, 1988; Pinnisch and McVetty, 1990). Therefore, as distance from the pollen source increases, bee activity becomes a major factor affecting yield (Hogarth *et al.*, 1995).

Hybrid seed yield and mean heterosis are severely decreased as A:R row ratios increase, due to increased distance between lines. Highest yields were obtained with a 1A:1R row ratio, which is completely impractical for mechanized hybrid seed production (McVetty *et al.*, 1995). Current row ratios of 3A:3R are used for hybrid seed production but yield two to four times less than the ideal 1A:1R row ratio (McVetty *et al.*, 1995). Yield could therefore be substantially increased if A and R-lines could be placed closer to each other.

Most bees forage along a row rather than crossing rows (Robinson, 1984); therefore, bees which depart a plant within a R-line row are more likely to move to another nearby R-line plant, rather than a distant A-line plant. This behavior further decreases the effectiveness of cross-pollination. Therefore, yield could also be increased if A and R-lines did not have to be segregated into separate rows, but rather could be randomly dispersed.

1.3 The importance of *Arabidopsis* as a model for flower development

Arabidopsis thaliana, due to its small stature, rapid life cycle, low repetitive DNA content and small, recently sequenced genome, has become an important laboratory tool in understanding plant science. Although very little is known about the genetic mechanisms controlling flower development in *B. napus*, reasonable progress has been made in understanding this process in *Arabidopsis*. However, the genetic control of flower development is now thought to be largely conserved in distantly related plant species (reviewed in Ma and dePamphilis, 2000). The process of flowering in *Arabidopsis* can be broken down into several stages including (1) the transition from vegetative to reproductive growth at the shoot apical meristem, (2) the formation of the inflorescence meristem, (3) the formation and identity determination of the floral organs, and (4) floral organ growth and differentiation (Okada and Shimura, 1994).

In the third stage of floral development, floral organs originate from the floral meristem which is composed of three layers of undifferentiated cells called the L1, L2, and L3 histogenic layers. Coordinated cell division and expansion between the cells of the three layers are necessary to generate the floral organs. Analysis of mutations affecting the formation and identity of floral organs has lead to a model of flower development called the ABC model (Coen and Meyerowitz, 1991). This model suggests that several sets of homeotic genes (A, B, and C) act alone or in combination to direct floral organ formation and identity in *Arabidopsis*. Depending on the spatial domain of gene activity, the identity of developing organs can be predicted. Where A is active, sepals arise; where A and B are active, petals are formed; where B and C are active, stamens arise; and where C alone is active, female reproductive structures arise. Although the identity of the sepals, petals, stamens (male reproductive organs) and gynoecium (female reproductive unit) can be predicted by this model, the nectarium develops independently (Davis *et al.*, 1993; Baum *et al.*, 2001). By altering the domains of gene activity, the identity of organs that arise can be altered accordingly, referred to as homeotic conversions.

1.4 Objectives

It was reasoned that current systems of *B. napus* hybrid seed production could be made 2 to 4-fold more efficient by developing an R-line that could not self-fertilize, and would therefore not require physical segregation from the A-line. This type of approach would minimize distance and row-segregation between A and R-lines, resulting in a predicted increase of yield and heterosis, while facilitating the mechanization of production. A female-sterile line of *B. napus*, serving as a pollen donor and a vector for restorer genes, could potentially serve as a suitable R-line with any current hybrid system. Based on the taxonomic affinity between *Arabidopsis* and *B. napus* and the general conservation of floral development across species, it was reasonable to assume that the *Arabidopsis* model of flower development could also be applied to *B. napus*.

Using this background as a fundamental rationale, the overall objective of this thesis was to examine the feasibility of creating female-sterile flowers, in both *Arabidopsis* and *B. napus*, while also identifying genes controlling flower development and testing the integrity of the ABC model of flower development in *B. napus*.

In Chapter 2 a detailed study of an existing female-sterile *Arabidopsis* mutant called *flo10-1* (Schultz *et al.*, 1991) or *superman* (*sup-2*) (Bowman *et al.*, 1992). As there were no known data available concerning pollen production in flowers rendered female-sterile, I was interested in comparing pollen quantity, viability, size, and germinability between the mutant female-sterile and wild-type plants. Moreover, *flo10-1* is female sterile due to the proliferation of additional stamens in place of the gynoecium. It was also of interest to compare the level of viability and production of the pollen of the additional stamens with that from the six stamens occupying the normal "wild-type" locations within *flo10-1* flowers.

To create female-sterile flowers, I wanted to specifically target the genetic alterations to the reproductive organs in order to restrict the phenotypical consequences to these regions of the flower and limit the biological implication on the whole plant. In *Arabidopsis*, the C class gene *AGAMOUS* (*AG*) is expressed primarily in the reproductive organs (Yanofsky *et al.*, 1990). In Chapter 3, I investigated the conservation of *AG* expression, by placing the regulatory elements of *AG* from

Arabidopsis thaliana into *Brassica napus* and analysed the subsequent expression pattern. I hypothesised that, based on the close taxonomic relatedness of the two species, the *AG* regulatory elements would direct a similar expression pattern in *B. napus*. Although examination of *AG* expression in the more distantly related *Linum usitatissimum* was carried out by my colleague, Rebecca H. Cross, the results of this work have been included in this thesis for completeness.

Previous work had shown that by ectopically expressing the class B gene *APETALA3* (*AP3*) of *Arabidopsis*, the organs that make up the gynoecium undergo a homeotic conversion to stamens, thus rendering the plant female-sterile (Jack *et al.*, 1994). In Chapter 4, the equivalent class B gene from *B. napus* (*BnAP3*) was cloned. I examined the effects of expressing *BnAP3*, under the control of the *AG* regulatory factors assessed in Chapter 4. I predicted that this approach would generate female-sterile flowers in plants of both *Arabidopsis* and *B. napus*. This procedure would also allow me to test for functional homology of *BnAP3* with *AP3*, as well as the integrity of the ABC model in *B. napus*.

Finally, another gene was isolated from *B. napus* (*BnSHP1*), which appeared to be expressed primarily in the gynoecium of *B. napus*. In Chapter 5 the sequence and function of this gene was compared with a putative ortholog from *Arabidopsis* (Ma *et al.*, 1991). Having the genomic sequence of the gene, I began to look for possible regulatory elements. Because the first intron of this gene was unusually large, I predicted that regulatory elements might exist within it, similar to the closely related *AG* gene (Sieburth and Meyerowitz, 1997).

1.5 Literature cited

Baum, S.F., Eshed, Y. and Bowman, J.L. (2001) The *Arabidopsis* nectary is an ABC independent floral organ. *Development*, (In Press).

Bowman, J.L., Sakai, H., Jack, T., Weigel, D., Mayer, U. and Meyerowitz, E.M. (1992) *SUPERMAN*, a regulator of floral homeotic genes in *Arabidopsis*. *Development*, **114**, 599-615.

Canola Council of Canada (1999) Canola Growers Manual. Winnipeg: Canola Council of Canada.

Coen. E.S. and Meyerowitz, E.M. (1991) The war of the whorls: genetic interactions controlling flower development. *Nature*, **353**, 31-37.

Davis, A.R., Haughn, G.W. and Sawhney, V.K. (1993) Preliminary investigation of the nectary in floral mutants of *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae). *Proc. Int. Symp. Polln. Trop.*, 9-14.

Endress, P. K. (1992) Evolution and floral diversity: The phylogenetic surroundings of *Arabidopsis* and *Antirrhinum*. *Int. J. Plant. Sci.* **153**, S106-S122.

Hogarth, C.R., Mendham, N.J. and Buzza, G.C. (1995) The effect of distance from pollen source on seed set and yield in apetalous hybrid canola seed production. *Proceedings 9th International Rapeseed Congress*, pp. 107-109.

Jack, T., Fox, G.L. and Meyerowitz, E.M. (1994) *Arabidopsis* homeotic gene *APETALA3* ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. *Cell*, **76**, 703-716.

Judd, W. S., Campbell, C. S., Kellogg, E. A. and Stevens, P. F. (1999) *Plant systematics: a phylogenetic approach*. Sunderland, Mass.: Sinauer Associates, Inc.

Lefort-Buson, M. and Dattee, Y. (1982) Genetic study of some agronomic characters in winter oilseed rape. (*Brassica napus* L.) I. Heterosis II. Genetic parameters. *Agronomie* **2**, 315-332.

Ma, H. and dePamphilis, C. (2000) The ABCs of floral evolution. *Cell*. **101**. 5-8.

Ma, H., Yanofsky, M.F. and Meyerowitz, E.M. (1991) *AGL1-AGL6*, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. *Genes Dev.*, **5**, 484-495.

McVetty, P. B. E. (1995) Review of performance and seed production of hybrid Brassicas. *Proceedings 9th International Rapeseed Congress*. pp. 98-103.

McVetty, P. B. E., Scarth, R., Rimmer, S. R. and Pinnisch, R. (1995) Hybrid canola seed production in Western Canada using the *pol* CMS system. *Proceedings 9th International Rapeseed Congress*. pp. 104-106.

Okada, K. and Shimura, Y. (1994) Genetic analysis of signaling in flower development using *Arabidopsis*. *Plant Mol. Biol.*, **26**, 1357-1377.

Pinnisch, R. and McVetty, P.B.E. (1990) Seed production of hybrid summer rape in the field using the *pol* cytoplasmic male sterility system: a first attempt. *Can. J. Plant Sci.*, **70**, 611-618.

Robinson, R.G. (1984). D istance f rom t he p ollen source and yields of male sterile sunflower and sorghum. *Can. J. Plant Sci.*, **64**, 857-861.

Schultz, A.S., Pickett, F.B. and Haughn, G.W. (1991) The *FLO10* gene product regulates the expression domain of homeotic genes *AP3* and *PI* in *Arabidopsis* flowers. *Plant Cell*, **3**, 1221-1237.

Sieburth, L.E. and Meyerowitz, E.M. (1997) Molecular dissection of the *AGAMOUS* control region shows that *cis* elements for spatial regulation are located intragenically. *Plant Cell*, **9**, 355-365.

Zasshi, I. (1988). Cross-pollination efficiency of insect pollinators. *Jap. J. Breed.*, **38**, 91-102.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A. and Meyerowitz, E.M. (1990). The protein encoded by the *Arabidopsis* homeotic gene *AGAMOUS* resembles transcription factors. *Nature*, **346**, 35-39.

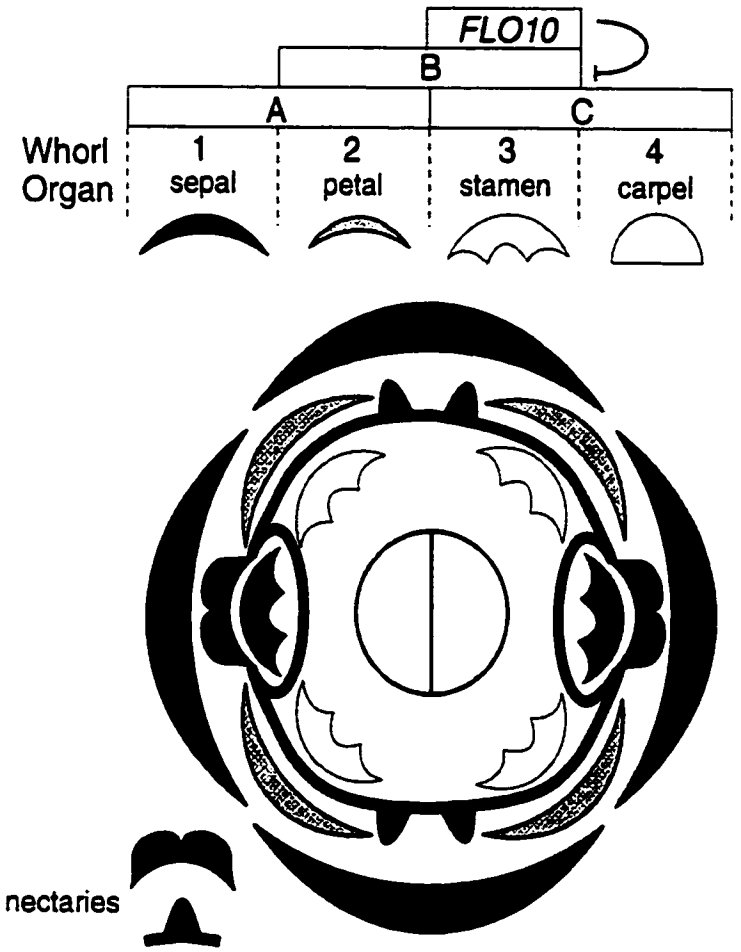
Chapter 2. THE ADDITIONAL STAMENS OF *FLO10-1* MUTANTS OF *ARABIDOPSIS THALIANA* ARE COMPROMISED IN PRODUCTION AND VIABILITY OF POLLEN

2.1 Introduction

The flower of *Arabidopsis thaliana* contains five types of floral organs arranged in concentric rings attached to a short stem or receptacle. These rings are referred to as whorls (Bowman, 1994) where the outermost or first whorl contains 4 sepals, the second whorl has 4 petals, the third whorl consists of 6 stamens, 2 of which are lateral and 4 of which are median, and the fourth whorl contains a central gynoecium, currently believed to consist of 2 fused carpels (figure 2.1) Separating the second and third whorls is the nectarium consisting of 6 nectaries, 2 of which are lateral and 4 of which are median (Davis, 1997). These floral organs originate from layers of undifferentiated cells in the floral meristem. Coordinated cell division and expansion between these cells are necessary to generate the floral organs, therefore cell-cell communication resulting in differential gene expression is required for normal development.

The formation and identity of 4 of these floral organs (sepals, petals, stamens, and carpels) can usually be predicted and explained with reasonable accuracy using the ABC model (Coen and Meyerowitz, 1991). This model suggests that 3 sets of homeotic genes act alone or in combination to correctly direct floral organ formation and identity in *Arabidopsis* (figure 2.1). Products of the class A genes (*APETALA1* and *APETALA2*) function alone in whorl 1 to direct sepal formation and in combination with class B gene products in whorl 2 to direct petal formation (Komaki *et al.*, 1988; Bowman *et al.*, 1989, 1991, 1993; K unst *et al.*, 1989; Hill and Lord, 1989; Jack *et al.*, 1992; Schultz and Haughn, 1993; Gustafson-Brown *et al.*, 1994; Jofuku *et al.*, 1994). Products of the class B genes (*APETALA3* and *PISTILLATA*) also function in combination with class C gene products in whorl 3 to direct stamen formation (Bowman *et al.*, 1989, 1991, 1993; Hill

Figure 2.1 A model depicting how the class A, B, and C homeotic genes function in the floral whorls to produce the sepals, petals, stamens, and carpels. *FLO10* functions in whorl 3 to block class B function in whorl 4. The typical arrangement of these organs, as well as the nectaries, in the flower of wild-type *Arabidopsis* is shown. The lateral stamens are shaded.


FLO10
B
A
C
Whorl
Organ
1
sepal
2
petal
3
stamen
4
carpel
nectaries

and Lord, 1989; Jack *et al.*, 1992). Products of the Class C gene (*AGAMOUS*) also function alone in whorl 4 to direct the formation of the gynoecium (Bowman *et al.*, 1989, 1991; Yanofsky *et al.*, 1990; Drews *et al.*, 1991).

The class B genes are partly controlled by a gene called *FLORAL MUTANT10* (*FLO10*; Schultz *et al.*, 1991) or *SUPERMAN* (*SUP*; Bowman *et al.*, 1992). *FLO10* is expressed primarily in whorl 3 (figure 2.1) where it is thought to maintain a boundary between differentiated cells of whorls 3 and 4, preventing expression of the class B genes in whorl 4 (Sakai *et al.*, 1995). A recessive mutation in the *FLO10* gene results in an increased number of stamens and a reduction in the gynoecium, the latter often being mosaics of both carpel and stamen tissues in the fourth whorl (Schultz *et al.*, 1991; Bowman *et al.*, 1992). It is believed that *flo10* mutations may disrupt this boundary resulting in collateral expression of both class B and class C genes in the fourth whorl (Sakai *et al.*, 1995), such that the phenotype predicted by the ABC model resembles that of the *flo10* phenotype.

Several *flo10* mutant alleles have now been isolated (Schultz *et al.*, 1991; Bowman *et al.*, 1992; Gaiser *et al.*, 1995) and all display similar phenotypes. Some of these *flo10* mutant alleles are weak, resulting in partially fertile flowers (e.g. *sup-5*), while others are stronger, producing more severely altered flowers containing several supernumerary or "additional" stamens in flowers that rarely set seed (e.g. *flo10-2* or *sup-1* and *flo10-1* or *sup-2*). Early studies with *FLO10* have addressed the effects of *flo10* mutations on floral organ identity, the degree of carpel to stamen conversion in the fourth whorl, and the development and location of additional stamens (Schultz *et al.*, 1991; Bowman *et al.*, 1992). More recently, mutations at the *flo10* locus have been shown to affect the normal development of the outer integuments of ovules (Gaiser *et al.*, 1995).

This study has focused on characteristics of the pollen produced by the additional stamens in *flo10-1* flowers. As there are few data available concerning this pollen, and microgametophyte production is a relatively expensive investment for the plant (Simpson and Neff, 1983), we were interested in comparing pollen quantity, viability, size, and germinability of the additional stamens with pollen from the six stamens occupying the normal "wild-type" locations within *flo10-1* flowers.

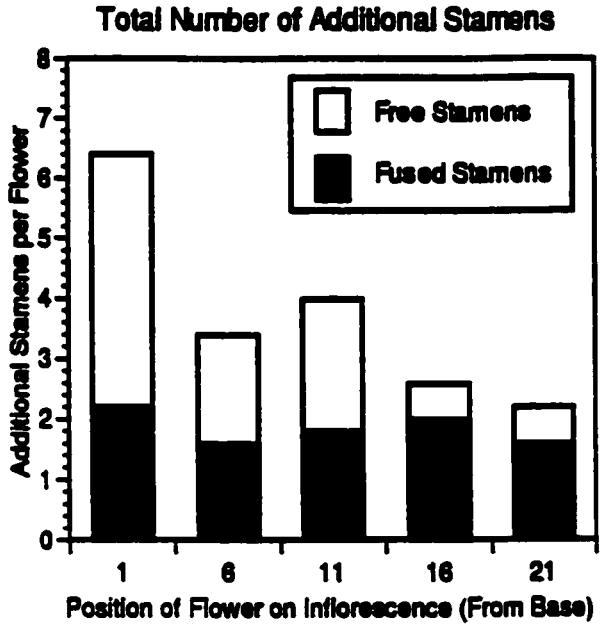
2.2 Results

2.2.1 Stamen number and organization

The number of stamens produced per flower was determined in *flo10-1* plants. In the third whorl, a mean of 3.92 ± 0.14 (S.E.) (n = 25) median stamens was produced, while both lateral stamens were always present. Although wild-type *A. thaliana* flowers ideally contain 6 stamens, 2 of which are lateral and 4 of which are median, many do not. Müller (1961) sampled several ecotypes and found the mean stamen number per flower to range from 4.1 (ecotype Limburg) to 5.3 (ecotype Dijon). Similarly, flowers of ecotype Columbia do not always contain a full complement of stamens, often lacking either one or both stamens at the lateral positions (Pylatuik, personal observation). Our results suggest that the "ideal" number of stamens occurs more frequently within the third whorl of *flo10-1* flowers than in wild-type flowers. In mature *flo10-1* flowers, additional stamens may exist either free, that is with the anther attached directly to the receptacle by a filament, or fused, either at the filament or the anther to an aberrant central organ. Two to 8 additional stamens have been reported in the fourth whorl of *flo10-1* (Schultz *et al.*, 1991), while as many as 20 additional stamens (assuming 6 wild-type) have been reported in the *flo10-2* (*sup-1*) mutant (Bowman *et al.*, 1992). In *flo10-1* plants, we report an average of 3.72 ± 0.87 (n = 25) additional anthers per flower, with a free to fused ratio of 1:1.

As figure 2.2 illustrates, the number of additional stamens was highest in flowers produced at the base of an inflorescence, tending to decrease in number acropetally. This tendency accords with that in *flo10-2* (*sup-1*) (Bowman *et al.*, 1992). Moreover, in *flo10-1*, the free to fused ratio of additional anthers was 2:1 in the first flower produced by the primary raceme, but decreased to 1:3 by the 16th and 21st positions (figure 2.2). The cause of this change in ratio is due to a decrease of free stamens, rather than an increase in fused stamens. The number of fused stamens was relatively constant between flower positions on the inflorescence, ranging from 1.6 to 2.2 per flower (figure 2.2). These results indicate a gradual decrease in male organs as new flowers are produced by the inflorescence meristem.

Figure 2.2 The average number of free and fused additional stamens (n=5) in *flo10-1* flowers originating from 5 different positions on the inflorescence.


Total Number of Additional Stamens
Free Stamens
Fused Stamens
Additional Stamens per Flower
0
1
2
3
4
5
6
7
8
1
6
11
16
21
Position of Flower on Inflorescence (From Base)

2.2.2 Morphology of *flo10-1* flowers

Representative features of *flo10-1* flowers (n = 18) examined by SEM are illustrated in figure 2.3a-i. In mature flowers, free additional stamens were preferentially located in axial positions between, and often slightly adaxial to, the paired third-whorl median stamens (figure 2.3a and b). Our results confirm those of Schultz *et al.* (1991) for this same mutant. These additional stamens were often associated with nectary tissue abaxial to the base of the filament. Nectary surfaces possessed stomata (figure 2.3b). When free stamens exceeded two in number, they also arose opposite the lateral sepals, adaxial to the third-whorl lateral stamens. However, in all flowers examined, free stamens, when present always occupied a median or axial position. When flowers contained only two free additional stamens, they both occurred in the median plane; if a flower contained only a single free additional stamen, it also occurred medially.

Despite the acropetal reduction in stamen number per flower in the primary raceme and shift from a predominance of free to fused stamens (figure 2.2), a distinctive pattern in the structure of the aberrant central organ was not obvious. The central organ in *flo10-1* flowers was usually a mosaic of carpelloid and staminoid tissue. Although this structure varied from being primarily carpelloid, bearing stigmatic papillae and external ovules (figure 2.3c), to primarily staminoid, possessing pollen-producing anthers (figure 2.3d), no distinct pattern was apparent with floral position on the inflorescence in our sample population. This conclusion is also supported in figure 2.2 where the mean number of fused anthers (i.e. staminoid tissue on the central aberrant organ) remained similar regardless of floral position on the inflorescence.

Two of the 18 flowers examined contained a filamentous structure originating from the third whorl (figure 2.3c and g). Epidermal cells of this long cylindrical structure resembled those of the stamen, topped with cells similar in morphology to those of anther lobes (figure 2.3h). Similar organs have been found in the third whorl of *apetala3* flowers (Bowman *et al.*, 1989), in the third whorl of *fl-54* flowers (Komaki *et al.*, 1988), in the second and third whorls of *ufo* mutants (Wilkinson and Haughn, 1995; Levin and Meyerowitz, 1995) and in wild-type flowers treated with the cytokinins

Figure 2.3 Morphology of *flo10-1* flowers. (a) View of the median side of a *flo10-1* flower. Additional stamens are preferentially located between and adaxial to pairs of third whorl median stamens and often are associated with nectary tissue. (b) A close-up view of the median side of a *flo10-1* flower. Additional stamens may also arise slightly abaxial to third whorl median stamens. Nectary tissue associated with additional stamens contains stomates on their surfaces. (c) View of an aberrant central organ that is predominantly carpelloid. The two carpels are separated at their summit and are each topped with stigmatic papillae (closed arrowhead). The tip of a filamentous structure can be seen (open arrowhead). (d) View of an aberrant central organ that is predominantly staminoid; an aberrant anther bearing stigmatic papillae (closed arrowhead) is attached to the receptacle by a filamentous stalk (arrow). Several pollen grains (small arrowheads) can be seen on the central organ, additional stamens and surrounding median stamens (not labeled). (e) The top of an aberrant central organ bearing exposed ovules showing a "hairdryer" morphology, attached by a funiculus; stigmatic papillae (large arrowhead) are also abundant. (f) A close-up of ovules on the central organ of a *flo10-1* flower. The micropyle of one ovule is evident. Pollen grains (small arrowheads). (g) View of the median side of a *flo10-1* flower. A filamentous structure (open arrowhead) is located in the third whorl where a median stamen would typically occur. (h) A close-up of the filamentous structure from (g). Epidermal cells toward the base of the stalk are similar to those of stamen filaments, whereas those toward the top of the stalk resemble epidermal cells of the anther. Stomates occur at the apex. (i) View of 2 stamens, located in the third whorl on the median plane, fused at the base of their filaments (arrow). Nectary tissue is associated with the fused stamens. (j) Pollen grains stained with fluoroscein diacetate fluoresce brightly when viable. Non-viable grains do not. as, additional stamen, co, central organ, f, funiculus, ls, lateral stamen, lse, lateral sepal, m, micropyle, ms, median stamen, mse, median sepal, n, nectary, nv, non-viable pollen, o, ovule, p, petal, s, stomata, and v, viable pollen. Scale bars = 100 μm.

a
ms
ms
se
n
b
s
c
co
d
ns
p
e
f
co
g
h
i
n
j

thidiazuron and benzylaminopurine (Venglat and Sawhney, 1994; 1996). Although such filamentous structures may occasionally be capped with stigmatic papillae (Venglat and Sawhney 1994; Wilkinson and Haughn, 1995; Bowman *et al.*, 1989), we were only able to identify stomata on their tips (figure 2.3h). In another flower, two stamens were fused together at the lower region of their filaments (figure 2.3i). This "double stamen" was located axially where a single median stamen would normally occur.

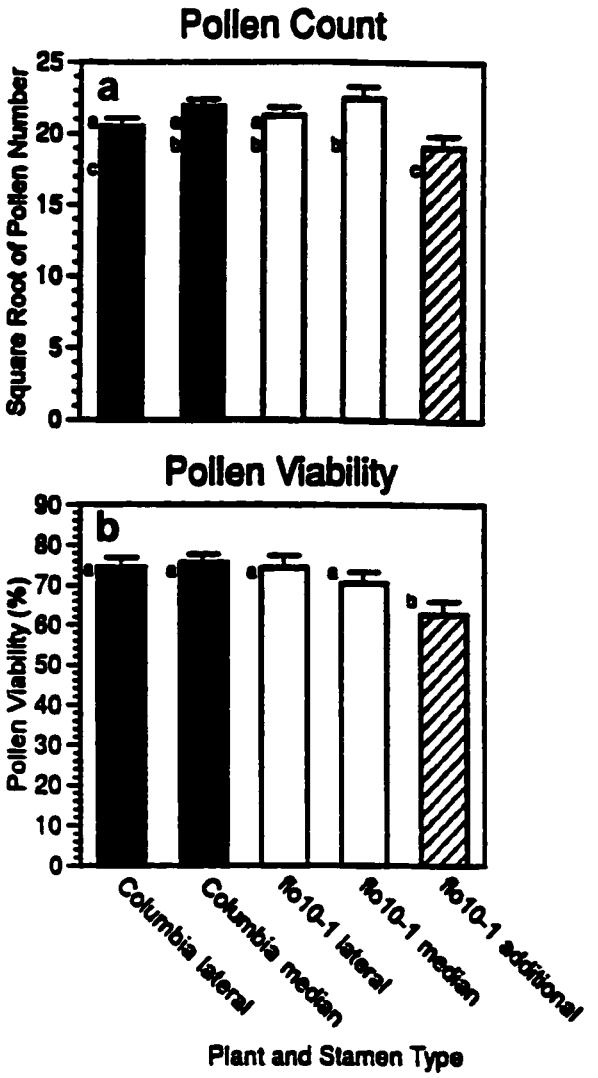
Ovules produced on the aberrant central organ of *flo10-1* were nearly radially symmetrical and tubular in shape (figure 2.3e and f). This "hairdryer" morphology has been described for *sup-5* in detail by Gaiser *et al.* (1995). These ovules are severely compromised in terms of viability (Gaiser *et al.*, 1995). With regards to pollen, we found no significant difference between viability of pollen produced by free additional anthers and those fused to the aberrant central organ (data not shown).

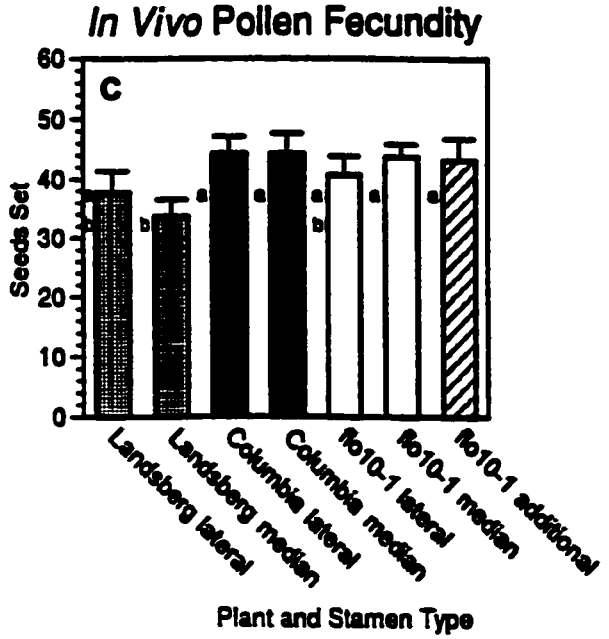
2.2.3 Pollen production

The amount of pollen produced per anther was assessed, for each stamen type, in both Columbia and *flo10-1* plants. As illustrated in figure 2.4a, lateral and median stamens produced similar amounts of pollen per anther in both Columbia ($p = 0.135$) and *flo10-1* ($p = 0.174$) plants, confirming previous findings in an unspecified ecotype (Preston, 1986). However, the quantity of pollen produced by the lateral (428.3 ± 19.7, $n = 23$) and median (485.2 ± 19.5, $n = 25$) anthers of Columbia was approximately twice that reported by Preston (1986). Between Columbia and *flo10-1* plants, significant differences were not found in pollen quantities produced by the lateral anthers ($p = 0.432$), or the median anthers ($p = 0.527$). However, the lateral anthers of Columbia produced less pollen than the median anthers of *flo10-1* (figure 2.4a). The additional anthers produced 18% less pollen than the lateral anthers ($p = 0.017$) and 28% less pollen than the median anthers ($p = 0.0002$) of *flo10-1* (figure 2.4a).

Variance between similar stamen types within a flower did not vary significantly from the estimated population variance in either Columbia or *flo10-1* plants ($p > 0.025$). Although free additional stamens were sampled preferentially from *flo10-1* flowers

Figure 2.4 Characteristics of pollen produced by various stamen types of *flo10-1* and wild-type flowers. Statistically similar values, determined by ANOVA, are indicated as a, b, or c. Error bars represent the standard error of the mean (n = 25). (a) Average number of pollen grains produced by each stamen type. Values were transformed to square roots to minimize the ratio of ranges. (b) Average viability (%) of pollen produced by each stamen type, as determined by FCR. (c) Seed set in *ap3-3* siliques following controlled hand-pollination using various sources of pollen.


Pollen Count
a
Square Root of Pollen Number
25
20
15
10
5
0
Pollen Viability
b
Pollen Viability (%)
90
80
70
60
50
40
30
20
10
0
Columbia lateral
Columbia median
flo10-1 lateral
flo10-1 median
flo10-1 additional
Plant and Stamen Type


In Vivo Pollen Fecundity
c
Seeds Set
60
50
40
30
20
10
0
Landsberg lateral
Landsberg median
Columbia lateral
Columbia median
flo10-1 lateral
flo10-1 median
flo10-1 additional
Plant and Stamen Type

whenever possible, five anthers fused to the central organ actually produced statistically similar amounts of pollen per anther as those of free anthers ($p = 0.13$).

Pollen production was also assessed for each anther type of Columbia and *flo10-1* plants with respect to the position on the primary inflorescence from which the flower originated. Interestingly, the quantity of pollen per anther was significantly lower (in all cases $p < 0.02$) in the first flower produced by the inflorescence meristem. In Columbia, anthers of first-formed flowers produced a mean of 300 ($n = 10$) pollen grains, while subsequent flowers produced a mean of 486 ($n = 40$) pollen grains. Likewise in *flo10-1*, first-formed flowers produced a mean of 243 ($n = 15$) pollen grains, but subsequent positions averaged 496 ($n = 60$) pollen grains. In 27 out of 30 possible comparisons (data not shown), pollen quantity per anther was not significantly different between subsequent flower positions (i.e. positions 6, 11, 16, and 21).

2.2.4 Pollen viability

The fluorochromatic reaction (FCR) was used to estimate pollen viability for each anther type of Columbia and *flo10-1*. As shown in figure 2.3j, viable pollen grains were identifiable by their bright flourescence under ultraviolet light (Heslop-Harrison and Heslop-Harrison, 1970). Illustrated in figure 2.4b, the viability of pollen produced by lateral and median anthers of both Columbia and *flo10-1* was not statistically different ($p > 0.7$ in both cases), averaging from 70.3 % to 75.7 %. These data agree with those of Altmann *et al.* (1994), who found mean viability (tested by germinating pollen grains of several diploid lines) to be 71.6 %. Furthermore, no statistical difference was found between Columbia and *flo10-1* with respect to viability of pollen produced by either the lateral ($p = 0.965$) or median ($p = 0.158$) stamens (figure 2.4b). However, the viability of pollen from the additional anthers of *flo10-1* (62.6 % ± 3.2 %, $n = 25$) was significantly lower than that from either the lateral ($p = 0.002$) or median ($p = 0.040$) anthers of *flo10-1* and from Columbia anthers ($p < 0.002$ for both anther types).

2.2.5 Pollen size

In several species, an increase in pollen size has been correlated with decreased quantity of pollen per anther (Price and Barrett, 1982; Mione and Anderson, 1992; Lau *et al.*, 1995; Vonhof and Harder, 1995) and increased pollen viability (Sharma *et al.*, 1996). Analysis of pollen from different anther types of Columbia, summarized in Table 2.1, showed pollen length to be statistically similar in both lateral and median anthers ($p = 0.741$), averaging 27.28 ± 0.39 μm. This measurement is comparable to those reported by Altmann *et al.*, (1994) who examined various lines of diploid *A. thaliana* and found their lengths to range from 26.6 - 28.8 μm (mean 27.66). Furthermore, pollen length from the three different anther types of *flo10-1* was statistically similar ($p > 0.50$ in all instances). However, all pollen types of *flo10-1* were significantly shorter ($P < 0.05$) than those of Columbia (Table 2.1).

Unlike pollen length, the width of pollen produced by all anthers examined was substantially greater (20.8 ± 0.31 μm) than that reported by Altmann *et al.* (1994) (mean = 15.41 μm). This disparity can be explained by the treatment of the material. Altmann *et al.* (1994) measured pollen in the absence of mounting media; therefore the pollen was probably measured in a dehydrated state. Glycerin jelly (used in this procedure) is reported to maintain both size and shape of pollen in a hydrated state (Reitsma, 1969). Expansion of Brassicaceae pollen occurs along the latitudinal axes during hydration (von der Ohe and Dustman, 1990). The length of the intercolpium is identical in dehydrated and hydrated grains, while the perimeter enlarges considerably during hydration, explaining the discrepancy between reports. The product of pollen length and width was used as an estimate of pollen size, which did not differ significantly between *flo10-1* additional and wild-type anthers ($p > 0.6$ in both cases). Mean pollen size from all anther types was 552.6 ± 27.1 ($n = 125$).

A crude estimate of anther size was determined for each stamen type to try to account for differences in pollen production. Pollen size was statistically similar in *flo10-1* stamen types, suggesting that smaller additional anthers may explain their reduced quantities of pollen. However, no significant difference in size could be resolved between the additional anthers and lateral or median anthers of *flo10-1* or

Table 2.1. Sizes of pollen grains from wild-type Columbia and *flo10-1* plants

Genotype	Anther type	Pollen length [a]	Pollen width [a]	Pollen size [b]
Columbia	lateral	27.2 ± 0.46	21.2 ± 0.35	576.3 ± 12.8
Columbia	median	27.4 ± 0.32	20.6 ± 0.34	563.7 ± 9.33
flo10-1	lateral	26.2 ± 0.29	20.8 ± 0.26	546.6 ± 10.9
flo10-1	median	25.9 ± 0.34	20.6 ± 0.36	534.2 ± 10.2
flo10-1	additional	26.2 ± 0.27	20.6 ± 0.22	542.1 ± 9.38

[a] The maximum length and width (in μm) of pollen grains was measured for 5 pollen grains from 5 anthers of each type (n=25).

[b] Pollen size was calculated individually as the product of length and width of the pollen grain (in μm^2).

Columbia. Anthers had a mean size (estimated as the product of the mean length of the two anther lobes and the maximum width of the anther) of 0.153 ± 0.014 mm^2 (n = 25) ranging from 0.145 ± 0.017 mm^2 (*flo10-1* additional) to 0.161 ± 0.014 mm^2 (*flo10-1* median).

2.2.6 Pollen fecundity

Although the FCR provides a useful assessment of pollen quality *in vitro* (Heslop-Harrison and Heslop-Harrison, 1970), we also sought to examine the ability of the pollen to germinate and effectively fertilize megagametophytes. Pollen fecundity was tested *in vivo* by pollinating male-sterile *ap3-3* plants (Jack *et al.*, 1992) with various *flo10-1*, Columbia and Landsberg anther types and counting the seed set. As figure 2.4c illustrates, quantities of seed matured per silique were similar. In 17 of 21 possible comparisons, the amount of seed produced by *ap3-3* siliques crossed with pollen from the various anthers of *flo10-1*, Columbia, and Landsberg, was statistically similar (figure 2.4c). This result is expected because variability due to different pollen viability would be minimized by the high pollen-ovule ratio presented in these crosses. Pollen from the median anthers of Landsberg often set less seed than pollen from other anthers when crossed with *ap3-3* (generated in Landsberg background). Landsberg pollen was not tested by FCR, therefore lower viability may explain the decrease in seed set. There is currently no evidence for incompatibility systems in *Arabidopsis* (Drescher and Kranz, 1987).

A mean of 41.1 ± 3.1 (n = 146) seeds were set per *ap3-3* silique, substantially greater than the 31.2 ± 2.82 (n = 5) ovules per flower (ecotype unknown) reported by Preston (1986). This observation may be explained by the increased number of carpels in the central gynoecium of *ap3-3*, the result of third whorl carpels fusing with each other and with the central gynoecium (Jack *et al.*, 1992). When harvesting the seeds produced by crossing with *ap3-3*, it was observed that most flowers contained a gynoecium with three locules (data not shown), all of which produced mature seeds. In 17 of 163 instances, *ap3-3* flowers produced less than 8 seeds each. These samples, which occurred randomly, were omitted from the data set. It is possible that these

flowers were damaged during handling and pollinating of the gynoecium, or growth of the silique may have been stunted by the labeling tape attached to them.

2.2.7 Seed germination

After testing the fecundity of pollen, the seeds produced by each *AP3-3* silique were assessed for fertility by determining percent germination. Seeds resulting from cross pollination by all plant and anther types were highly viable (99.9 % ± 0.1 %, n = 163 siliques), with no significant difference between any plant or anther type ($p > 0.05$). Seeds from 3 siliques were omitted due to fungal contamination of the seeds and culture media. Several cultured seedlings from each cross were transplanted into pots and grown to maturity. As illustrated in figure 2.5a-e, plants resulting from *flo10-1* and Columbia pollen were phenotypically normal and equally vibrant. Plants resulting from Landsberg *erecta* pollen retained the *erecta* phenotype (figure 2.5f and g).

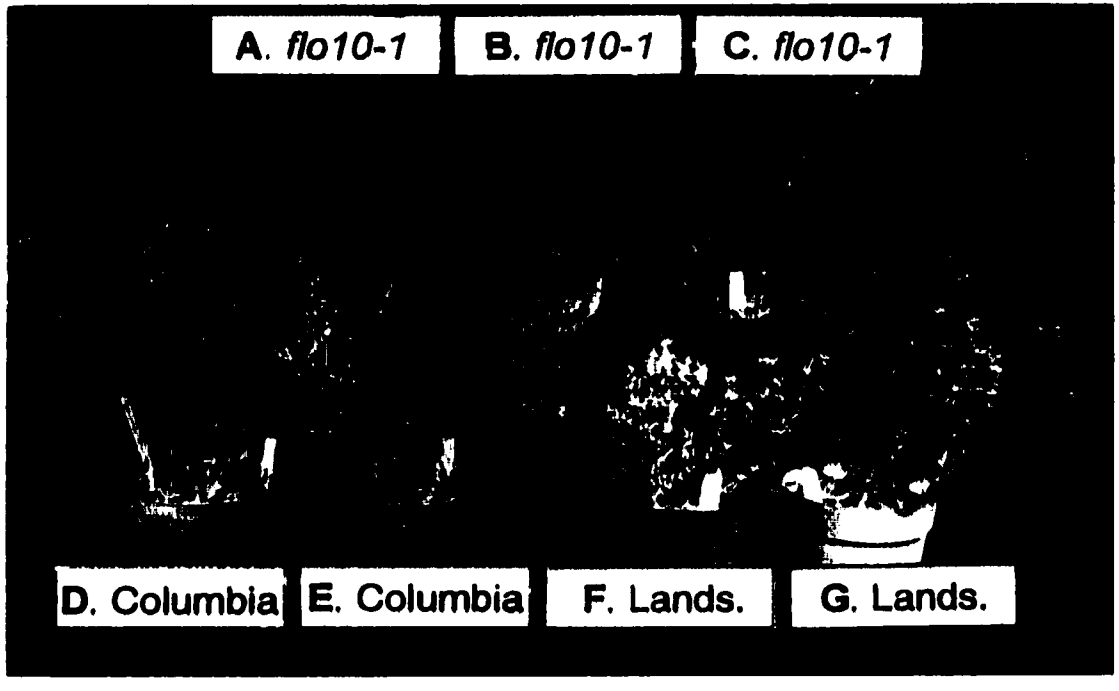
2.3 Discussion

2.3.1 Characteristics of pollen from third whorl stamens are not compromised in *flo10-1* flowers

In this study we determined that the additional stamens of *flo10-1* flowers produce significantly less pollen than stamens of the third whorl. Pollen produced by the additional stamens is also less viable via FCR than that of the third whorl. Nevertheless, pollen and anther size remained constant in all stamen types of *flo10-1* flowers. Therefore, anther or pollen size can not account for these differences in pollen productivity and viability. Perhaps decreased quantity and/or viability of pollen produced by additional stamens may be due to fewer microsporocytes or the existence of abnormal sporogenous cells within the anther.

Interestingly, pollen produced by the third whorl stamens of *flo10-1* plants did not differ in amount or viability from pollen of wild-type stamens. This suggests that the third whorl is not compromised, in terms of pollen production and viability, by

Figure 2.5 Mature plants from seeds harvested from *ap3-3* siliques following pollination from various sources of pollen. (a) Pollen from median stamens of *flo10-1*. (b) Pollen from lateral stamens of *flo10-1*. (c) Pollen from additional stamens of *flo10-1*. (d) Pollen from median stamens of Columbia. (e) Pollen from lateral stamens of Columbia. (f) Pollen from median stamens of Landsberg. (g) Pollen from lateral stamens of Landsberg.


A. flo10-1
B. flo10-1
C. flo10-1
D. Columbia
E. Columbia
F. Lands.
G. Lands.

altered fourth whorl activities due to the *flo10-1* mutation. However, microgametophytes from *flo10-1* stamens are slightly shorter than wild-type pollen grains and appear to be slightly smaller in size. As pollen is relatively expensive for plants to produce (Simpson and Neff, 1983), the nutritional demand on *flo10-1* flowers to produce additional pollen in fourth whorl stamens may outweigh any nutritional surplus provided from decreased carpel formation. This overall shift in nutritional demand by the flower may result in a reduction of pollen size in third whorl stamens without compromising pollen production or viability in the third whorl.

Another key finding in this study was that the initial flower borne on the primary raceme produced much less pollen per anther than later-formed flowers. This was a general phenomenon, occurring consistently in both *flo10-1* and wild-type plants. Although a sufficient explanation for this result can not currently be offered, other unique properties have been associated with the first-formed flower on an inflorescence. We have observed that first formed flowers of *flo10-1* plants may occasionally be phenotypically normal, producing viable selfed seeds, within their siliques, which germinate to produce plants that display the *flo10-1* phenotype. This phenomenon conflicts with our finding that generally, there is an acropetal decrease in male dominance in the inflorescence of *flo10-1* plants.

2.3.2 A function for FLO10 in whorl 3?

Our results suggest that lateral stamens are produced more commonly in the third whorl of *flo10-1* flowers than in wild-type flowers. The function of FLO10 is to prevent the expression of class B genes in whorl 4 (Sakai *et al.*, 1995), although our study suggests that it may also function partially in whorl 3, resulting in repressed stamen formation. Some wild-type plants such as the Enkheim, Limburg, and Dijon ecotypes rarely contain a full complement of stamens (Müller, 1961) while others such as the Landsberg (Smyth *et al.*, 1990), Wassilewskija (Running and Meyerowitz, 1996) and Columbia (Pylatuik, personal observation) ecotypes contain a full complement of stamens more frequently (approx. 75%). In all wild-type ecotypes, a reduction of stamen number is the result of one or both of the lateral stamens missing from the third

whorl. In the *flo10-1* plants we examined, lateral stamens were always present, resulting in a full complement of third whorl stamens. Therefore it is possible that, by whatever means *FLO10* acts to repress stamen formation in whorl 4, it may also function non-autonomously in whorl 3, partially repressing lateral stamen formation. Widely known is the fact that stamens occur as 2 sets (e.g. Davis, 1997), the lateral o nes p ossibly arranged in a whorl exterior to the median stamens. Furthermore, in the wild-type flower the lateral stamen primordia arise slightly later than the median ones (Hill and Lord, 1989). Our results support the hypothesis of two separate sets of stamens, each of which may have different regulatory thresholds in response to FLO10. Using this model, we can explain the normal function of FLO10 in plants as blocking class B gene expression completely in the central whorl containing the gynoecium, while a non-autonomous activity, possibly mediated by decreased cell proliferation in whorl 3, may explain the natural variation in number of the relatively-late developing laterals.

2.3.3 *flo10-1* adds to the growing evidence of bilateral symmetry

Previous studies of *flo10-2* (*sup-1*) did not suggest any regular positioning of additional stamens (Bowman *et al.*, 1992), while other studies on *flo10-1* indicated a pattern of additional stamen positioning analogous to that of the third-whorl stamens, but rotated 90 degrees (Schultz *et al.*, 1991). Our studies partially support the possibility that such an analogous pattern occurs interior to the third-whorl stamens in *flo10-1* flowers. We found that one free additional stamen regularly occurred between and slightly interior to each pair of median stamens of the third whorl. In keeping with the model proposed by Schultz *et al.* (1991), these additional fourth-whorl stamens in the median plane would be analogous to the lateral third-whorl stamens, if such a pattern existed.

Although the spatial distribution of these additional stamens suggests analogy to the third whorl stamens, developmental aspects do not. First, in wild-type flowers, the median or long stamen primordia are initiated slightly before the lateral or short stamen primordia (Hill and Lord, 1989) and the same is true of the third-whorl stamens of *flo10-1* (Schultz *et al.*, 1991). However, additional stamen primordia in *flo10-1* flowers

initiate first in positions analogous to, but rotated 90 degrees from, those of the third-whorl lateral stamens (Schultz *et al.*, 1991). Second, when stamens fail to develop in wild-type flowers, it is usually the lateral stamens that are absent (Hildebrand, 1879; Müller, 1961; Pylatuik, personal observation), suggesting that the median stamens are produced preferentially to lateral ones. In *flo10-1*, additional stamens arise preferentially between and slightly interior to pairs of median stamens rather than opposite and interior to lateral third-whorl stamens. Analogous third- and fourth-whorl patterns would predict the opposite result, that is, we would expect stamens arising between and interior to the third-whorl median stamens to be compromised. This observation can be viewed as a preferential sidedness for *flo10-1* mutations to manifest themselves within the *Arabidopsis* flower. Evidence therefore suggests that in *flo10-1*, third- and fourth-whorl stamens of *flo10-1* occupying the same planes of the flower are similar in terms of developmental sequence and locational frequency.

Sided bias exists in a wide variety of floral mutations in *Arabidopsis*. In *apetala3* and *pistillata* mutants, although all third-whorl organs are carpelloid, the median organs are more affected than the laterals (Bowman, 1994). Similarly in *apetala2* mutants, median organs of the first whorl are mosaics of sepal and carpel tissue, whereas lateral organs may be carpelloid, wild-type, or lacking (Kunst *et al.*, 1989; Bowman et al., 1991). Coupled with the fact that additional stamens arise preferentially on the median, rather than lateral, side of the flower, it appears that the median side of the *Arabidopsis* flower is more susceptible to phenotypic aberrations than the lateral side.

In *Arabidopsis* flowers containing x-ray - induced yellow ch-42 sectors, 87% of the chimeric sector boundaries produced in the first whorl were on the median side of the flower, while the remaining 13% were on the lateral (Furner, 1996). Similarly in the fourth whorl, sector boundaries mainly occurred on the median rather than the lateral side (Furner, 1996). Sector analysis using *Arabidopsis* plants that carried the *Ac* transposon from maize between the constitutive 35S promoter and the GUS reporter gene showed flowers having median sepals and stamens that were often dissected (Bossinger and Smyth, 1996). These authors found that the four petals also frequently displayed a median sector boundary (i.e. 2 chimeric petals on one side, 2 on the other).

Furner (1996) suggested that the tendency for sector boundaries to arise in the medial plane is due to differences in organ primordia cell number. In keeping with this theory, higher cell number in median organ primordia may also account for a greater susceptibility to phenotypic aberrations in the medial plane of floral mutants, including our present observations in *flo10-1*.

2.4 Experimental procedures

2.4.1 Plant material

Arabidopsis thaliana (L.) Heynh. ecotype Columbia was purchased from Lehle Seeds (Round Rock, Texas); ecotype Landsberg, homozygous for the *erecta* mutation, was a gift from Dr. S.P. Venglat (Plant Biotechnology Institute, Saskatoon, Saskatchewan); *flo10-1* (Schultz *et al.*, 1991), also called *sup-2* (Bowman *et al.*, 1992) is a floral mutant generated in the Columbia background (Schultz *et al.*, 1991) and was supplied by the Arabidopsis Biological Resource Center (Ohio State University, Columbus, Ohio); and *apetala3-3* (*ap3-3*), a floral mutant generated in a Landsberg background homozygous for the *erecta* mutation (Jack *et al.*, 1992), was a gift from Dr. E. M. Meyerowitz (California Institute of Technology, Pasadena, California). Following one week of vernalization at 4°C with no illumination, all plants were grown in a Conviron® (Winnipeg, Manitoba, Canada) growth chamber on Terra-lite Redi-earth® (W.R. Grace & Co. Canada Ltd., Ajax, Ontario, Canada) at a continuous temperature of 20°C and a relative humidity of 20%. Plants were grown under a photoperiod of 16 hours of continuous fluorescent illumination supplemented with incandescent light (265 $\mu E\ m^{-2}\ sec^{-1}$) cycled with 8 hours of darkness.

2.4.2 Scanning electron microscopy (SEM)

Every fifth flower, starting with the first flower produced at the base of the primary inflorescence was sampled from each of three plants of *flo10-1*. A total of five flowers from each plant were collected during anthesis. These flowers were fixed and

processed for scanning electron microscopy (SEM) using a procedure modified from Davis *et al.* (1986). Samples were fixed in a 2% glutaraldehyde, 25 mM Na phosphate solution (pH 6.8) and dehydrated in a graded acetone series. A Philips SEM 505 at 30kV was used to examine the tissues, which were photographed using Polaroid 665 positive/negative film.

2.4.3 Pollen quantity per anther

Every fifth flower, starting with the first flower produced by the primary raceme, was sampled from five plants each of Columbia and *flo10-1*. A total of five flowers from each plant were collected approximately one day prior to anthesis. Using a dissecting microscope, one of each anther type: median, lateral, and "additional" (*flo10-1* only), was collected from each flower providing a total of 25 samples of each anther type per plant genotype. Whenever possible, additional stamens that were not fused to central gynoecial tissue were selected for the analysis. Anthers were dissected in a drop of water on a microscope slide, covered by a coverslip, and all pollen grains immediately counted at 100-400 X magnification using a compound light microscope.

2.4.4 Pollen viability test

Samples of pollen were collected from each anther type during anthesis using the same pattern and method as described for pollen counts. Pollen viability was determined for samples that were pooled from all anthers of a particular type in each flower. Three of the 5 plants sampled were the same as those used for pollen counts; however, in this case every fifth flower was sampled starting with the second flower on the primary inflorescence. During this procedure the number of each anther type per flower was also recorded. The fluorochromatic reaction (FCR) was used to assess the viability of the pollen from Columbia and *flo10-1*; a 1:50 volumetric dilution of 2 mg/ml fluorescein diacetate (Shivanna and Rangaswamy, 1992) with 0.5 M sucrose solution was found to yield standardized results. Observations of the FCR were made with fluorescence optics and appropriate filters using a Zeiss epifluorescence microscope at 160-400 X magnification.

2.4.5 *In vivo* pollen fecundity test

Samples of pollen were collected from each anther type of the Columbia, Landsberg, and *flo10-1* genotypes approximately one day following anthesis using the same pattern and method as described for pollen counts. The pollen produced by these flowers was applied liberally to receptive stigmas of fourth whorl carpels of male sterile *ap3-3*, by brushing each stigma with two dehisced anthers representing each anther type. Anthers bearing large quantities of pollen were selected for these experiments. The *ap3-3* plants were grown to maturity in isolation from other *Arabidopsis* plants, to prevent undesirable cross pollination which increases in male-sterile lines (Trnêná *et al.*, 1987). The ripe siliques were harvested separately into labeled microcentrifuge tubes and stored dry at room temperature (70-90 days) until the seeds were counted.

2.4.6 Seed germination test

Following counting, seeds produced by pollinating the *ap3-3* plants with Columbia, Landsberg, and *flo10-1* plants were surface sterilized (Mourad *et al.*, 1995), before culturing in 100 mm petri plates on 1.0 % agar-solidified minimal medium (Haughn and Somerville, 1986) containing 2 % sucrose. Petri plates were sealed with Parafilm®. Germination was scored after one week of vernalization at 4°C in darkness followed by one week of growth under continuous fluorescent illumination (60 $\mu E\ m^{-2}\ sec^{-1}$) at 23°C. Several seedlings derived from each pollen type were selected from the plates, transplanted into pots, and grown to maturity under the same conditions described above for plant material.

2.4.7 Determination of anther and pollen size

Five flowers were selected randomly each from Columbia and *flo10* plants and all anther types were sampled per flower. Pollen grains were immediately dissected from mature anthers into a drop of glycerin jelly (Johansen, 1940), a cover slip was placed on top, and the jelly allowed to solidify. Pollen grains were measured at 200 X

magnification using a compound microscope with a scaled ocular. Five pollen grains from each anther were measured for a total of 25 pollen grains per anther type.

During this procedure one anther of each type was also removed from 5 randomly-selected flowers approximately one day prior to anthesis. These anthers were mounted in water on a microscope slide, covered with a coverslip, and measured at 40 X magnification using a microscope with a scaled ocular.

2.4.8 Analysis of data

Differences between pollen counts, viability, germination, size, and seed germination were tested by one-way analysis of variance (ANOVA) using the Statview® statistics program (Abacus Concepts Inc., Berkeley, CA). Prior to analysis, pollen-count data required a square-root transformation to achieve the lowest ratio of ranges.

2.5 Summary

flo10-1 (superman-2) is a floral mutant in *Arabidopsis thaliana* that normally produces female-sterile flowers. This phenotypic aberration results from a combination of increased stamen number and a reduced or abnormal gynoecium that is non-functional. The flowers of *flo10-1* contain two lateral and four median stamens, as seen in wild-type plants; however, they also contain several additional stamens. All stamen types have been examined with respect to frequency and location within the flower. The amount of pollen produced from each of the three types of stamens of *flo10-1* and the viability of this pollen was also examined and compared to wild-type (cv. Columbia) to determine the consequences of this mutation on male fertility. Both the lateral and median stamens of *flo10-1* and wild-type plants produced similar amounts of pollen per stamen and demonstrated no significant difference in viability. Per stamen, the additionals of *flo10-1* produced significantly less pollen than those of the laterals and medians. Furthermore, the pollen produced from these additional stamens was significantly less viable. Although less abundant and viable, pollen produced by additional stamens can effectively fertilize ovules, producing normal, healthy plants.

2.6 Literature cited

Altmann, T., Damm, B., Frommer, W.B., Martin, T., Morris, P.C., Schweiser, D., Willmitzer, L. and Schmidt, R. (1994) Easy determination of ploidy level in *Arabidopsis thaliana* plants by means of pollen size measurement. *Plant Cell Rep.*, **13**, 652-656.

Bossinger, G. and Smyth, D.R. (1996) Initiation patterns of flower and floral organ development in *Arabidopsis thaliana*. *Development*, **122**, 1093-1102.

Bowman, J.L. (1994) *Arabidopsis, An atlas of morphology and development.* New York: Springer-Verlag.

Bowman, J.L, Alvarez, J., Weigel, D., Meyerowitz, E.M. and Smyth, D.R. (1993) Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. *Development*, **119**, 721-743.

Bowman, J.L., Sakai, H., Jack, T., Weigel, D., Mayer, U. and Meyerowitz, E.M. (1992) *SUPERMAN*, a regulator of floral homeotic genes in *Arabidopsis*. *Development*, **114**, 599-615.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. (1989) Ge nes dir ecting f lower development in *Arabidopsis*. *Plant Cell*, **1**, 37-52.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. (1991) Genetic interactions among floral homeotic genes of *Arabidopsis*. *Development*, **112**, 1-20.

Coen. E.S. and Meyerowitz, E.M. (1991) The war of the whorls: genetic interactions controlling flower development. *Nature*, **353**, 31-37.

Davis, A.R. (1997) And the nectaries make two more...eight sets of floral parts in the wild-type flower of *Arabidopsis thaliana*. *Am. J. Bot.*, **84** (Suppl), 40.

Davis, A.R., Peterson, R.L. and Shuel, R.W. (1986) Anatomy and vasculature of the floral nectaries of *Brassica napus* (Brassicaceae). *Can. J. Bot.*, **64**, 2508-2516.

Drescher, W. and Kranz, A.R. (1987) First evidence for entomogamous hybridization in *Arabidopsis thaliana* (L.) Heynh. *Arabidopsis Inf. Serv. No 23*, pp 41-45.

Drews, G.N., Bowman, J.L. and Meyerowitz, E.M. (1991) Negative regulation of the *Arabidopsis* homeotic gene *AGAMOUS* by the *APETALA2* product. *Cell*, **65**, 991-1002.

Furner, I.J. (1996) Cell fate in the development of the *Arabidopsis* flower. *Plant J.*, **10**, 645-654.

Gaiser, J.C., Robinson-Beers, K. and Gasser, C.S. (1995) The *Arabidopsis SUPERMAN* gene mediates asymmetric growth of the outer integument of ovules. *Plant Cell*, **7**, 333-345.

Gustafson-Brown, C., Savidge, B. and Yanofsky, M.F. (1994) Regulation of the *Arabidopsis* floral homeotic gene *APETALA1*. *Cell*, **76**, 131-143.

Haughn, G.W. and Somerville, C. (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. *Mol. Gen. Genet.*, **204**, 430-434.

Heslop-Harrison, J. and Heslop-Harrison, Y. (1970) Evaluation of pollen viability by enzymatically induced fluorescence; intracellular hydrolysis of fluoroscein diacetate. *Stain Technol.*, **45**, 115-120.

Hildebrand, F. (1879) Vergleichende Untersuchungen über die Saftdrüsen der Cruciferen. *Jahrb. Wiss. Bot.*, **12**, 10-40.

Hill, J.P. and Lord, E.M. (1989) Floral development in *Arabidopsis thaliana*: A comparison of the wild-type and the homeotic *pistillata* mutant. *Can. J. Bot.*, **67**, 2922-2936.

Jack, T., Brockman, L.L. and Meyerowitz, E.M. (1992) The homeotic gene *APETALA3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. *Cell*, **68**, 683-697.

Johanson, D.A. (1940) *Plant Microtechnique*. New York: Mcgraw-Hill.

Jofuku, K.D., den Boer, S.G.W., Van Montagu, M. and Okamuro, J.K. (1994) Control of *Arabidopsis* flower and seed development by the homeotic gene *APETALA2*. *Plant Cell*, **6**, 1211-1225.

Komaki, M.K., Okada, K., Nishino, E. and Shimura, Y. (1988) Isolation and characterisation of novel mutants of *Arabidopsis thaliana* defective in flower development. *Development*, **104**, 195-208.

Kunst, L., Kienz, J.E., Martinez-Zapater, L. and Haughn, G.W. (1989) *AP2* gene determines the identity of perianth organs in flowers of *Arabidopsis thaliana*. *Plant Cell*, **1**, 1195-1208.

Lau, T.C., Lu, X., Koide, R.T. and Stephenson, A.G. (1995) Effects of soil fertility and mycorrhizal infection on pollen production and pollen grain size of *Cucurbita pepo* (Cucurbitaceae). *Plant Cell Environ.*, **18**, 169-177.

Levin, J.Z. and Meyerowitz, E.M. (1995) *UFO*: An *Arabidopsis* gene involved in both floral meristem and floral organ development. *Plant Cell*, **7**, 529-548.

Mione, T. and Anderson, G.J. (1992) Pollen-ovule ratios and breeding system evolution in *Solanum* section *Basarthrum* (Solanaceae). *Am. J. Bot.*, **79**, 279-287.

Mourad, G., Williams, D. and King, J. (1995) A double mutant allele, *csr1-4*, of *Arabidopsis thaliana* encodes an acetolactate synthase with altered kinetics. *Planta*, **196**, 64-68.

Müller, A. (1961) Zur Charakterisierung der Blüten und Infloreszenzen von *Arabidopsis thaliana* (L.) Heynh. *Kulturpflanze*, **9**, 364-393.

Preston, R.E. (1986) Pollen-ovule ratios in the Cruciferae. *Am. J. Bot.* **73**, 1732-1740.

Price, S.D. and Barrett, S.C.H. (1982) Tristyly in *Pontederia cordata* (Pontederiaceae). *Can. J. Bot.*, **60**, 897-905.

Reitsma, T. (1969) Size modification of recent pollen grains under different treatments. *Rev. Palaeobot. Palynol.*, **9**, 175-202.

Running, M.P. and Meyerowitz, E.M. (1996) Mutations in the *PERIANTHA* gene of *Arabidopsis* specifically alter floral organ number and initiation pattern. *Development*, **122**, 1261-1269.

Sakai, H., M edrano, L .J. a nd M eyerowitz, E .M. (1995) Role of *SUPERMAN* in maintaining *Arabidopsis* floral whorl boundaries. *Nature*, **378**, 199-203.

Schultz, A.S., Pickett, F.B. and Haughn, G.W. (1991) The *FLO10* gene product regulates the expression domain of homeotic genes *AP3* and *PI* in *Arabidopsis* flowers. *Plant Cell*, **3**, 1221-1237.

Schultz, E.A. and Haughn, G.W. (1993) Genetic analysis of the floral initiation process (FLIP) in *Arabidopsis*. *Development*, **119**, 745-765.

Sharma, M.L., Singh, P. and Lal, M. (1996) Floral behavior in *Saccharum officinarum* hybrids and *S. spontaneum* clones. *Ind. J. Agric. Sci.*, **66**, 455-458.

Shivanna, K.R. and Rangaswamy, N.S. (1992) *Pollen Biology, A Laboratory Manual.* New Delhi: Narosa Publishing House.

Simpson, B.B. and Neff, J.L. (1983) Evolution and diversity of floral rewards. In *Handbook of Experimental Pollination Biology*. (C.E. Jones and R.J. Little. ed.) New York: Van Nostrand Reinhold. pp. 142-159.

Smyth, D.R., Bowman, J.L. and Meyerowitz, E.M. (1990) Early flower development in *Arabidopsis*. *Plant Cell*, **2**, 755-767.

Trnêná, L., Škáová, M., Relichová, J. and Cetl, I. (1987) High outcrossing rates in a partially fertile line and their significance in the genetic structure of populations in *Arabidopsis thaliana* (L.) Heynh. *Arabidopsis Inf. Serv. No. 23*, pp. 31-40.

Venglat, S.P. and Sawhney, V.K. (1994) Ectopic formation of trichomes and stomata in floral organs of *Arabidopsis thaliana* induced by thidiazuron. *Can. J. Bot.*, **72**, 671-677.

Venglat, S.P. and Sawhney, V.K. (1996) Benzylaminopurine induces phenocopies of floral meristem and organ identity mutants in wild-type *Arabidopsis* plants. *Planta*, **198**, 480-487.

Von der Ohe, K. and Dustmann, J. H. (1990) Raster-elektronenmikroskopische studien an pollen aus Bienenhonig. III. Der harmomegathie-mechanismus und seine auswikungen auf die exine-strukturen am beispiel unterschiedlicher pollentypen. *Apidologie*, **21**, 293-302.

Vonhof, M.J. and Harder, L.D. (1995) Size-number trade-offs and pollen production by papilionaceous legumes. *Am. J. Bot.* **82**, 230-238.

Wilkinson, M.D. and Haughn, G.W. (1995) *UNUSUAL FLORAL ORGANS* controls meristem identity and organ primordia fate in Arabidopsis. *Plant Cell*, **7**, 1485-1499.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A. and Meyerowitz, E.M. (1990) The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. *Nature*, **346**, 35-39.

Chapter 3. ELEMENTS REGULATING *AGAMOUS* EXPRESSION ARE CONSERVED BETWEEN *ARABIDOPSIS THALIANA, BRASSICA NAPUS* AND *LINUM USITATISSIMUM*[*]

3.1 Introduction

Angiosperm flowers represent the most complex reproductive structures in land plants. Flowers typically consist of distinct whorls of organs arranged in concentric rings. From outside to in, these include sepals, petals, stamens and carpels. Moreover, in many species, nectary tissue occurs in strategic locations (e.g., at the base of the stamens) that enhance pollination effected by food-seeking flower visitors. Much of the morphological diversity between flowers of different species is in the number and patterning of organs; however, the identity of the organs within the specified whorls rarely changes. Many genes that function to control the number and pattern of organs, as well as their identity, have now been identified. One of the first flowering genes to be cloned was the *AGAMOUS* (*AG*) gene from *Arabidopsis* (Yanofsky *et al.*, 1990). The product of *AG* is a MADS box-containing transcription factor that functions to control, in combination with other transcription factors, the identity of the reproductive organs (i.e. stamens and carpels), as well as establish the determinacy of the flower (Yanofsky *et al.*, 1990).

AG gene expression is intricately controlled throughout plant development by regulators upstream in the signaling cascade. In the early stages of flower development, *AG* is ectopically expressed in the floral meristem in the regions where reproductive organs will arise. In later flower development this expression is confluent throughout the young reproductive organs (Drews *et al.*, 1991). As the reproductive organs mature, *AG* expression is limited to certain tissues within each organ type (Bowman *et al.*, 1991). Later in development, expression of *AG* extends outside the reproductive organs into the nectaries (collectively, the nectarium; Davis *et al.*, 1998) where high levels of

[*] *L. usitatissimum* work including figures 3.2 and 3.3 carried out by Ms. Rebecca Cross during her M.Sc. studies.

expression are observed (Bowman *et al.*, 1991). The *cis*-acting regulatory components required for *AG* expression are located within the second intron of *AG* which is approximately 3 kb in length (Sieburth and Meyerowitz, 1997). Furthermore, these *cis*-acting elements can be divided into regions which direct expression to the stamens and regions that direct expression to the gynoecium (Deyholos and Sieburth, 2000).

The flowers of *Brassica napus* (rapeseed or Argentine canola) share the same ground plan as those of *Arabidopsis* (Endress, 1992). The flower is bi-laterally symmetrical, containing four sepals, four petals, six stamens (two short lateral and four long median), and a gynoecium consisting of two locules in the lateral axis (Smyth *et al.*, 1990). The nectarium consists of two prominent lateral outgrowths adaxial (abaxial in *Arabidopsis*) to the lateral stamens and two prominent (four prominent in *Arabidopsis*) median outgrowths abaxial to the median stamens (Davis, 1994; Davis *et al.*, 1986). *Linum usitatissimum* (flax) is also a dicotyledonous plant but it produces radially symmetrical flowers, having organs in groups of five. The alternating sepals and petals are unfused while the extreme base of the stamens is fused to form a short staminal ring. The nectarium is comprised of five flattened swellings (nectaries), one per abaxial stamen base on the staminal ring, opposite to the sepals (Brown, 1938). The ovaries of the five carpels are fused into a gynoecium with five unfused stigmas and styles. The fruit formed is a boll that can contain up to 10 seeds, two in each locule (Moss, 1983).

Currently, over 15 species of land plants, including monocot and dicot angiosperms as well as gymnosperms, have been found to contain genes homologous to *AG* (Hasebe, 1999). As such, the involvement of *AG* in controlling reproductive organ formation was likely established before the divergence of angiosperms and gymnosperms (Hasebe, 1999). Support comes from several examples where the ectopic expression of *AG* homologs from one species confers identity changes in the same reproductive organs in another species (Mandel *et al.*, 1992; Rigola *et al.*, 2001; Rutledge *et al.*, 1998; Tandre *et al.*, 1995). These results suggest that the downstream control elicited by *AG* has been highly conserved between species. However, proper organ and flower formation also depends on the correct upstream control of *AG*. As such, our interest was to place the *cis*-acting elements of *AG* from one species (i.e. *A.*

thaliana) into other species to examine the conservation of upstream regulatory *trans* factors on the *Arabidopsis AG* controlling elements. *Arabidopsis AG* expression was examined in *B. napus*, a species within the same family (Brassicaceae) as *Arabidopsis* and in the more distantly related *L. usitatissimum*, a species within a different subclass (Rosidae) from *Arabidopsis* (Dilleniidae) (Judd *et al.*, 1999).

3.2 Results

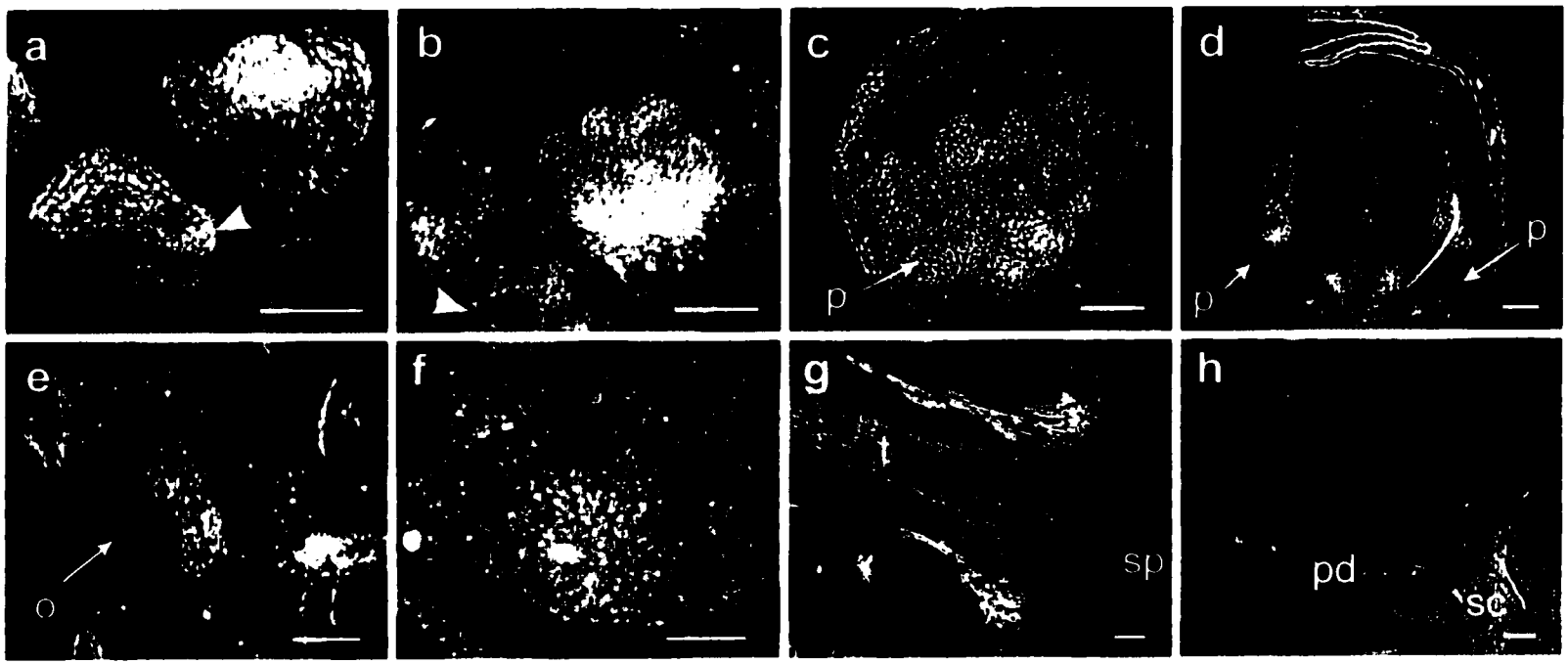
3.2.1 Selection and PCR confirmation of transformants

Following *Agrobacterium*-mediated transformation with the *AG::GUS* construct (pMD992), seven *B. napus* and six *L. usitatissimum* plants were identified as resistant to kanamycin. All plants were confirmed as *AG::GUS* transgenic through PCR analysis for the *NPTII* gene. Staining of intact tissues showed that in *B. napus*, GUS activity was limited to the reproductive organs, nectarium, and stems of the inflorescences (racemes). In *L. usitatissimum*, GUS activity was limited to flowers, primarily in the reproductive organs and the nectarium. The intensity of GUS staining varied between lines. Flowers from plants with the most intense GUS staining were selected for investigation at the cellular level. Variability was observed in the staining pattern among individual flowers of the same plant, however, the staining patterns described below represent those seen in more than 80% of the flowers examined.

3.2.2 GUS activity during early development of *B. napus* flowers

GUS staining patterns in flowers of *AG::GUS* transgenic *B. napus* plants were viewed using dark-field illumination, where GUS activity appeared red or pink on a green to brown background of tissue. GUS activity first appeared in young floral buds shortly after the four sepal primordia were initiated (figure 3.1a). GUS activity was limited to the undifferentiated region of cells at the center of the flower and was not found in the surrounding sepal primordia (figure 3.1a). At this early stage of *B. napus*

Figure 3.1 *B. napus* flowers showing *AG* directed GUS activity and viewed under dark-field illumination. (a-d) Longitudinal sections of floral buds. (a) Floral buds, at the developmental stage in which sepal primordia appear as mounds on the flanks of the floral meristem (arrowheads in a and b), showed no GUS activity. GUS activity was first detected in the central region of the floral meristem after the sepal primordia began to enlarge. (b-d) Once the sepals had grown to enclose the buds, GUS activity was limited to the developing stamens, gynoecia, and the region of the floral meristem below these structures. (c,d) GUS activity was not present in the sepals or petals (p). (e) Transverse section of a gynoecium. GUS activity was detected only in the septum and was absent (or very low) in ovary walls, placentae, and ovules (o). (f) Transverse section of an anther. GUS activity was detected only in the connective tissue, and was absent or very low in anther walls, tapetum, and microsporogenous tissue. (g) Longitudinal section of a mature stigma and style. High levels of GUS activity were detected in the stigma and stigmatic papillae (sp) and the transmitting tract (t) of the style. Strands of xylem appeared white. (h) Longitudinal section through an inflorescence. High levels of GUS activity were seen in the inflorescence scape (sc), but were absent or much lower in the pedicels (pd) of developing buds. Scale bars, 100 μm.


a
b
c
d
p
p
p
e
f
g
h
o
sp
pd
sc

floral development (see Polowick and Sawhney, 1986), the onset and patterning of GUS staining was similar to that of *Arabidopsis* flowers (Deyholos and Sieburth, 2000) at stage 3 (Smyth *et al.*, 1990). Prior to this stage of development, floral buds did not display GUS activity (figure 3.1a and b).

As the sepals enclosed the developing floral meristem, GUS activity was detected throughout stamen and gynoecia primordia and the underlying receptacle, but not in the sepals (figure 3.1b). At this stage, *B. napus* flowers were developmentally similar to stage 6 *Arabidopsis* buds and the GUS staining pattern, as directed by the *AG* intron 2 controlling elements, was the same in the two species (Deyholos and Sieburth, 2000). As the primordia of stamens and the gynoecium increased in size, GUS activity remained confined to the reproductive organs and the central tissue of the receptacle, while newly formed petal primordia, in addition to the sepals, did not stain for GUS activity (figure 3.1c). The *B. napus* flower shown in figure 3.1c is comparable to stage 8 buds of *Arabidopsis*, in which the *AG::GUS* construct conferred the same expression pattern (Deyholos and Sieburth, 2000).

3.2.3 GUS activity during late development of *B. napus* flowers

Intact mature flowers of transgenic *AG::GUS B. napus* plants showed intense GUS staining in the gynoecium, particularly the stigma and style, the connective of anthers, filaments, and the nectarium (data not shown). A large amount of GUS activity was also observed in the stem directly below the inflorescence meristem (figure 3.1h).

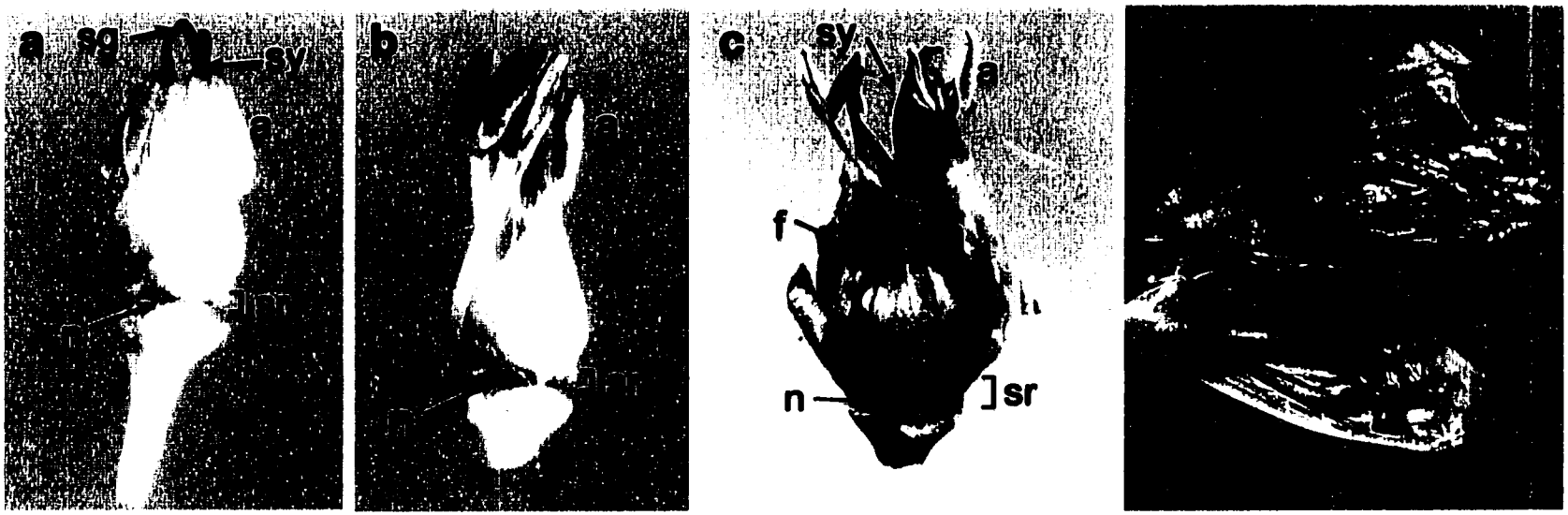
In sectioned material, GUS activity remained confined to the reproductive organs of buds greater than approximately 1.0 mm in length and became limited to the central tissues of the gynoecium and the connective of the anthers. Lower activity was detected in the filaments of the stamens (figure 3.1d). At this stage of development the stigma and style of the gynoecium are just beginning to differentiate. Based on the onset of stigma and style differentiation, buds at a comparable stage of development (stage 9-10), in *Arabidopsis* plants transformed with *AG::GUS*, showed GUS activity throughout the gynoecium, including the ovules, placentae and ovary wall (Deyholos and Sieburth, 2000).

Transverse sections of nearly mature *B. napus* buds showed GUS activity to be confined to the septum of the gynoecium, without any expression in the ovules, ovary wall, or the placentae (figure 3.1e). In the anthers of *Arabidopsis* flowers, GUS activity was expressed equally throughout the connective, tapetum and the filaments (Deyholos and Sieburth, 2000). However, in the anthers of transgenic *B. napus* flowers, GUS activity was limited to the connective and was not expressed in the locules, tapetum, or microsporogenous tissue (figure 3.1f). In mature *B. napus* flowers, levels of GUS staining were very high in mature stigmas and equally high in the transmitting tract of the style (figure 3.1g). Similar *AG::GUS* constructs (pAG-I::*GUS*) in *Arabidopsis* flowers directed high levels of GUS activity in the stigma, but this activity was much lower in the transmitting tract of the style (Sieburth and Meyerowitz, 1997). Finally, in both *Arabidopsis* (Deyholos and Sieburth, 2000) and *B. napus* (figure 3.1h), GUS activity was found to be very high throughout the inflorescence with undetectable levels in the pedicels of flowers.

3.2.4 GUS activity in *L. usitatissimum* flowers

In all *AG::GUS* transgenic *L. usitatissimum* plants, GUS activity was restricted to the flowers. *AG::GUS* lines 1-4 had low levels of GUS activity found only in the stigmas and styles. This pattern of GUS expression did not vary over the different developmental stages of the flowers. Comparatively, *AG::GUS* lines 5 and 6 had GUS activity in the anthers, distal regions of the filaments, staminal ring, and nectarium, in addition to the stigmas and distal portion of the styles (figure 3.2a, b and c). In the *AG::GUS* lines 5 and 6, the pattern of flower staining also differed with developmental stage (figure 3.2a and b). In young buds where the anthers were below the tips of the stigmas, GUS activity was present in the stigmas, styles and nectarium, as well as at very low levels in the anthers (figure 3.2a). As the buds matured and the stamens elongated to the same height as the stigma tips, GUS activity increased throughout these tissues (figure 3.2b). In a fully mature and open flower, GUS activity was very high within the stigmas, styles, parts of the staminal ring and nectarium (figure 3.2c). However, in these mature flowers, GUS activity within the anthers slightly decreased,

Figure 3.2 *L. usitatissimum* flowers of lines 5 and 6 showing *AG*-directed GUS activity. (a-c) A series of flowers showing developmental changes in the pattern of GUS activity. (a) Young bud with the anthers below the stigmas showed GUS activity in the stigmas and styles with very low activity in the anthers, staminal ring, and nectarium. (b) As buds mature, i.e. anthers at the same height as stigmas, GUS activity was more pronounced in the anthers, staminal ring, and nectarium as well as the stigmas and styles. (c) In mature flowers, GUS activity was intense in the stigmas, styles, staminal ring and nectarium, but less active in the anthers and filaments. (d) GUS activity was also present in the petal vasculature in some T_1 flowers from *AG::GUS* transgenic lines 5 and 6. sg, stigma, sy, style, a, anther, f, filament, sr, staminal ring, n, nectarium.

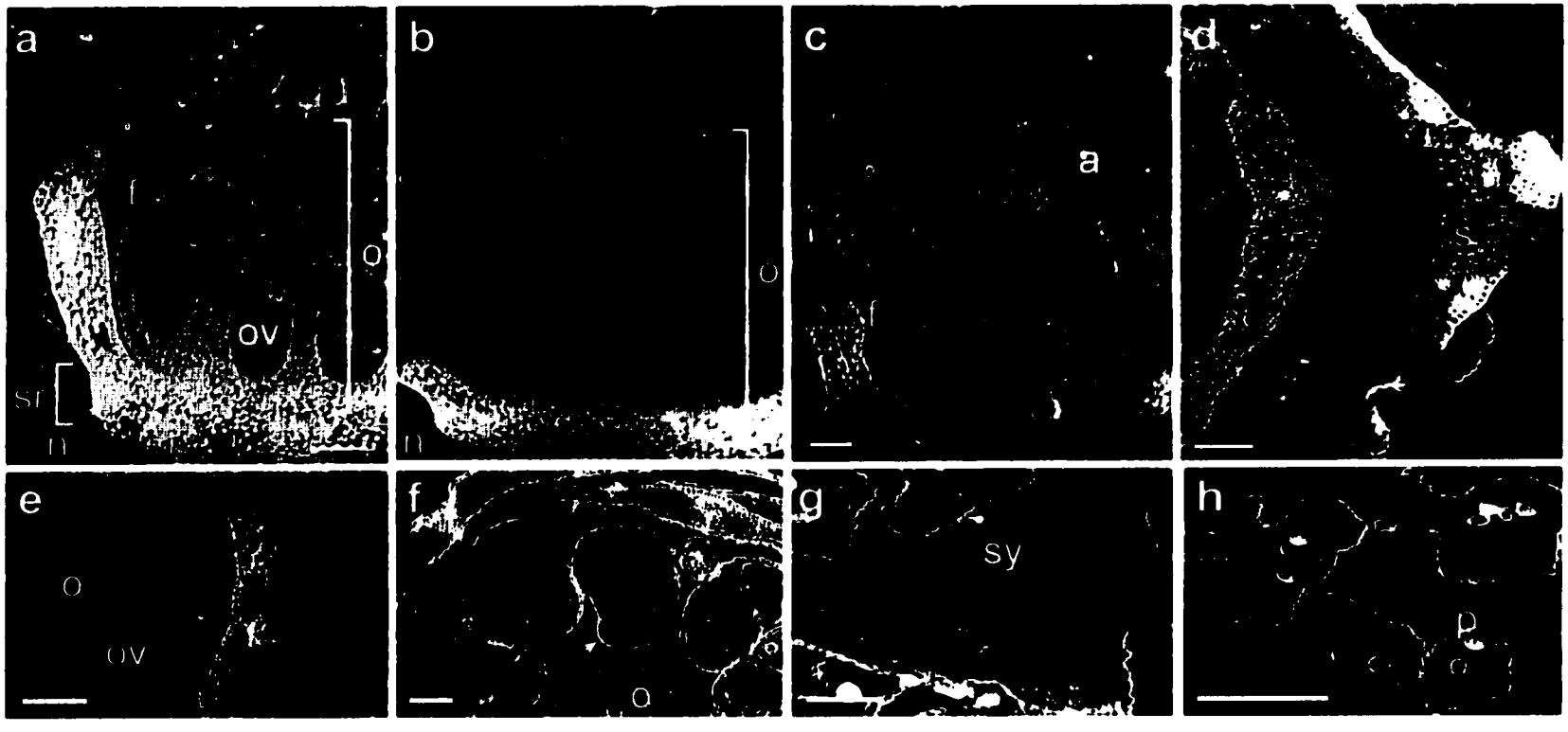

a
sg
sy
b
c
sy
a
f
h
n
]sr

while in the filaments, it increased (figure 3.2c) when compared with the younger flowers (figure 3.2a and b). In a few mature flowers collected from the T_1 progeny of the *AG::GUS* lines 5 and 6, the petal vasculature was moderately stained (figure 3.2d). Wild type flowers showed no GUS activity.

A selection of flowers and buds at various developmental stages and showing high GUS activity were embedded, sectioned, and observed under dark-field illumination. GUS activity was observed in the ovary but not the ovules of flowers where the stamens were the same height as the stigmas (figure 3.3a and e). In these flowers, GUS activity in the receptacle and the ovary base was found to be intense (figure 3.3a and b) but decreased distally (figure 3.3a). Intense GUS activity was observed in the stigmas and styles of flowers (figure 3.3g) from the onset of GUS expression until maturation.

The short staminal ring at the base of the stamens was intensely stained, both in the nectaries and regions between the nectaries (figure 3.3a and b). The filaments of stamens of young buds appeared more darkly stained in longitudinal section (figure 3.3c) than they appeared to be in the intact flowers (figure 3.2a). The anthers showed strong activity throughout the endothecium, tapetum and pollen itself, but not the epidermis (figure 3.3c, f and h). GUS activity was observed in the whole pollen grain, but was absent in the exine (figure 3.3h). Interestingly, some flowers from *AG::GUS* lines 5 and 6 showed light GUS activity in the sepals (figure 3.3d).

Figure 3.3 Sections of *L. usitatissimum* flowers showing *AG* directed GUS activity, viewed under dark-field illumination. (a-f) GUS activity appears as orange or pink; (g-h) intense GUS activity appears blue or purple. (a-c) Longitudinal sections of floral buds. (d-h) Transverse sections of floral buds. (a) GUS activity present in the ovary (o), including the wall and septae, a nectary (n), and the staminal ring (sr) in a bud where anthers are equal in length to the stigmas. No GUS activity was observed in the ovules (ov). (b) A bud with anthers below the stigmas, showed intense staining of a nectary (n) and the ovary base (o). (c) GUS activity in the anthers (a) and filaments (f). (d) The sepals (s) of some buds (particularly those of line 6) showed GUS activity. (e) GUS activity present in the ovary (o) and lack of activity in the ovule (ov). (f) GUS activity throughout anthers, except the epidermis (arrowhead), and the pollen. (g) Intense GUS activity in the styles (sy). (h) *AG* expression in pollen (p) except the exine. Scale bars, 100 μm.


a
f
o
ov
sf
n
b
o
n
c
a
f
d
e
o
ov
f
o
g
sy
h
p
o

3.3 Discussion

3.3.1 *Arabidopsis AG* expression is limited primarily to whorls 3 and 4 in *B. napus* and *L. usitatissimum*

In both *B. napus* and *L. usitatissimum*, as in *Arabidopsis*, the *cis* elements contained within the second intron of the *AG* gene directed GUS activity primarily to the organs of the third and fourth whorls. This expression pattern is consistent with the C class function of the AGAMOUS protein in contributing to flower development and, more specifically, reproductive organ development (Coen and Meyerowitz, 1991). Due to its highly conserved nature, it is likely that AGAMOUS function was established early in the evolution of plants (Hasebe, 1999) and plays an essential role in controlling the formation of reproductive organs. Our results suggest that, like the AGAMOUS protein, the regulatory components, which direct the general spatial expression of *AG*, have been equally conserved. It is our conclusion that in these three species, the joint requirement of correct protein function and spatial regulation of *AG* in flower development constitutes a strong selective pressure, acting to maintain the inter-family conservation of the *AG* gene.

3.3.2 *Trans*-acting elements controlling early *AG* expression: conserved and not conserved

Both temporally and spatially, *AG* expression in the early stages of flower development, as indicated by GUS activity, is identical in *B. napus* and *Arabidopsis* and remains identical until the gynoecial tube begins to elongate (stage 8 of *Arabidopsis* bud development). These data suggest that the *trans*-acting elements responsible for directing early *AG* expression have been conserved between *B. napus* and *Arabidopsis*. Furthermore, the corresponding *cis*-acting elements of the *AG* second intron also confer the same developmental regulation of expression, spatially and temporally, in both species.

Conversely, in *L. usitatissimum*, a species more distantly related to *Arabidopsis*, the *AG cis*-elements do not appear to be sufficient to trigger expression in the early stages of development. It is possible that *trans*-acting factors, which function to promote early *AG* expression via the *AG* second intron *cis*-elements in flowers of *Arabidopsis* and *B. napus*, are not conserved with those of *L. usitatissimum*. We conclude that the mechanism promoting early *AG* expression in flowers has diverged between *L. usitatissimum* (subclass Rosidae) and the two Brassicaceaen species (subclass Dilleniidae).

3.3.3 Similarities of *AG* expression in *B. napus* and *L. usitatissimum*

3.3.3.1 *Ovules*

Unlike *Arabidopsis*, where the second intron of *AG* directs expression throughout developing ovules until stage 12 of bud development (Deyholos and Sieburth, 2000), this element did not direct expression to the ovules of *B. napus* and *L. usitatissimum* during any stage of development. Previous studies have shown that in *Arabidopsis*, *AG* is required during early ovule development in order to direct ovule identity (Western and Haughn, 1999). Later in ovule development, just prior to fertilization, *AG* expression is limited to the endothelium of the ovules (Bowman *et al.*, 1991).

It is possible that *trans*-acting factors, which function to promote *AG* expression in the ovules of *Arabidopsis*, via the *AG* second intron *cis* elements, are not conserved with those of *B. napus* and *L. usitatissimum*. This is an intriguing possibility, particularly for *B. napus*, which is closely related to *Arabidopsis*. Two possibilities could explain this lack of expression: (1) the function of *AG*, required in *Arabidopsis* for ovule development, is either unnecessary in *B. napus* and *L. usitatissimum* or it has been replaced by another gene in these species. As such, the *trans*-acting regulatory components controlling *AG* ovule expression have probably been lost in these species. Alternatively, (2) if *AG* expression is required for proper ovule development in *B. napus* and *L. usitatissimum*, our results would suggest that both the *cis*- and *trans*-acting factors controlling *AG* ovule expression have diverged to the point at which the components of

the different species examined here are no longer compatible. Determining the necessity for *AG* expression in the ovules of these species examined would help clarify which explanation is more likely.

3.3.3.2 *Stigma and Nectarium*

As in *Arabidopsis*, the *AG* second intron directed expression throughout the stigma of both *B. napus* and *L. usitatissimum*. *AG* is highly expressed in the nectarium of *Arabidopsis* (Bowman *et al.*, 1991), and like that of the stigma, the *AG* second intron also directed expression throughout the nectarium of both *B. napus* and *L. usitatissimum*. It was previously suggested that both stigma and nectary-specific *cis* elements resided within sequences upstream of the first intron (Sieburth and Meyerowitz, 1997). Our results, and those of Deyholos and Sieburth (2000), show that in all three species, the sequence upstream of the first intron of the *AG* gene is not required for its expression in the stigma. This upstream sequence is also not required for expression in the nectary tissue of *B. napus* and *L. usitatissimum*. These findings suggest that redundant stigma and nectary-specific *cis* elements exist in both the upstream sequence and the second intron of *AG*. Additionally, our results suggest that the *trans* factors that act upon these elements have been conserved between these three species.

The identity and development of the reproductive organs can be predicted by the ABC model of flower development (Coen and Meyerowitz, 1991) and are dependent on the C class function of *AG*. It has been concluded however, that the nectarium develops independently of the ABC model (Davis *et al.*, 1993; Baum *et al.*, 2001). The high levels of expression directed by the *AG cis*-acting elements in the nectarium of all three species suggest a conserved function in this tissue. It is therefore possible that *AG* may have both an ABC-dependent and ABC-independent function in the development of flowers.

3.4 Experimental procedures

3.4.1 Plant material

The *Brassica napus* L. cv. Westar T_0 generation of transformed lines was grown in the Phytotron Facility (College of Agriculture, University of Saskatchewan) on Terra-lite Redi-earth® (W.R. Grace & Co. Canada Ltd., Ajax, Ont.) with a 16 h/8 h light/dark cycle (230 mmol photons $\cdot m^{-2} \cdot s^{-1}$) and a 23°C/18°C day/night temperature cycle. All *Linum usitatissimum* L. cv. Normandy plants were grown in Terra-lite Redi-earth® under greenhouse conditions supplemented with sodium lights with a 16 h/8 h light/dark cycle and 22°C/18°C day/night temperature cycle. Plants of *L. usitatissimum* were fertilized with Plant-Prod 20-20-20 (50 mg in 4 L) weekly. Tissues harvested from all plants for the purpose of DNA isolation and PCRs were collected on ice and subsequently stored at –80°C.

3.4.2 Plant transformation

The *AG::GUS* construct (pMD992 (see figure B.1) - generously supplied by Dr. L. E. Sieburth; Deyholos and Sieburth, 2000) was used to transform *B. napus* using the pC2760 *Agrobacterium* strain containing the LBA4404 Ti binary plasmid by the method of Moloney *et al.* (1989). *L. usitatissimum* hypocotyls were transformed with pMD992 in pC2760 *Agrobacterium* using a method modified from Dong and McHughen (1993). Seven day-old hypocotyls were cut into 0.5 cm sections and dipped in an *Agrobacterium* solution. The hypocotyls were co-cultivated with the *Agrobacterium* on MS1 medium (Dong and McHughen, 1993) without selection for seven days, and then transferred to MS1 with selection for the generation of callus. To generate shoots, callus was placed on MS3 shooting medium, modified from Moloney *et al.* (1989) in which the benzyladenine had been replaced with 1 mg/L trans-zeatin and the Timentin concentration had been increased to 300 mg/L. When shoots were approximately 1-2 cm in length they were excised, all callus carefully removed, and the shoots placed in jars containing MS5 rooting medium, modified from Wijayanto and McHughen (1999)

by using full strength MS salts. After approximately 3 weeks each plantlet was uprooted, placed in moist Redi-earth and covered with plastic wrap. The wrap, after covering the plant for 2 – 3 days, was removed gradually over a further 2 - 3 days.

Transformed *B. napus* and *L. usitatissimum* plants were identified based on their resistance to kanamycin and a subsequent PCR specific for the *NEOMYCINPHOSPHOTRANSFERASEII* (*NPTII*) gene using the primers: NPTII-1 (5' GAGGCTATTCGGCTATGACTG) and NPTII-2 (5' ATCGGGAGCGGCGATACCG TA).

3.4.3 GUS Staining and Microscopy

Tissues were stained for GUS activity and processed through an ethanol series as described by Sieburth and Meyerowitz (1997). Tissues used for histological analysis were prepared by embedding in Paraplast (Oxford L abware, S t. L ouis, M o., U .S.A.) immediately following the ethanol series. Sections (10 μm) were cut on a Leitz-Wetzlar rotary microtome, mounted on slides, de-waxed by two overnight incubations in xylene, and mounted with Permount (Fisher S cientific). GU S s taining w as v isualized us ing dark-field illumination. Sections on slides were digitized using a CanoScan 2700F (Canon U.S.A. Inc., Lake Success, NY). Brightness, contrast, and gamma levels were adjusted using Adobe Photoshop (Adobe Systems, Mountain View, CA).

3.5 Summary

To investigate the conservation of upstream regulatory elements controlling *AGAMOUS* (*AG*) expression, we placed the *cis*-acting elements of *AG* from *Arabidopsis thaliana* into *Brassica napus* and *Linum usitatissimum* and analysed the subsequent expression patterns in each species. Spatially, the expression patterns in *B. napus* and *L. usitatissimum* resembled those of *AG* in *Arabidopsis*, in that expression was confined primarily to the reproductive organs and nectarium. Temporally, patterns of *AG* expression in *B. napus* were identical to *AG* expression in *Arabidopsis* during e arly development, ho wever *AG* w as no t e xpressed dur ing early flower development in *L.*

usitatissimum. Within organs, tissue-specific expression patterns were not conserved between species. Unlike *Arabidopsis*, *AG* expression was not observed in the ovules of *B. napus* and *L. usitatissimum*. These results suggest that although regulatory factors controlling the localized expression of *AG* have been conserved between these species, those controlling temporal and tissue-specific expression have not been conserved.

3.6 Literature cited

Baum, S.F., Eshed, Y. and Bowman, J.L. (2001) The *Arabidopsis* nectary is an ABC independent floral organ. *Development* (In Press).

Bowman, J.L., Drews, G.N. and Meyerowitz, E.M. (1991) Expression of the *Arabidopsis* floral homeotic gene *AGAMOUS* is restricted to specific cell types late in flower development. *Plant Cell*, **3**, 749-758.

Brown, W.H. (1938) The bearing of nectaries on the phylogeny of flowering plants. *Proc. Am. Phil. Soc.*, **79**, 549-594.

Coen, E.S. and Meyerowitz, E.M. (1991) The war of the whorls: Genetic interactions controlling flower development. *Nature*, **353**, 31-37.

Davis, A.R. (1994) Features of the nectary in wild-type and some mutant flowers of *Arabidopsis thaliana* (L.) Heynh. var. Columbia. In *Arabidopsis. an atlas of morphology and development.* (Bowman, J .L. ed.) New York: Springer-Verlag, pp. 172-177.

Davis, A.R., Haughn, G.W. and Sawhney, V.K. (1993) Preliminary investigation of the nectary in floral mutants of *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae). *Proc. Int. Symp. Polln. Trop.*, 9-14.

Davis, A.R., Peterson, R.L. and Shuel, R.W. (1986) Anatomy and vasculature of the floral nectaries of *Brassica napus* (Brassicaceae). *Can. J. Bot.*, **64**, 2508-2516.

Davis, A.R., Pylatuik, J.D., Paradis, J.C. and Low, N.H. (1998) Nectar-carbohydrate production and composition vary in relation to nectary anatomy and location within individual flowers of several species of Brassicaceae. *Planta*, **205**, 305-318.

Deyholos, M.K. and Sieburth, L.E. (2000) Separable whorl-specific expression and negative regulation by enhancer elements within the *AGAMOUS* second intron. *Plant Cell*, **12**, 1799-1810.

Dong, J-Z and McHughen, A. (1993) An improved procedure for production of transgenic flax plants using *Agrobacterium tumefaciens*. *Plant Science*, **88**, 61-71.

Drews, G.N., Bowman, J.L. and Meyerowitz, E.M. (1991) Negative regulation of the *Arabidopsis* homeotic gene *AGAMOUS* by the *APETALA2* product. *Cell*, **65**, 991-1002.

Endress, P.K. (1992) Evolution and floral diversity: The phylogenetic surroundings of *Arabidopsis* and *Antirrhinum*. *Int. J. Plant. Sci.*, **153**, S106-S122.

Hasebe, M. (1999) Evolution of reproductive organs in land plants. *J. Plant Res.*, **112**, 463-474.

Judd, W.S., Campbell, C.S., Kellogg, E.A. and Stevens, P.F. (1999) *Plant Systematics: a phylogenetic approach*. Sunderland, Mass.: Sinauer Assoc. Inc.

Mandel, M.A., Bowman, J.L., Kempin, S.A., Ma, H., Meyerowitz, E.M. and Yanofsky, M.F. (1992) Manipulation of flower structure in transgenic tobacco. *Cell*, **71**, 133-143.

Moloney, M.M., Walker, J.M. and Sharma, K.K. (1989) High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. *Plant Cell Reports*, **8**, 238.

Moss, E.H. (1983) *Flora of Alberta* 2nd e dn. R evised: J . G. P acker. U niversity o f Toronto Press, Toronto, On. pp. 393.

Polowick, P.L. and Sawhney, V.K. (1986) A scanning electron microscopic study of the initiation and development of floral organs of *Brassica napus* (cv. Westar). *Am. J. Bot.*, **73**, 254-263.

Rigola, D., Pè, M.E., Mizzi, L., Ciampolini, F. and Sari-Gorla, M. (2001) *CaMADS1*, an *AGAMOUS* homologue from hazelnut, produces floral homeotic conversion when expressed in *Arabidopsis*. *Sex. Plant Reprod.*, **13**, 185-191.

Rutledge, R., Regan, S., Nicolas, O., Fobert, P., Côté, C., Bosnich, W., Kauffeldt, C., Sunohara, G., Séguin, A. and Stewart, D. (1998) Characterization of an *AGAMOUS* homologue from the conifer black spruce (*Picea mariana*) that produces floral homeotic conversions when expressed in *Arabidopsis*. *Plant J.*, **15**, 625-634.

Sieburth, L.E. and Meyerowitz, E.M. (1997) Molecular dissection of the *AGAMOUS* control region shows that *cis* elements for spatial regulation are located intragenically. *Plant Cell*, **9**, 355-365.

Smyth, D.R., Bowman, J.L. and Meyerowitz, E.M. (1990) Early flower development in *Arabidopsis*. *Plant Cell*, **2**, 755-767.

Tandre, K., Albert, V.A., Sundas, A. and Engström, P. (1995) Conifer homologues to genes that control flower development in angiosperms. *Plant Mol. Biol.*, **27**, 69-78.

Western, T.L. and Haughn, G.W. (1999) *BELL1* and *AGAMOUS* genes promote ovule identity in *Arabidopsis thaliana*. *Plant J.*, **18**, 329-336.

Wijayanto, T. and McHughen, A. (1999) Genetic transformation of *Linum* by particle bombardment. *In Vitro Cell. Dev. Biol. Plant.*, **35**, 456-465.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A. and Meyerowitz, E.M. (1990) The protein encoded by the *Arabidopsis* homeotic gene *AGAMOUS* resembles transcription factors. *Nature*, **346**, 35-39.

Chapter 4. TARGETED B-CLASS EXPRESSION TO THE REPRODUCTIVE ORGANS OF *ARABIDOPSIS* FLOWERS PROVIDES EVIDENCE FOR A FOUR-ORGAN ORIGIN OF THE GYNOECIUM

4.1 Introduction

Arabidopsis thaliana and *Brassica napus* are related species belonging to the Brassicaceae family. Members of this family share a remarkably stable floral ground plan containing 4 types of floral organs arranged in concentric rings attached to a short stem or receptacle. These rings are referred to as whorls where the outermost or first whorl contains 4 sepals, the second whorl 4 petals, the third whorl 6 stamens, 2 of which are lateral and 4 of which are median, and the fourth whorl is believed to contain 2 carpels fused to form the central gynoecium (Smyth *et al.*, 1990). Separating the second and third whorls of *Arabidopsis* is a ring of nectary tissue, consisting of 2 prominent lateral outgrowths abaxial (adaxial in *B. napus*) to the lateral stamens and 4 prominent (2 prominent in *B. napus*) median outgrowths abaxial to the median stamens (Davis, 1994; Davis *et al.*, 1986). Although this floral ground plan is variable with respect to stamen number, the presence or absence of petals, the union of sepals, and the arrangement of nectary tissue, the gynoecium is essentially free of any deviation from the ground plan (Endress, 1992).

Floral organs originate from layers of undifferentiated cells (histogenic layers) in the floral meristem. Coordinated cell division and expansion between these cells are necessary to generate the floral organs. Therefore, cell-cell communication resulting in differential gene expression is required for normal development. The formation and identity of 4 of these floral organs (sepals, petals, stamens, and carpels) can usually be predicted and explained with reasonable accuracy using the ABC model (Coen and Meyerowitz, 1991). This model suggests that several sets of homeotic genes act alone or in combination to correctly direct floral organ formation and identity in *Arabidopsis*.

Products of the class A genes (*APETALA1* and *APETALA2*) function alone in whorl 1 to direct sepal formation and in combination with class B gene products in whorl 2 to direct petal formation (Komaki *et al.*, 1988; Bowman *et al.*, 1989, 1991b, 1993; Kunst *et al.*, 1989; Hill and Lord, 1989; Jack *et al.*, 1992; Schultz and Haughn, 1993; Gustafson-Brown *et al.*, 1994; Jofuku *et al.*, 1994). Products of the class B genes (*APETALA3* (*AP3*) and *PISTILLATA* (*PI*)) also function in combination with the class C gene product in whorl 3 to direct stamen formation (Bowman *et al.*, 1989, 1991b, 1993; Hill and Lord, 1989; Jack *et al.*, 1992). The product of the class C gene (*AGAMOUS* (*AG*)) also functions in whorl 4, to direct carpel formation (Bowman *et al.*, 1989, 1991b; Yanofsky *et al.*, 1990; Drews *et al.*, 1991). Recently class D and class E functions have been added to the model. Products of the class D genes (*SPATULA* (*SPT*) and *CRABS CLAW* (*CRC*)) function in combination with *AG* in whorl 4 to direct carpel development (Alvarez and Smyth, 1997), while the product of the class E genes (*SEPALLATA1, 2,* and *3*) functions in combination with class B and C genes in whorls 2, 3, and 4 to direct petal, stamen, and carpel development, respectively (Honma and Goto, 2001; Theissen et al., 2000; Theissen, 2001).

Previous studies have demonstrated that the ectopic expression of *AP3* (Jack *et al.*, 1994) or the ectopic expression of both *AP3* and *PI* (Krizek and Meyerowitz, 1996), under the control of the constitutive 35S promoter of cauliflower mosaic virus, can cause a homeotic conversion of carpels to stamens in whorl 4. While these studies established the role of these genes as regulators of petal and stamen identity in *Arabidopsis* flowers, they provide little insight to the morphological basis of stamen to carpel conversion. Furthermore, the possibility of other subtle consequences caused by the ectopic expression of these genes in other tissues cannot be disregarded.

The objective of this study was to target the expression of the B class gene *BnAP3* of *B. napus*, a homolog to *AP3*, to the fourth whorl of developing flowers and investigate the morphological and anatomical changes that result in the flower. By utilizing the *AG* enhancer elements (Deyholos and Sieburth, 2000) to target *BnAP3* expression to the reproductive organs, any observed effects could be attributed solely to the localized misexpression of *BnAP3* in the flower. Previous research had suggested that the action of *AP3* in the fourth whorl might be dosage dependent (Jack *et al.*, 1994).

By placing *BnAP3* under the control of the *AG* controlling elements, which direct a far lower level of expression than the 35S CaMV promoter (Yanofsky *et al.*, 1990; Odell *et al.*, 1985), more precise increments of transcriptional expression levels could be exerted with varying transgene insertion events. The use of the *B. napus AP3* gene for these studies reduced the possibility of transgene silencing in *Arabidopsis*.

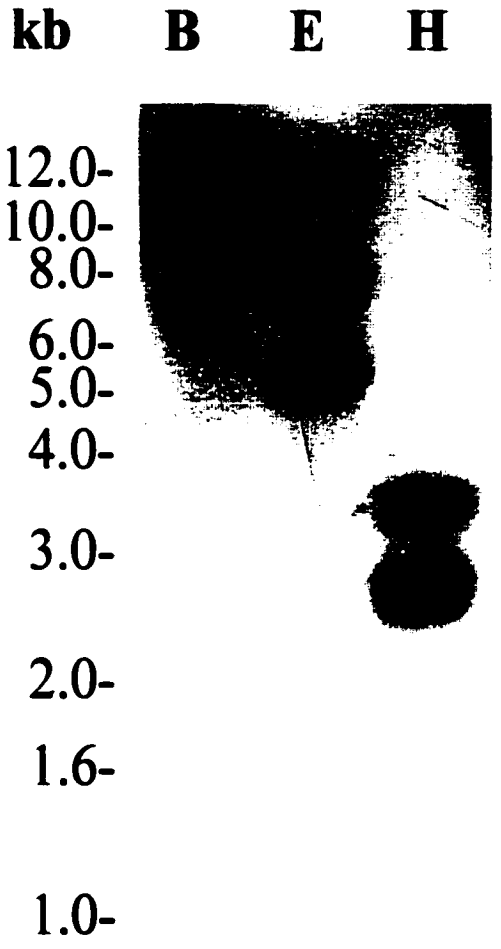
4.2 Results

4.2.1 Isolation and characterization of *BnAP3*

Screening of the *B. napus* cDNA library with the *A. thaliana AP3* probe yielded a single clone, pBAP3-1, which was highly similar to the 3' region of *AP3* of *A. thaliana* (Jack *et al.*, 1992). 5' RACE was used to retrieve the ~250 bp 5' region predicted, by sequence alignment, to be missing from the pBAP3-1 clone (see experimental procedure). Two products of 5' RACE were identified: pBAP3#9 and pBAP3#14. The nucleotides of the overlapping region of pBAP3#9 and pBAP3-1 were identical, while that of pBAP3#14 and pBAP3-1 were 96% similar. Two orthologs of *AP3* have previously been identified in *B. oleracea* L. var. *italica* (Carr and Irish, 1997). The nucleotide sequences of both pBAP3#9 and pBAP3-1 were highly similar to the *B. oleracea Boi2 AP3* cDNA (97%) while pBAP3#14 was less similar to *Boi2 AP3* (94%), but 99% similar to *Boi1 AP3*. These results suggest that at least two copies of *AP3* exist within the *B. napus* genome which would be expected since *B. napus* is an amphidiploid, its genome originating from *B. oleracea* and *B. rapa* (U, 1935; Erickson *et al.*, 1983). Southern analysis of *B. napus* genomic DNA probed with *BnAP3* DNA lacking the MADS box suggests that up to 3 copies of the *BnAP3* gene occur within the *B. napus* genome (figure 4.1).

The predicted amino acid sequence of *BnAP3* (accession #AF124814, Appendix A.1) shares 98% identity with that of *Boi2AP3*, 95% with *AP3*, and 49% with that of *OsMADS16*, the *AP3* ortholog from rice (*Oryza sativa*) (figure 4.2). The MADS box of *BnAP3* is identical to that of *Boi2AP3*, 98% similar to that of *AP3*, and 77% similar to that of *OsMADS16* while the K region of *BnAP3* is 97% similar to that of *Boi2AP3* and

Figure 4.1 Southern analysis for *BnAP3* copy number using genomic DNA collected from wild-type *B. napus* plants digested with *Bam*HI (B), *Eco*RI (E), and *Hin*DIII (H). A probe specific for the *BnAP3* gene (excluding the MADS box) was hybridized to the digested DNA and washed to 0.2X SSPE at 65°C. Southern analysis suggests that up to 3 copies of *BnAP3* exist within the *B. napus* genome.


kb
B
E
H
12.0-
10.0-
8.0-
6.0-
5.0-
4.0-
3.0-
2.0-
1.6-
1.0-

AP3, and 49% similar to that of *OsMADS16* (figure 4.2). *BnAP3*, like *Boi2AP3*, has a small deletion, unimportant for function (Carr and Irish, 1997), at amino acid positions 194-201 compared to *AP3* and *OsMADS16* (figure 4.2).

RNA isolated from various tissues and amplified through RT-PCR with primers specific for *BnAP3* showed that *BnAP3* RNA is present in flowers, and also in non-floral tissues. RT-PCR produced strong signals specific for the 672 bp *BnAP3* mRNA in floral buds and carpels, both unfertilized and fertilized (figure 4.3). The detection of *BnAP3* in buds is consistent with its role in determining the identity and development of petals and stamens. The presence of a signal in apical meristems collected 28 hours following light induction to flowering (TSAMs) suggests that *BnAP3* is expressed very early in flower development. This is in agreement with the finding that in *Arabidopsis*, *AP3* is first expressed at early stage 3 in flower development, coinciding with the initiation of sepal primordia (Hill *et al.*, 1998). *AP3* transcripts are detected late in ovule development within the integuments of *Arabidopsis* (Jack *et al.*, 1992) and *BnAP3* may also be similarly expressed in *B. napus*, accounting for its presence in the gynoecium. *BnAP3* was also detected in non-floral tissues, generating a strong signal in roots and a weak signal in bracts (figure 4.3). *BnAP3* was not detected in either leaves or stems. Neither *AP3* (Jack et. al., 1992) nor the rice ortholog *OsMADS16* (Moon *et al.*, 1999) has been detected in non-floral tissues. These previous studies however, were based on Northern analysis, which is not as sensitive as RT-PCR, for detecting transcripts. Whether or not *BnAP3* serves a functional role in these tissues remains to be determined.

4.2.2 Phenotype correlates with transgene insertion events

Following *Agrobacterium*-mediated transformation with the AG+BAP3 construct (figure 4.4), several *Arabidopsis* plants were identified as resistant to kanamycin. These plants were designated as wild-type, or weakly, or strongly mutant based on their ability to set seed. Of the wild-type plants, all flowers produced normal siliques and set seed. The flowers of weakly mutant plants set very little seed and produced a few (~5-10) seed-bearing siliques that could be found at any position along

Figure 4.2 Predicted amino acid identities between the *BnAP3*, *Boi2AP3*, *AP3*, and *OsMADS16* coding regions. *BnAP3* and *Boi2AP3* show 98% identity and are both predicted to encode a protein of 224 amino acids in length. *BnAP3* has 95% identity to *AP3* and 49% identity to *OsMADS16*, which encode proteins of 232 and 223 amino acids, respectively. The MADS box region is indicated with a solid box that extends from amino acids 2 to 57. The K box region, indicated with a broken box, extends from amino acids 88 to 154.

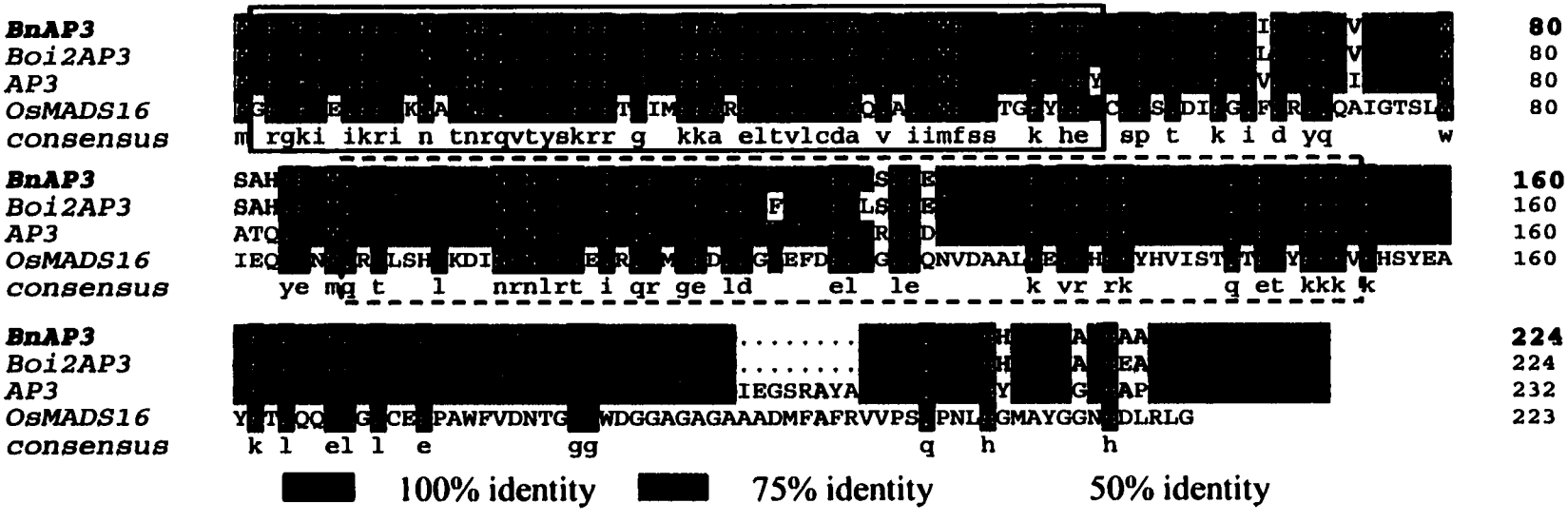

BnAP3
Boi2AP3
AP3
OsMADS16
consensus
80
160
224
100% identity
75% identity
50% identity

Figure 4.3 Expression profile of *BnAP3* in tissues collected from *B. napus* as determined using RT-PCR. *BnAP3* transcripts were not detected in leaves or stems. *BnAP3* transcripts (672 bp in length) were detected in roots, bracts, TSAMs (shoot apical meristems collected 28 hours following light induction to flowering), floral buds, and young and mature gynoecia. Actin transcripts (approximately 580 bp in length) served as a positive control and were detected in all tissues.

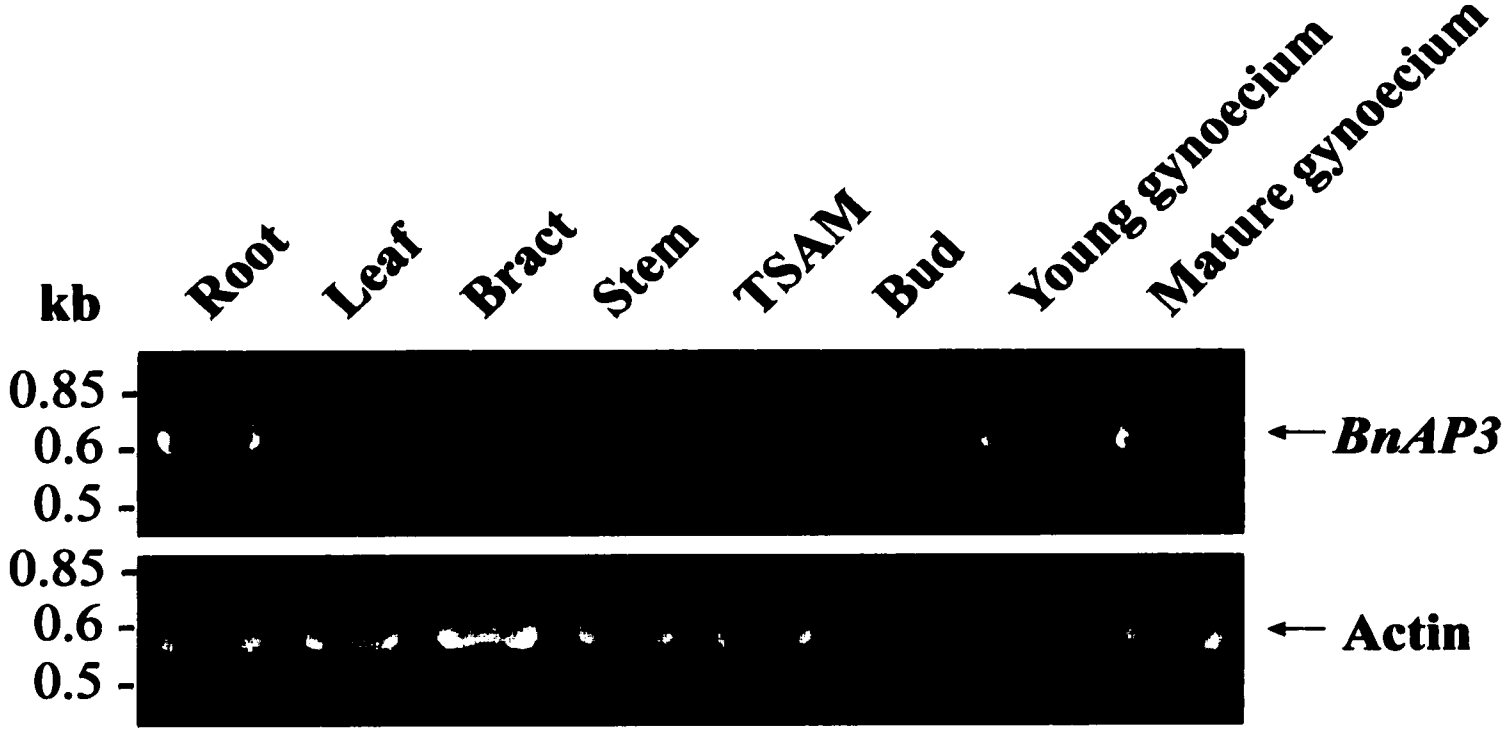
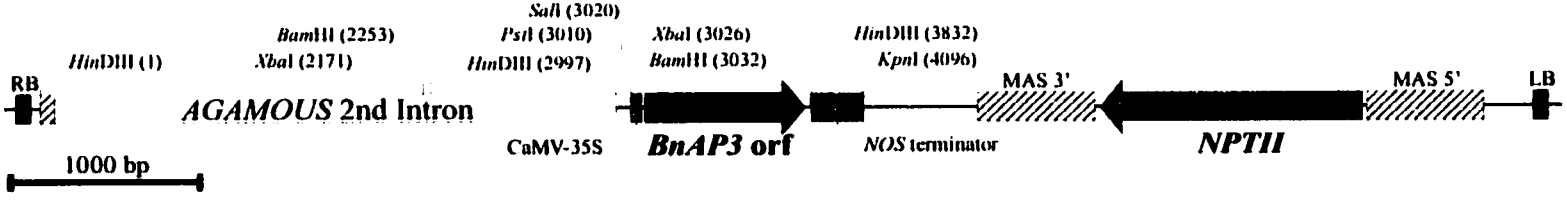

kb
Root
Leaf
Bract
Stem
TSAM
Bud
Young gynoecium
Mature gynoecium
0.85
0.6
0.5
BnAP3
0.85
0.6
0.5
Actin

Figure 4.4 The *AG::BnAP3* construct. Cis-acting elements required to direct the correct expression of *AGAMOUS* are contained within the 3 kb second intron while the TATA box is provided in the CaMV 35S minimal promoter (Sieburth and Meyerowitz, 1997). The (CaMV::*BAP3* orf::*NOS* terminator) cassette was generated in pBluescript KS+ and directionally cloned into pMD992 at the *Pst*I and *Kpn*I restriction sites. *NPTII* is a selectable marker, present as part of the pCGN1547 binary vector, which confers kanamycin resistance.

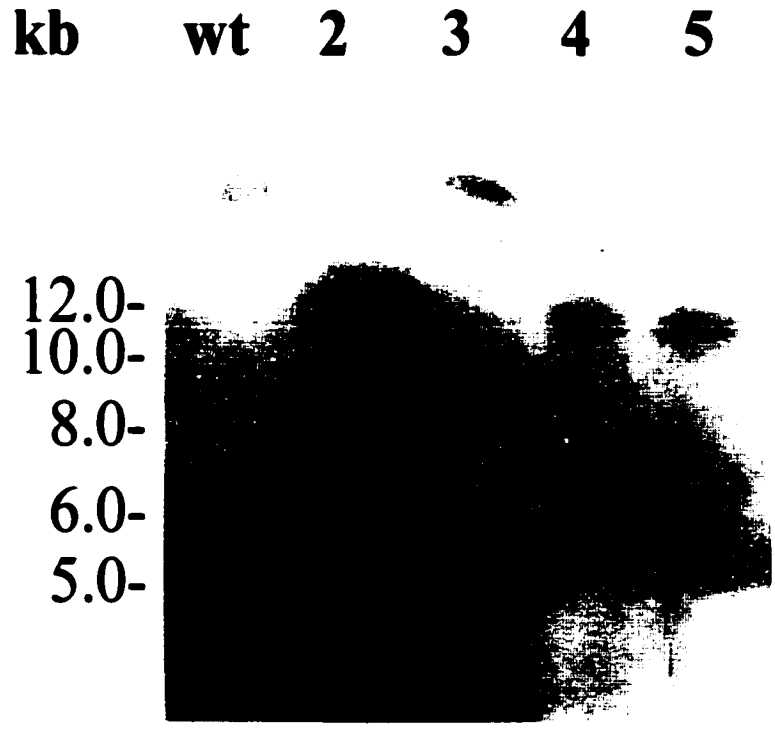

SalI (3020)
BamHI (2253)
PstI (3010)
XbaI (3026)
HinDIII (3832)
HinDIII (1)
XbaI (2171)
HinDIII (2997)
BamHI (3032)
KpnI (4096)
RB
MAS 3'
MAS 5'
LB
AGAMOUS 2nd Intron
CaMV-35S
BnAP3 orf
NOS terminator
NPTII
1000 bp

the inflorescence. The flowers of strongly mutant plants rarely set seed and produced only one or two seed-bearing siliques that were located acropetally on the inflorescence. In subsequent generations, the severity of floral alterations did not correlate with position on the inflorescence. Transgene integration was assessed by Southern analysis for each arbitrarily assigned class of transgenic plant (figure 4.5). Plant 2, which was designated as strongly mutant, had 4 transgene inserts; plant 3, designated as weakly mutant, had three transgene inserts; and plants 4 and 5, designated as wild-type, contained only one transgene insert each (figure 4.5). The number of inserts correlated positively with the degree of phenotypic abnormalities seen in the transgenic plants.

4.2.3 *AG::BnAP3* does not affect floral whorls 1, 2, or 3

Mature flowers (n = 11 for plant 2; n = 10 for plant 3;) of *Arabidopsis* T_0, ranging in development between stages 11 – 16 (Smyth *et al.*, 1990), were examined by SEM. All flowers contained four wild-type sepals and four wild-type petals displaying normal shape, size, and surface morphology on both the abaxial and adaxial surfaces. Of the flowers examined, 90% contained the full complement of stamens, two short lateral and four longer median stamens. Ten percent of flowers contained only five stamens, lacking one at a lateral position. In flowers missing a lateral stamen, a distinct region of undifferentiated cells, unoccupied by any other structure, was found at the receptacle where the missing lateral stamen would normally be located. This arrangement suggests that the missing stamen was aborted early in development. In *Arabidopsis*, ecotype Columbia, flowers quite often do not contain a full complement of stamens, often lacking either one or both stamens in the lateral plane (Pylatuik *et al.*, 1998). All stamens were wild-type in appearance, displaying typical anther and filament cellular arrangement and morphology. Located between the second and third whorls, abaxial to the stamens, nectary tissue was consistently present in all flowers examined and was never seen to develop in regions adaxial to the second and third whorl boundaries. Although lateral portions of the nectarium (Davis *et al.*, 1998) were always present, medial proliferations of the nectary tissue, typically arising abaxial to each of the median stamens, were not always present. These observations are consistent with

Figure 4.5 Southern analysis of genomic DNA collected from a selection of wild-type (wt) and *AG::BnAP3 Arabidopsis* plants (2-5), digested with *BamHI*. A probe specific for the *AG::BnAP3* construct was hybridized to the digested DNA. Southern analysis indicated at least 4 transgene insertion events for plant 2, 3 transgene insertion events for plant 3, and only one transgene insertion in plants 4 and 5.


kb
wt
2
3
4
5
12.0-
10.0-
8.0-
6.0-
5.0-

the previously reported nectary phenotype of wild-type flowers (Davis, 1994) and *35S::AP3* flowers (Baum *et al.*, 2001).

4.2.4 *AG::BnAP3* triggers the production of four organs in floral whorl 4

In all but two flowers examined by SEM (n = 21 of T_0) and all but three flowers examined by LM (n = 32 of T_1), the wild-type gynoecium (figure 4.6a) of whorl 4 (w4) was replaced with four distinct organs (figures 4.6b, c and 4.7c), two of which arose in the lateral axis, immediately adaxial to each lateral stamen, and two of which arose in the median axis, central and adaxial to each of the two pairs of median stamens (figures 4.6d, 4.7b and c). No flowers contained more than four organs in w4.

Only five flowers (n = 53 of both T_0 and T_1) contained less than four organs in w4. Both flowers examined by SEM (T_0) were missing an organ from the median axis. One of these flowers contained a distinct region of enlarged cells occupying the receptacle where a median organ would have otherwise occurred. The second of these flowers contained a lateral organ, the base of which was attached to the receptacle spanning both the median and lateral axes. Originating from the median axis, this organ displayed a pronounced fringe of marginal tissue extending the length of the organ (figure 4.6e). The marginal tissue consisted of files of elongated cells lacking stomata and similar in appearance to that of the margin of fusion in the wild-type gynoecium.

4.2.5 *AG::BnAP3* causes floral indeterminacy

Many flowers displayed additional whorls of organs arising from the receptacle interior to the quartet of w4 organs. The degree of floral indeterminacy resulting from expression of the *AG::BnAP3* construct was correlated with transgene insertion events. In plant 3 (weakly mutant with three transgene inserts), 90% of flowers examined (n=10) contained four whorls while 10% contained a fifth whorl (w5). In plant 2 (strongly mutant and containing four transgene insertion events), 91% of flowers examined (n=11) contained at least five whorls of organs (figure 4.6c), and 9% contained an additional sixth whorl (w6) (figure 4.6f).

Figure 4.6 Scanning electron micrographs of *Arabidopsis* flowers from wild-type (a) and *AG::BnAP3* plants (b-k). (a) Vertical view of a stage 12 wild-type flower. (b) Vertical view of a stage 11 flower displaying a mild phenotype with four organs (numbered 1–4) interior to the whorl 3 (w3) stamens. One lateral stamen did not develop. (c) Median view of whorl 4 (w4) and whorl 5 (w5) organs from a flower showing a strong phenotype. The proximal median organ of w4 was removed for clarity. (d) Median view showing successive organs in the lateral axis (left side) of a flower with a strong phenotype. Acropetally these organs are the lateral nectary (ln), stamen (ls), w4 organ (l(4)), and w5 organ (l(5)). A median organ arises aligned and adaxial to the median pair of stamens (right of center). Sepals and petals have been removed. (e) Flower with a mild phenotype containing only three organs in w4. Note the prominent ridge of tissue arising from the median plane, extending the length of the organ (arrowhead). (f) Two unfused whorl 6 (w6) organs each arising from the lateral plane, with w4 and w5 organs removed. Successive whorls are separated by stalks (arrows). A prominent ridge of tissue is visible on one w6 organ (arrowhead). Fourth (g) and fifth (h) whorls are distinctly separated by a prominent stalk (arrow). Filaments which subtend all of the w4 organs of this flower are visible (g), while none of the w5 organs contained a proximal filament (h). (i) W5 organs are interior and aligned with w4 organs in both the median and lateral planes. (j) Unfused organs of w5 arise predominantly from the lateral axis, but often have a prominent ridge congenitally fused along one margin which originates in the median plane (arrowhead). (k) Occasionally the inner-most whorl produced a fused gynoecium reduced in size (5). ms, median stamen, ls, lateral stamen, ln, lateral nectary, l, lateral organ, m, median organ. Scale bars, 100 μm.

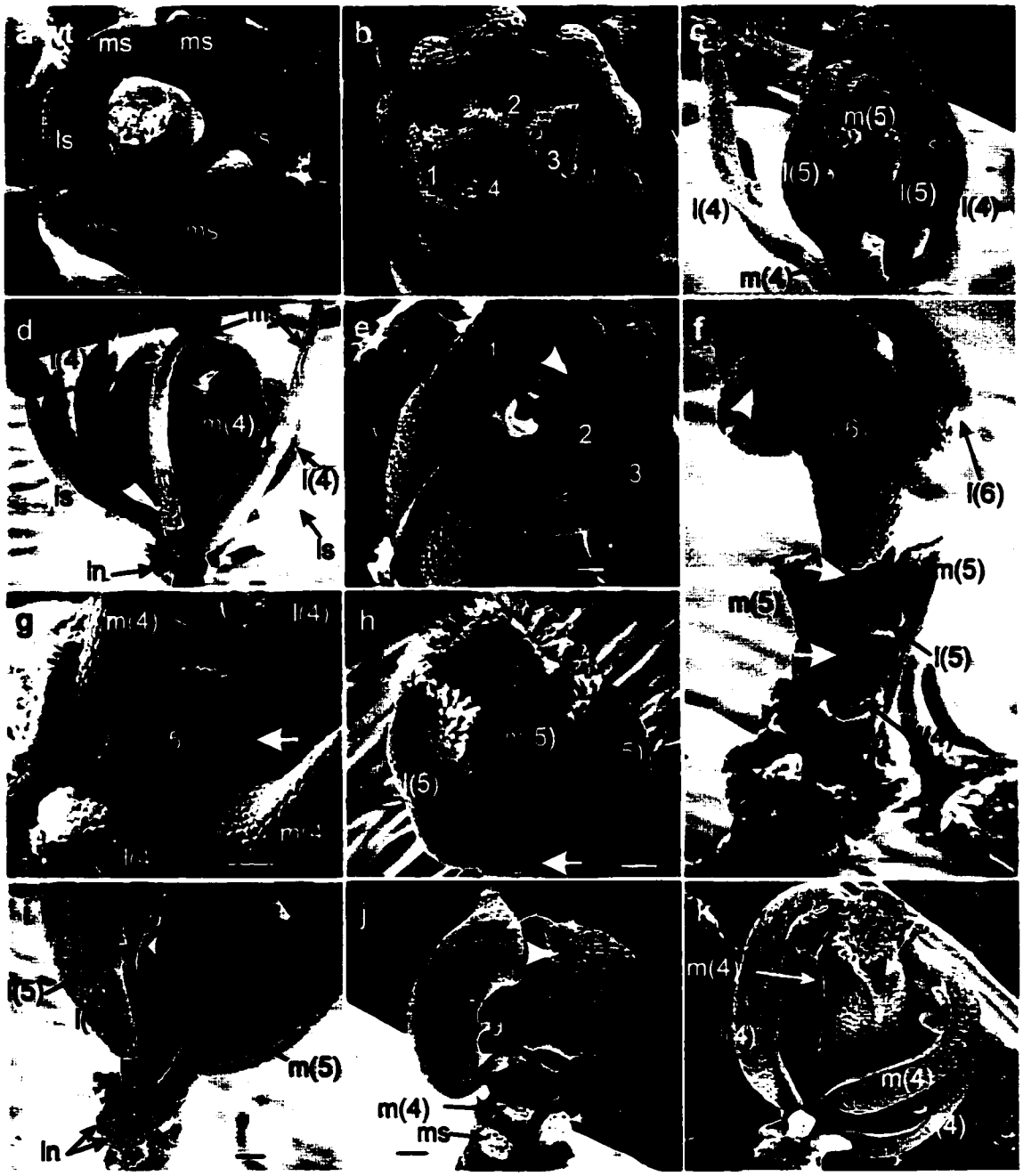
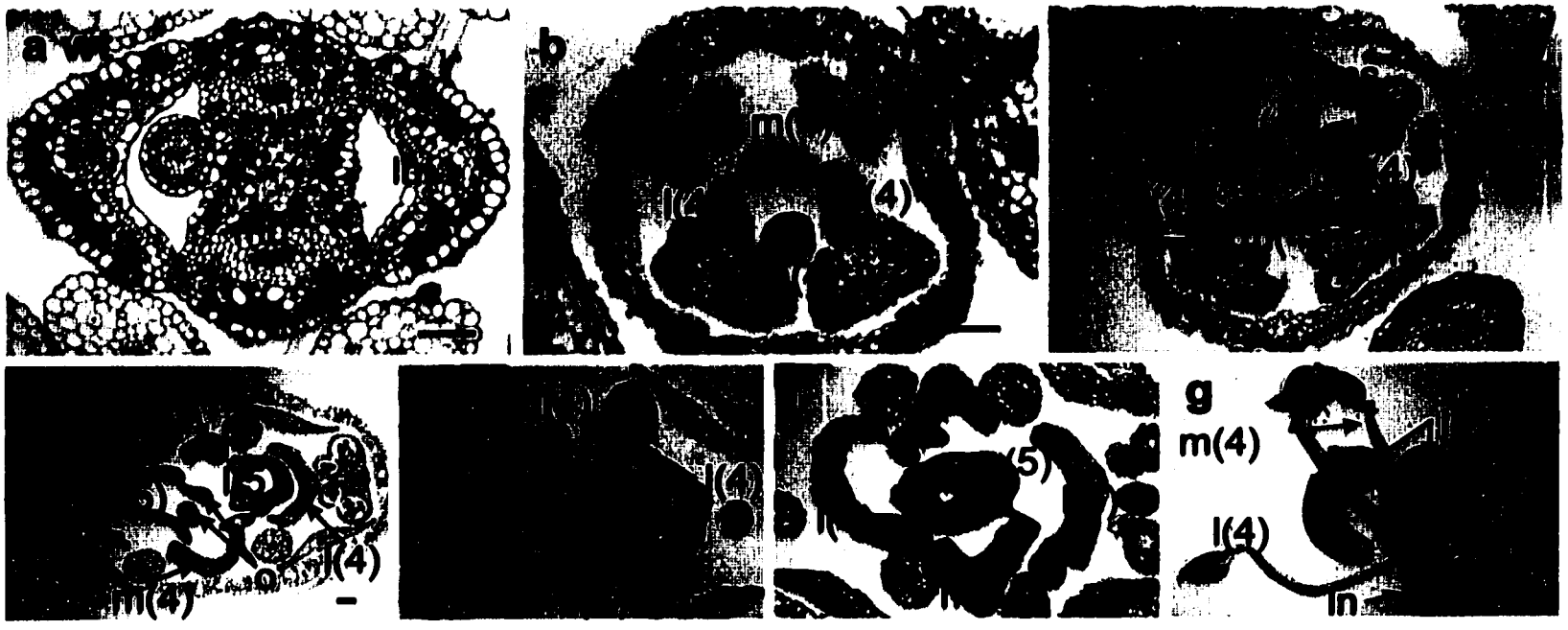

ms
ms
ls
ms
b
2
1
3
4
c
m(5)
l(5)
l(5)
l(4)
l(4)
m(4)
d
l(4)
m(4)
ls
l(4)
ls
ln
e
1
2
3
f
l(6)
m(5)
m(5)
l(5)
g
m(4)
l(4)
m(4)
h
l(5)
l(5)
m(5)
ln
j
m(4)
ms
k
m(4)
m(4)

Figure 4.7 Light micrographs of transverse sections of wild-type (a) and *AG::BnAP3* (b-f) *Arabidopsis* flowers and buds stained with toluidine blue. Sections (a-f) are arranged such that the lateral axis is horizontal and the median axis is vertical. A whole mount flower (g) was stained in phloroglucin-HCl and viewed in the median plane. (a) Wild-type gynoecium which occupies whorl 4 (w4). The lateral (lb) and median (mb) vascular bundles occupy the valves and placentae, respectively. (b) Stage 7 bud (mild phenotype) with four distinct primordia emerging from the center of the flower. In the median axis, one w4 organ differed from the others, appearing more cylindrical in shape (bottom). (c) Stage 9-10 bud (mild phenotype) with four distinct organs produced adaxial to the w3 stamens. Two organs arose adaxial and aligned to each pair of median stamens, while the other two organs arose in the lateral plane, adaxial and aligned to the lateral stamens. Only one lateral stamen developed. Each w4 organ contained microsporogenous tissue in a pair of microsporangia located near the margins (arrowhead). (d) A mature bud with four unfused w4 organs and two unfused w5 organs. Both w5 organs arose in the lateral plane. Note the ovules (arrows) produced by one of the w5 organs. (e) A mature bud displaying four w4 organs and two w5 organs fused along one margin. Note the ovules (o) produced by w4 and w5 organs. (f) Mature bud displaying four w4 organs and a fused structure in w5 resembling a gynoecial tube. (d-f) Flowers displaying strong phenotypes. Organs that arose from the median plane were often variable in appearance while those in the lateral plane were more consistent in appearance. This observation was attributed to the fact that median organs of w4 were often subtended by filaments which appeared circular in these sections. (g) Mature flower displaying two lateral and two median stamen-like w4 organs. The elongated filaments of these organs each contained a single vascular bundle that extended to the apex of the organ. The two w5 organs that arose from the lateral axis were more carpel-like although they still contained 2 pollen sacs which arose from each margin. A lateral w4 and w5 organ (left) became detached from the receptacle during mounting. ms, median stamen, ls, lateral stamen, ln, lateral nectary, l, lateral organ, m, median organ. Scale bars: A-F, 50 μm; G, 500 μm.


a
b
m(4)
l(4)
g
m(4)
l(4)
ln

Successive w4, w5 and w6 organs were often distinctly separated by a prominent stalk (figure 4.6f-h) referred to as a gynophore by Alvarez and Smyth (1999). Of the mutant flowers which displayed additional whorls, half contained a w5 which produced a quartet of organs, two in the median axis and two in the lateral axis (figure 4.6c and h). This second quartet of organs originated adaxial and aligned with the base of the w4 quartet of organs (figure 4. 6c, d a nd i). T he o ther ha lf o f t he m utant flowers w ere comprised of two organs in w5 or w6. This second diad of organs always arose in the lateral axis either unfused (figures 4.6f, j, 4.7d and g), fused along one margin (figure 4.7e), or completely fused (figure 4.7f) into a reduced gynoecium (figure 4.6k). Unfused w5 and w6 organ pairs often displayed a pronounced fringe of marginal tissue which extended the length of the organ and originated from the median axis (figure 4.6f and j), similar to that previously described (figure 4.6e). Nectary tissue was not present adaxial to wild-type positions, similar to observations in *35S::AP3* plants (Baum *et al.*, 2001).

4.2.6 *AG::BnAP3* causes conversion of carpels to stamens

Individual organs produced in w4-w6 of *AG::BnAP3* plants displayed properties of both stamens and carpels. These organs ranged from being very carpel-like to very stamen-like (figure 4.8a-h). Organs of w4 were usually the most stamen-like while w5 organs were more carpel-like (figure 4.7g). In the weakest conversion, organs displayed many of the characteristics of the wild-type gynoecium. For example, organs were boat-shaped similar to carpel valves, with a concave adaxial surface (figures 4.8a and 4.9d top) and displayed a reduced amount of distinctive marginal tissue from which a few ovules arose (figures 4.7d and 4.8a). A large megasporocyte (megaspore mother cell) was detectable within some ovules (figure 4.9i). On the abaxial epidermis, valve-like organs were comprised of cells morphologically identical to those of the wild-type ovary wall, with epicuticular wax appearing as specks on the surface (figure 4.8h). Chronologically, stomata on these surfaces developed in a similar manner to those of wild-type ovary walls and appeared later in flower development at stage 14-15 (figure 4.8h). The distal region of organs typically displayed a broad ridge of stylar tissue

Figure 4.8 Scanning electron micrographs of organs produced in the fourth and subsequent whorls of *Arabidopsis AG::BnAP3* flowers. (a) Adaxial view of a whorl 4 (w4) organ which displayed a prominent distal ridge of stigmatic papillae (arrowhead)

and a few ovules (o) along the margins. (b) Abaxial view of a twisted organ similar to that in (a), from the flower in (o). The prominent distal ridge of stylar tissue was continuous with the marginal tissue which extended to the base of the organ. (c) Adaxial view of an organ with two abnormal ovule-like outgrowths along its margins and a prominent invagination (arrowhead) along the proximal region of its margin. (d) Adaxial view of an organ displaying one marginal outgrowth and two prominent marginal invaginations at the distal portion of the organ. Note the short stalk or filament at the basal region of the organ. (e) Abaxial view of an organ subtended by a prominent basal filament. (f) Adaxial view of an organ similar to (e), tipped at its apex with stigmatic papillae. Both a pollen sac (arrowhead) and an ovule (o) existed at the distal region of this organ. (g) Adaxial view of a nearly wild-type stamen displaying a distinct anther (a) and filament. (h) Cells on the abaxial surface of w4 organs, typical of those that occurred on the ovary wall of wild-type carpels, were interspersed with stomata (st) and had epicuticular specks of wax. (i) A weak phenotype displaying four organs fused along the basal region of their margins. (j) Marginal outgrowth of an organ resembling a funiculus at its proximal region and tipped with stigmatic papillae at its apex. (k) Apex of a w4 organ displaying stylar tissue with a prominent cluster of stomata converging at the tip. (l) Transverse view of the base of a fractured w4 median organ showing a single vascular bundle (arrowhead) in its center. (m) Lateral view of a typical filament generated in the proximal region of a w4 organ. Epidermal cells on the abaxial surface (ab) were similar to those produced on the abaxial surface of ovary walls and were interspersed with stomata. Cells of the marginal surface (m) were filed similar to those found at the margin of fusion in a wild-type gynoecium; however, they had prominent cuticular thickenings similar to those of the stylar tissue or anthers and filaments of wild-type stamens. Cells were interspersed with fewer stomata than those of the abaxial surface. Cells of the adaxial surface (ad) resembled those of wild-type filaments and did not include stomata. (n) W4 of a stage 7 bud which displayed dimorphic lateral and median organ primordia. (o) Median view of a stage 15 flower showing dimorphic median and lateral organs. Lateral organs were sessile to the receptacle while median organs were subtended by a filament. l, lateral organ, m, median organ, f, filament. Scale bars, 100 μm.

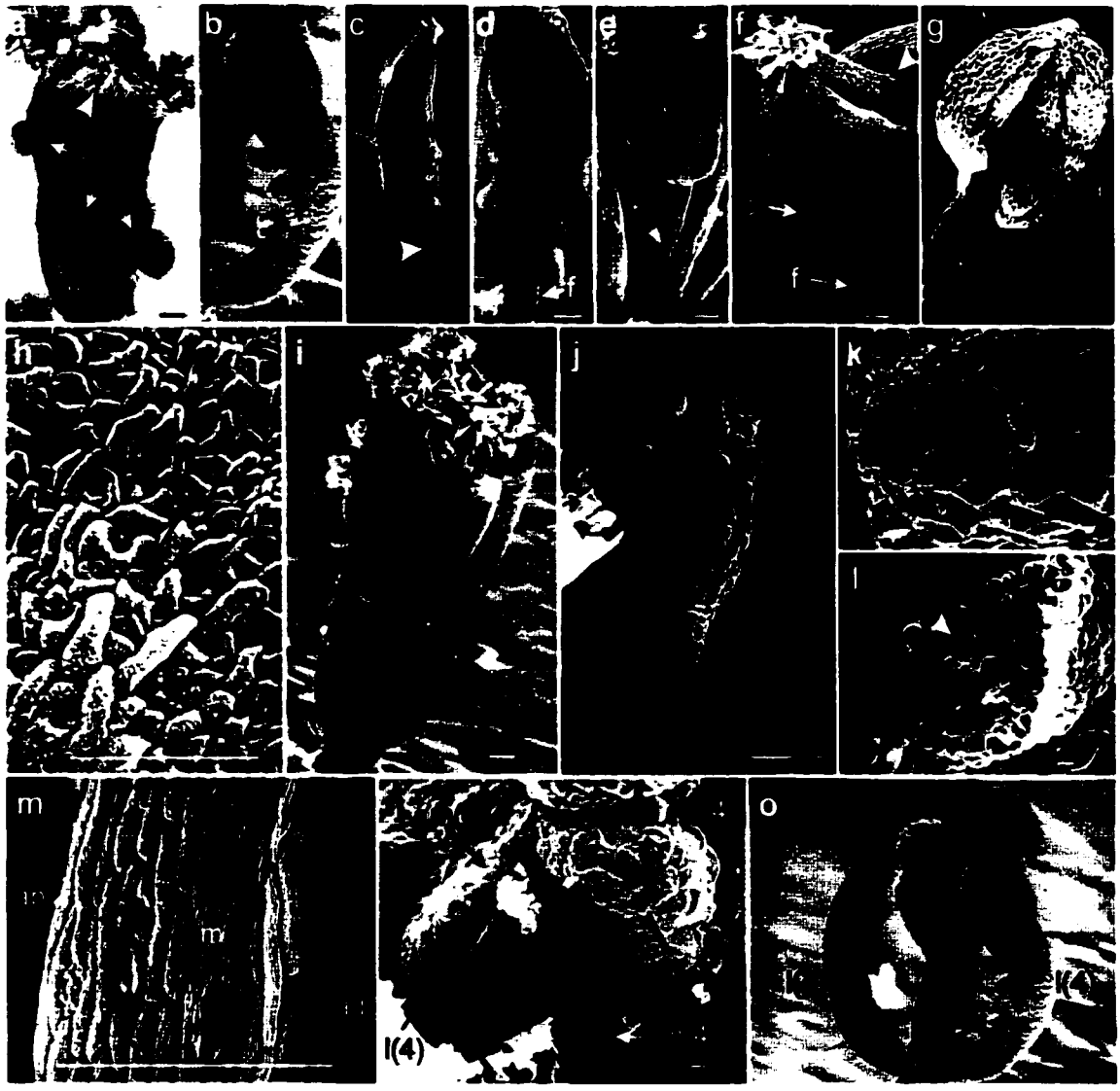
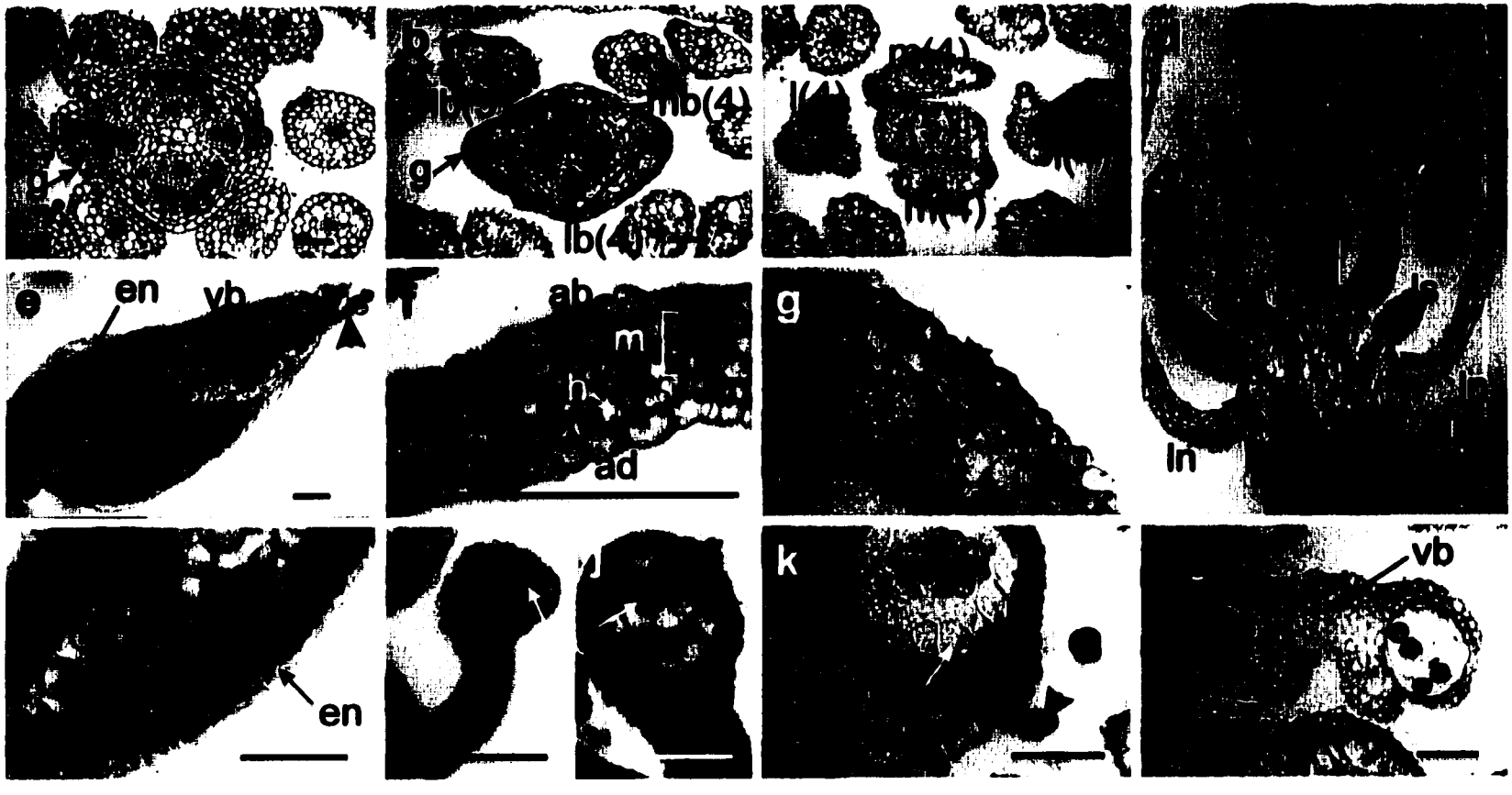

a
b
c
d
e
f
g
h
i
j
k
l
m
m
m
l(4)
o

Figure 4.9 Light micrographs of wild-type (a) and *AG::BnAP3* (b-l) *Arabidopsis* flowers stained with toluidine blue (except e and h, stained with phloroglucin-HCl). (a-c) Transverse sections oriented such that the lateral axis is horizontal. (b) Four prominent vascular bundles occurred in the stalk or gynophore (g), similar to that seen in the gynophore of wild-type flowers (a). Flowers with more than four whorls (c) had additional vascular tissue interior to the whorl 4 (w4) bundles (b). (c) In sections approximately 50 – 70 μm higher in the floral bud, lateral and median vascular bundles were traced to the lateral and median w4 organs. (d) Longitudinal section through a complete flower viewed from the median plane. Vascular bundles to the lateral w4 organs shared the same trace as w3 lateral stamens. (e) Mature w4 organ with a single vascular bundle (vb) extending to the apex tipped with stigmatic papillae (arrowhead) bearing pollen. The bundle has widened just below the apex. (f) Transverse section of a w4 organ that was carpel-like in appearance and composed of six cell layers, typical of wild-type gynoecial valves, making up the abaxial epidermis (ab), three layers of mesophyll (m), a hypodermal layer of narrow elongated cells (h), and an adaxial epidermis (ad). (g) Transverse section of a stamen-like organ. There was no distinct adaxial hypodermal layer, although the sub-epidermal layer on the abaxial surface (arrowhead) was distinctly different from the mesophyll. (h) Mature w4 organ like that of (e), displayed a distinct endothecium surrounding the pollen sac. (i) Ovule produced on the margin of a w4 organ contained a distinct megaspore mother cell (arrow). (j) Pollen sac of a w4 organ contained microsporogenous tissue and a tetrad of microspores (arrow). (k) Microsporangium of a w4 organ contained a tapetum (arrow). Note the adjacent stigmatic papillae (arrowhead). (l) W4 organ with two microsporangia. Pollen grains produced within the pollen sac on the right were nearly mature and the tapetum had degenerated. Note the distinct sub-epidermal layer on the abaxial surface of the w5 organ (arrowhead). mb, median bundle, lb, lateral bundle, ln, lateral nectary, ls, lateral stamen, l, lateral organ, m, median organ, en, endothecium. Scale bars, 50 μm.


g
mb(4)
g
lb(4)
mb(4)
lb(4)
e
en
vb
ab
m
h
ad
g
ln
en
k
vb

which became continuous with the replum-like tissue at the margins of the organ (figure 4.8b). This stylar tissue was often tipped with a broad ridge of stigmatic papillae (figure 4.8a), but the stigmatic papillae were never seen to extend along the replum to the basal portion of the organ. Often partial fusion between organs occurred along the basal region of the margins (figure 4.8i).

As the transformation of carpel to stamen became more evident, the number of ovules produced at the marginal tissue decreased and the ovules produced were not fully differentiated, often appearing as short filiform outgrowths (figure 4.8c and d). Two of these outgrowths appeared to comprise a funiculus tipped with stigmatic papillae (figure 4.8j), rather than an ovule. Stylar tissue and stigmatic papillae also became less abundant with the transformation of carpel to stamen (figure 4.8c). Rather than forming a broad ridge, stylar tissue and stigmatic papillae became localized as small patches at the tips or distal regions of the organs (figures 4.8f, 4.9e and k). Occasionally, organs were tipped only with stylar tissue and displayed a group of stomata at the apex (figure 4.8k) rather than stigmatic papillae.

Often small invaginations on the adaxial surface, superficially resembling pollen sacs, were evident, both proximally and distally, along the margins of w4-w5 organs (figure 4.8c). Morphologically, more prominent transformations to stamen-like organs had invaginations along both margins of the organ (figure 4.8d), accompanied by a narrowed basal portion attaching to the receptacle as a stalk or filament (figure 4.8d-g).

The filaments of these stamen-like organs were comprised of elongated and filed cells of several types (figure 4.8m). The abaxial epidermis of the filament was devoid of stomata until later in development when epicuticular wax was also deposited as specks (figure 4.8m), similar to that of the abaxial surface of ovary walls. Along the lateral margins of the filament, cells contained prominent cuticular thickenings similar to cells of the style or replum tissue and were interspersed with stomata, while cells on the adaxial surface became more enlarged and displayed less prominent cuticular ridges (figure 4.8m). One organ (figure 4.8g) was converted to the point at which its various structures, including the filament, exhibited all the epidermal cell types of a wild-type stamen (Bowman and Smyth, 1994). The filaments of w4 organs, like those of wild-type

stamens, underwent substantial elongation late in the development of the flower (figure 4.7g).

The degree of carpel to stamen conversion, like that of floral determinacy, was also correlated with transgene insertion events. Only 10% of the w4, w5, or w6 organs of the examined flowers from line 3 (weak mutant) contained filaments, whereas 55% of the organs produced by the flowers of line 2 (strong mutant) had filaments. Additionally, the w4 organs of line 3 flowers were more likely to be fused along the margins, displayed a more prominent ridge of stylar tissue and stigmatic papillae, and produced ovules in greater numbers. These conversions, more subtle than those produced by ectopic expression of *AP3*, allow some insight into the process of organ differentiation and the ontogeny of the carpel.

4.2.7 Dimorphism between median and lateral fourth whorl organs

The organs that developed in the median axis often displayed morphological characteristics that differed to those of the organs which arose in the lateral axis. The most obvious of these differences was the presence or absence of a subtending filament. Filaments were twice as likely to occur in the median axis (29% of organs) than in the lateral axis (13%) (figures 4.7d,e and 4.8o). In general, organs arising from the median axis tended to appear more stamen-like. The morphological differences between lateral and median organs were evident as early as stage 7 (figures 4.7b and 4.8n). At this early stage in development, organs in the lateral axis developed as elliptical primordia, which were quite large in comparison to their small circular median counterparts which developed between the margins of the two larger lateral organ primordia (figures 4.7b and 4.8n).

4.2.8 Histology of fourth whorl organs

Transverse sections of *AG::BnAP3* flowers revealed four prominent vascular bundles in the gynophore (figure 4.9b) correlating with each of the w4 organs. The presence of four prominent vascular bundles is consistent with that seen in the

gynophore of the wild-type gynoecium (figure 4.9a). Unlike wild-type flowers (figure 4.7a) where the two lateral bundles enter the valves, and the two median bundles enter the placentae of the gynoecium (Lawrence, 1951), in the mutant flowers, each of these four bundles can be traced to the four organs produced in w4 (figures 4.8l and 4.9c). In the stalk-like gynophore, further vascular bundles arose (figures 4.7g and 4.9b) to supply w5 organs with a single bundle each (figures 4.7g and 4.9c). In wild-type flowers however, no further bundles occurred in the central pith region of the gynophore (figure 4.9a). Longitudinal sections showed that laterally, the vascular bundles entering the w4 organs shared the same source as the bundles of the short stamens (figure 4.9d), in a similar arrangement to that seen in wild-type flowers (Davis, 1994). The single vascular bundle of a w4 organ (figures 4.8l and 4.9l) often extended its entire length (figure 4.7g), terminating at the distal end which could be tipped with stigmatic papillae (figure 4.9e). This arrangement contrasts with wild-type stamens wherein the vasculature ends within the connective tissue of the anther (personal observation) and stigmatic papillae are normally absent. Moreover, the dilated grouping of xylem tracheary elements near the tip of these stamen-like organs (figures 4.7g and 4.9e) resembles the dilation typically found in the medial stigmatic region of the Brassicaceae (figure 3.1g).

In organs that most closely resembled the valve portions of the wild-type gynoecium, transverse sections showed that each organ was composed of 6 cell layers similar to those of wild-type ovary walls. These layers, previously described in Alvarez and Smyth (1999), include one layer of abaxial epidermal cells, three layers of mesophyll cells, a layer of thin elongated cells, and a layer of adaxial epidermal cells (figure 4.9f). In organs which demonstrated some transition from carpel to stamen organization, the typical six-layer pattern of cells was lost as the organ became thicker (figure 4.9g). The layer of thin elongated cells, which would be later lignified in wild-type mature fruit, was no longer differentiated and appeared much like the mesophyll cells, while a distinct hypodermal layer of cells at the abaxial surface was produced (figure 4.9g and l, arrowhead). It is probable that along the margins of the organ, surrounding the pollen sacs, this distinct abaxial hypodermal layer gave rise to the endothecium, often seen in these organs (figure 4.9h).

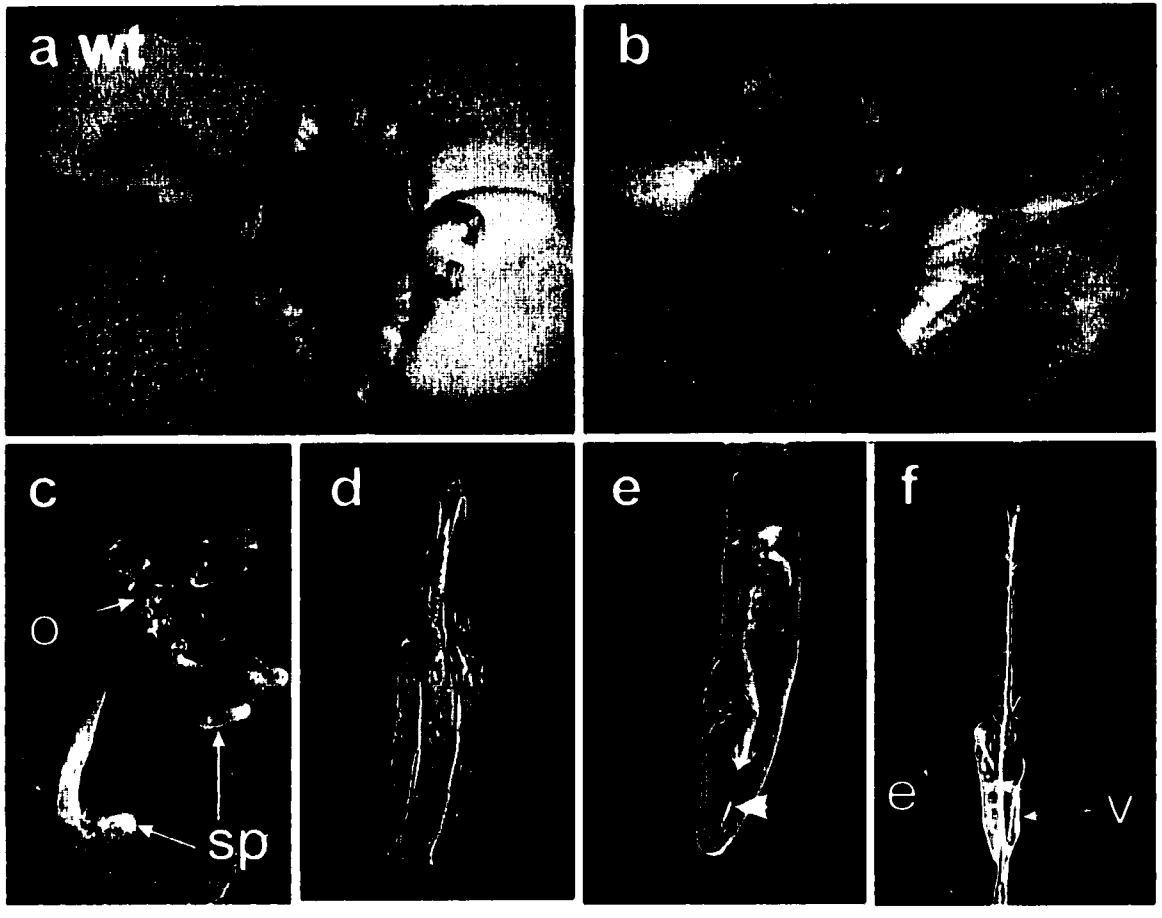
W4 and w5 organs with stamen-like features maximally possessed two microsporangia (figures 4.7c, g, 4.9e and l), and therefore developed only half the microsporangia of the wild-type tetrasporangiate third-whorl stamens abaxial to them (figure 4.7c). Microsporangia produced within the chimeric organs (figure 4.8d, f and g) contained meiotically active tissue (figure 4.9J) which eventually developed into pollen grains (figure 4.9e and l) that resembled wild-type microgametophytes. The locules of these microsporangia were lined by a tapetum (figure 4.9k) which degraded as the pollen grains matured and attained a distinct exine (figure 4.9l). At maturity, pollen sacs of the mutant w4 organs were surrounded by a distinct endothecium (figure 4.9e and h) which presumably contributed to the dehiscence occasionally observed in these organs. Stigmatic papillae were sometimes found at the margin of organs coinciding with the presence of a pollen sac in the interior of the organ (figure 4.9k).

4.2.9 *AG::BnAP3* alters fourth whorl development in *B. napus*

Several *B. napus* lines transformed with the *AG::BnAP3* construct were identified and transgene insertion events analyzed. Most of the flowers of transgenic plants were wild-type in morphology (figure 4.10a) with only a few flowers per plant displaying phenotypic alterations. The frequency with which flowers displayed abnormalities increased acropetally. Unlike *Arabidopsis* plants, the degree of phenotypic abnormalities did not correlate with transgene insertion events (data not shown).

In flowers that displayed abnormalities, the outer three whorls were largely unaffected. This result was expected because the *AG* controlling elements produced an expression pattern in *B. napus* (Pylatuik *et al.*, submitted) similar to that previously reported in *Arabidopsis* (Deyholos and Sieburth, 2000). Sepals and petals were not affected, both being wild-type in arrangement, number, and morphology. Although stamens were wild-type in morphology, a small number of flowers contained more than the typical complement of six stamens. Several lines produced a few flowers (1-5 per plant) which had one or two extra (w3) stamens, often arising in the median axis, adaxial and central to a pair of median stamens. In flowers with an increased number of

Figure 4.10 *B. napus* wild-type (a) and *AG::BnAP3* (b-f) flowers and siliques. (a) and (b) are oriented such that the median axis is horizontal. (b) The wild-type gynoecium arrangement was replaced with 4 aberrant organs in an *AG::BnAP3* flower (compare with a), while the outer whorls of sepals, petals, and stamens remained wild-type in number, organization, and appearance. (c) Whorl 4 (w4) organs produced ovules (o) on their margins and the apices were tipped with a broad ridge of stigmatic papillae (sp). (d) A w4 silique composed of 4 fused organs. (e) A w5 silique within a w4 silique, viewed from the lateral plane (corresponds to the region identified as e' in f). The internal silique was subtended by a prominent gynophore (arrowhead) and is composed of two valves (v), the second of which can be seen when viewed from the median plane (f).


a wt
b
c
o
sp
d
e
f
e`
v

stamens, w4 organs appeared unaffected. The position of these mildly affected flowers was typically basipetal on the inflorescence.

The most severely affected flowers tended to occur acropetally on the inflorescence. Although the outer 3 whorls of these flowers were normal, the w4 gynoecium was replaced with several abnormal carpeloid organs (figure 4.10b). The w4 organs were often four in number, unfused, displayed naked ovules at their margins and their distal regions were tipped with a broad ridge of stigmatic papillae (figure 4.10c). Flowers displaying four fused carpels in the fourth whorl were also produced (figure 4.10d). In one flower, the mature silique that appeared normal externally was found to contain a second bi-valved silique internally (figure 4.10e and f), subtended by a prominent gynophore (figure 4.10e). This arrangement is similar to that observed with w3 and w4 carpeloid organs produced in *ap3-3* mutant flowers of *Arabidopsis* (Jack *et al.*, 1992). Although seeds were produced in the w4 silique, no seeds were produced by the w5 silique. A similar phenotype, produced by high temperature stress, was previously described for cv. Westar of *B. napus* by Polowick and Sawhney (1988). These authors reported that the internal silique was produced from an individual ovule, as an outgrowth of the placenta, rather than as an outgrowth of the receptacle determined here.

4.3 Discussion

4.3.1 *B. napus APETALA3 (BnAP3)* is a B-class gene involved in flower development

BnAP3 has high sequence similarity to other known B-class genes such as *AP3* of *Arabidopsis*, *Boi2AP3* of *B. oleracea*, and *OsMADS16* of *O. sativa*. Its expression pattern, as determined by RT-PCR, was very similar to the expression patterns of *AP3* (Jack *et al.*, 1992) and *OsMADS16* (Moon *et al.*, 1999). The fact that *BnAP3* transcripts were detected in non-floral tissues, unlike *AP3* and *OsMADS16*, could be due to the higher sensitivity of RT-PCR vs. Northern analysis. The detection of *BnAP3* in non-floral tissues does not necessarily suggest an essential function in these tissues, since *AP3* is normally expressed in the integuments of ovules but is not essential for viable

seed production (Jack *et al.*, 1992). It is of interest to note that *BnAP3* transcripts were detected in seedling roots of *B.napus*. Several other MADS-box genes have also been shown to be transcribed in roots (Rounsley *et al.*, 1995; Alvarez-Buylla *et al.*, 2000).

Ectopic expression of *AP3* in *Arabidopsis* was found to be sufficient to cause a replacement of the gynoecium in whorl 4 (w4) with stamens (Jack *et al.*, 1994). We have shown that a similar effect resulted when *BnAP3* is placed under the control of the *AG* controlling elements in *Arabidopsis*, and to a lesser extent, when *AG::BnAP3* was introduced into *B. napus*. Collectively, this evidence suggests that *BnAP3* is homologous to *AP3* and acts to partially specify B-class function in flowers of *B. napus*.

4.3.2 Whorl 4 expression of *BnAP3* results in floral indeterminacy

Control over floral determinacy in *Arabidopsis* is typically regarded as being a function of the *AG* gene. When *AG* is mutated the floral meristem continues to proliferate and, together with a loss of the *AG* organ identity function, subsequent whorls of sepals and petals are produced creating a flower within a flower (Yanofsky *et al.*, 1990). Ectopic expression of both B-class genes, *AP3* and *PI*, in w4 was found to promote cellular proliferation (Krizek and Meyerowitz, 1996), in a manner antagonistic to *AG*. While our research complements these data, we have also demonstrated that increased expression of *BnAP3* alone in w4 is sufficient to affect cellular proliferation. Our data also suggest that cellular proliferation responds in a dosage-dependent manner, as the number of whorls produced in transgenic flowers was positively correlated with the number of *AG::BnAP3* transgene insertion events. We propose that the dosage effect of *BnAP3* also accounted for the difference in phenotypic extremes observed between *Arabidopsis* and *B. napus*. *B.napus* is an allotetraploid with up to three copies of the *BnAP3* gene in its genome, while *Arabidopsis* has a much smaller genome and contains only one copy of *AP3* (Jack *et al.*, 1992). At least three transgene integration events were required before any effect was seen on floral development in *Arabidopsis*. Southern analysis on several *AG::BnAP3* transgenic *B. napus* lines showed a maximum of four transgene integration events (data not shown). By comparison with *Arabidopsis*, this gene dosage would be expected to produce only minor effects, in *B. napus*.

4.3.3 Targeted expression of *BnAP3* to whorl 4 alters organ fate

The *AG* controlling elements used in this work have previously been shown in *Arabidopsis* to target expression, starting in late stage 3 of flower development, to whorls 3 and 4 only (Deyholos and Sieburth, 2000). Previous studies have demonstrated that ectopic expression of *AP3* in *Arabidopsis* causes a homeotic conversion of carpels to stamens (Jack *et al.*, 1994; Krizek and Meyerowitz, 1996). We have shown that targeted misexpression of *BnAP3*, solely to whorls 3 and 4, also caused a homeotic conversion of carpels to stamens demonstrating that localized *BnAP3* expression alone is sufficient to alter the developmental fate of w4. *BnAP3* was not expressed until late stage 3 of flower development when placed under the control of the *AG* controlling elements. Therefore, our results suggest that w4 organ fate and floral determinacy remain somewhat plastic up to this stage of flower development.

No w4 organs were produced which were completely converted to stamens, suggesting that some aspects of carpel identity, already determined by late stage 3 of development, cannot be dedifferentiated by the post-stage 3 introduction of *BnAP3*. This lack of conversion is particularly true of tissues derived from the L1 histogenic layer of cells (see later). An incomplete conversion to stamens could be attributed to the *BnAP3* expression lagging behind that of *PI* in *AG::BnAP3* plants. *PI* is expressed in w4 only during the early stages of flower development however, the added post-stage 3 expression of *AP3* in w4 could be sufficient to maintain effective levels of expression of both genes by an autoregulatory circuit (Jack *et al.*, 1994), even though wild-type *PI* expression has been shown to decline between stages 3 and 5 in w4 (Jack *et al.*, 1994). Because the timing of increased *BnAP3* expression coincided with decreased *PI* expression in w4, it is conceivable that *BnAP3* expression lagged that of *PI*, particularly in the L1-derived tissues resulting in an incomplete conversion to stamen cells in these tissues. Alternatively, transgene dosage of *BnAP3*, previously suggested to play a role in phenotypic variability (Jack *et al.*, 1994; Goto and Meyerowitz, 1994), may not have been high enough to trigger a complete w4 conversion to stamens.

4.3.4 L1-derived tissues remain carpeloid in whorl 4 organs of *AG::BnAP3* flowers

The floral meristem of *Arabidopsis* is comprised of three cell lineages or histogenic layers, L1, L2, and L3, which ultimately contribute, in varying degrees, to the floral organs (Jenik and Irish, 2000). In the wild-type gynoecium, L1 contributes the epidermis on both the abaxial and adaxial surfaces of the carpels, the stigmatic papillae, and the transmitting tract of the style. The L2 and L3 layers contribute the hypodermal layers and the inner cell layers, respectively (Jenik and Irish, 2000). In wild-type stamens a similar fate map occurs; the L1 giving rise to the epidermis, the L2 giving rise to the connective tissue, endothecium, sporogenous tissue, and tapetum, and the vascular tissue arising from the L3 (Jenik and Irish, 2000).

From SEM observations it was clear that a large number of w4, w5, and w6 organs became stamen-like in appearance, contained filaments and produced pollen sacs on their margins. However, the epidermal cell types still remained very carpel-like in appearance. Organs were often tipped at their distal ends with stigmatic papillae and cells with cuticular thickenings similar to wild-type stylar cells. The abaxial surfaces of organs displayed epidermal cells with epicuticular wax deposited as specks. Stomata present in these regions developed over the same time course as those found in wild-type ovary walls. Furthermore, the margins of these organs often displayed cell types similar to the replum of wild-type carpels. This effect was clearly seen in the filaments which subtended the distal portion of the transgenic organs. Epidermal cell types of the abaxial, marginal, and adaxial surfaces of the filaments mirrored the corresponding regions of wild-type carpels.

However, the inner tissues of these organs, as examined by light microscopy, resembled wild-type stamens. The conspicuous hypodermal layer of narrow elongated cells on the adaxial surface, characteristic of wild-type carpels, was often missing. The layers of mesophyll cells increased in number giving rise to connective tissue and resulting in a thickening of the organ. One or both margins often contained microsporangia that consisted of microsporogenous tissue surrounded by a layer of tapetal cells. Upon maturation, these organs displayed a lignified hypodermal

endothecium surrounding the pollen sacs. Mature pollen grains were often found within these pollen sacs.

These results clearly show that the majority of w4 organs of *AG::BnAP3* flowers were stamen-like in tissues derived from the L2 and L3 histogenic layers, but carpel-like in those tissues derived from the L1 layer. This outcome may be the result of differences between the levels of *PI* expression in the different histogenic layers leading to fluctuations in the *PI/AP3* autoregulatory circuit described in the previous section. Alternatively, *AG* controlling elements are known to direct differential expression in specific stamen and carpel tissues during later stages of development (Bowman *et al.*, 1991a). In stamens, *AG* expression is highest in the connective tissues (L2 and L3 derived) and lower in the walls of anthers and filaments (L1 derived) (Bowman *et al.*, 1991a). This transgenic *AG* expression pattern of *BnAP3* likely explains the differences in carpel to stamen conversion observed between these different tissue types, as *AP3* is expressed throughout all tissue types of wild-type stamens (Jack *et al.*, 1992). Furthermore, recent studies of *Arabidopsis* flowers chimeric for *AP3* activity, show identical intermediate phenotypes in w3 organs when *AP3* is active in L2 and L3 derived tissues, but inactive in L1 derived tissue (Jenik and Irish, 2001). These authors found that loss of *AP3* activity in L1 derived tissue also causes poorly defined anthers and filaments, carpeloid epidermal cells, and only two microsporangia produced per anther. We therefore conclude that the intermediate organs formed in w4, w5, and w6 of *AG::BnAP3* plants are the result of no or very low *BnAP3* expression in the L1 derived tissues.

4.3.5 *BnAP3* can suppress D class function

The organs of w5 and w6 often had a closer resemblance to carpels than did organs of w4. However, many standard carpeloid features such as stigmatic papillae, style, septum, transmitting tract, and ovules were significantly reduced in organs produced in all whorls interior to the w3 wild-type stamens. Congenital fusion of the organs was also reduced. The products of *SPATULA* (*SPT*) and *CRABS CLAW* (*CRC*), expressed in w4 and designated as D class genes (Alvarez and Smyth, 1997), together

with *AG*, specify aspects of carpel identity. Their functions have been attributed to the development of stigmatic papillae, style, septum, transmitting tract, ovules and the congenital fusion between organs (Alvarez and Smyth, 1999). As such, these aforementioned tissues are reduced in *SPT/CRC* double mutants along with a reduction of congenital fusion between w4 organs (Alvarez and Smyth, 1999).

Examination of w4-w6 organs from *AG::BnAP3* transgenic plants by both SEM and LM showed that they closely resembled the w4 organs of *SPT/CRC* double mutants. These observations support the suggestion that B function normally acts to prevent the action of SPT and CRC in w2 and w3 (Alvarez and Smyth, 1999).

4.3.6 Four whorl 4 organs: evidence for a four-organ gynoecium

The floral ground plan of *A. thaliana*, *B. napus* and the Brassicaceae in general is remarkably stable (Endress, 1992). Within the family this floral ground plan shows natural variations with respect to stamen number, the presence or absence of petals, and the union of sepals. However, the gynoecium is practically free of any deviations from this ground plan (Endress, 1992). The gynoecium is generally thought to consist of two carpels congenitally fused and positioned in the lateral plane (Brückner, 2000). Both carpels are thought to have fertile and marginal placentae which fuse to form the replum in the median plane, from which the ovules are produced. There are four prominent vascular bundles within the gynoecium, two bundles in the lateral axis arising in each carpel valve and two bundles in the median axis arising from each placental region. Each lateral bundle belongs to each valve, while those of the placentae are thought to be the product of fused ventral bundles from each carpel. However, several four organ theories as well as a six organ theory have been proposed to explain the ontogeny of the Brassicaceaen gynoecium (reviewed in Brückner, 2000). Although there are variations, the basic four organ theory suggests that the gynoecium is the product of four organs, arranged in 2 dimerous whorls, with the organs of the 2 whorls differing in shape (Brückner, 2000). The organ pair in the lateral axis forms the valves, while the organ pair in the median axis forms the placentae. In this theory each organ contributes an independent vascular bundle to the gynoecium.

The introduction of *BnAP3* into w4 of *A. thaliana* and *B. napus* resulted in dramatic changes to the gynoecium. The most notable and consistent of these changes was the production of four organs. The locations from which these organs arose were highly predictable, two invariably in the lateral axis where wild-type valves of the gynoecium would have developed and two in the median axis, the location where the placental regions would have developed. The presence of four organs in place of the gynoecium in *AG::BnAP3* plants did not alter the vascular arrangement of four prominent vascular bundles at the base of w4, which also exists in the wild-type flower.

There are a number of models that would explain the production of four organs in w4 of *AG::BnAP3* flowers. The first is that organs which typically give rise to the gynoecium increased in number, from two to four, by a process of duplication or dédoublement. This model assumes that the phylogenetic starting point of the gynoecium was two organs. The concept of dédoublement has been used to describe the evolvement of four median stamens of the third whorl from a phylogenetic starting point of two (reviewed in Endress, 1992). It is possible that in w4, *BnAP3* could double organ number, similar to the theorized evolvement of four median stamens in w3. Strong evidence opposing this model comes from the placement of the four organs. In accordance with all current 2 carpel theories, each carpel primordium arises from the lateral axis. If dédoublement of these organs were to have occurred, the model dictates that the 4 organs of *AG::BnAP3* flowers would have arisen alternate and interior to the lateral stamens and median stamen-pairs. The two additional w4 organs however, arose opposite and interior to the median stamens. Based on the placement of these organs, dédoublement is doubtful as a suitable explanation.

The second model which might explain the observation of four organs in w4 is that the change induced by the *AG::BnAP3* construct was a reflection of the floral indeterminacy function of *BnAP3*. This model also assumes that the phylogenetic starting point of the gynoecium was two carpels. Floral indeterminacy is the result of increased cellular proliferation from the center of the floral meristem. In *AG::BnAP3* flowers, it is possible that the lack of determinacy resulted in the production of multiple whorls, each containing two organs arising in positions alternate and adaxial to outer whorls. In this situation, four organs would not be produced in w4; rather, the two

additional organs would be formed in a new inner whorl. Support for this model comes from the high number of flowers where fifth or sixth whorls appeared to contain only two organs, displaying various degrees of fusion. When completely fused, the result was a structure that closely resembled a wild-type gynoecium (figures 4.6k and 4.7f). However, the presence of prominent median ridges along one margin of several unfused organs contradicts this model. These ridges arose from the median region of the receptacle from which predicted fourth organ(s) were missing. Although it appeared that only two organs were present, the ridge may be indicative of a vestigial median organ fused to the lateral organ. Prominent ridges were never seen when a whorl contained a complete quartet of organs and as such, one might actually have been observing four organs rather than two in w5 and w6.

A third model suggests that the presence of four organs is a product of the organ identity function of *BnAP3*, assuming that the phylogenetic starting point of the gynoecium was four organs. When the development of these four organs was changed from carpeloid to stamenoid, fusion and differentiation of the median and lateral organs broke down, and the four separate organs from which the gynoecium is comprised, developed independently. The observations presented here support this last model. (1) The dimorphism between organs that arose in the median axis versus those that arose in the lateral axis of the same flower. The lateral organs were often more "valve"-like and were attached to the receptacle in a sessile, non-stalked manner. On the contrary, a proximal filament often subtended organs in the median axis. (2) Median organs did not appear to exist in a whorl distinguishable from that of the lateral organs. Subsequent lateral-median quartets of organs adaxial to the w4 organs were often separated by distinct stalks similar to the gynophores of Alvarez and Smyth (1999). (3) Both the lateral and median w4 organs arose from the receptacle simultaneously, while subsequent lateral-median quartets of organs of w5 and w6 arose later in development (see also Krizek and Meyerowitz, 1996). (4) On the rare occasion that flowers displayed only three obvious organs in w4, one lateral organ often contained a prominent ridge of tissue which arose from the median region from which the predicted fourth organ was missing. This ridge of tissue may have represented the missing median organ.

The phylogenetic basis of the Brassicaceaen carpel cannot be determined by vasculature and ontogeny, within the fourth whorl of *AG::BnAP3* plants, alone. The most regular feature of these transgenic flowers however, was the production of four organs in w4. This is a striking observation considering the recognized stability of the gynoecial ground plan. Either of the last two models could fit within the framework of a four organ phylogenetic starting point for gynoecium development. It is inconsequential to the theory of a four organ gynoecium, whether the gynoecium is comprised of a single whorl containing a quartet of organs or whether the gynoecium is comprised of two whorls each containing a diad of organs. However, our observations support the former.

An alternate explanation for our observations is that the gynoecium is comprised of only two lateral organs, and that the median organs consistently present in *AG::BnAP3* flowers are a product of increased cell proliferation rather than preformed growth centers derived from the placental regions of the gynoecium. Several mutants have been described in which increased cellular proliferation in the floral meristem has been observed. Two of these mutants, *CLAVATA1* (*CLV1*) and *CLAVATA3* (*CLV3*), increase organ number in all four whorls, particularly the innermost whorls (Clark *et al.*, 1993; 1995). In these mutants, carpels are initiated as a ring of organs around a central dome of meristematic tissue which continues to produce successive whorls of organs. The number of carpels produced in w4 of *CLV* mutants is not restricted to four and can average greater than six (Clark *et al.*, 1995). Although *AG::BnAP3* flowers occasionally produced less than four organs in w4, they never exceeded four. Our observations suggest that the increase in w4 organ number of *AG::BnAP3* flowers is not simply due to increased cell proliferation. Instead, these data suggest that w4 organs of *AG::BnAP3* flowers were produced from four defined growth points within the fourth-whorl, probably corresponding to four predetermined growth points of the wild-type gynoecium.

4.4 Experimental Procedures

4.4.1 Plant material

Arabidopsis thaliana ecotype Columbia was purchased from Lehle Seeds (Round Rock, Tex.) and *Brassica napus* cultivar Westar was a gift from Dr. W. Keller, Plant Biotechnology Institute, National Research Council, Saskatoon. All *Arabidopsis* plants were grown in a Conviron® (Winnipeg, Man.) growth chamber as described in Pylatuik *et al.* (1998). Tissues harvested from *Arabidopsis* plants were immediately placed on ice and subsequently stored at –80°C.

All *B. napus* plants grown for tissue collection and the T_0 generation of transformed lines were grown in the Phytotron Facility (College of Agriculture, University of Saskatchewan) on Terra-lite Redi-earth® (W.R. Grace & Co. Canada Ltd., Ajax, Ont.) with a 16 hour/8 hour light/dark cycle (230 mmol photons $\cdot m^{-2} \cdot s^{-1}$) and a 23°C/18°C day/night temperature cycle. Plants grown for the collection of transitional shoot apical meristems (TSAMs - apical meristems harvested 28 hours following light induction to flowering) were cultivated as described in Bonham-Smith *et al.* (1992). The T_1 generation of transformed *B. napus* were grown under greenhouse conditions supplemented with sodium lights with a 16 hour/8 hour light/dark cycle and continuous temperature of 22°C. Tissues harvested from *B. napus* plants for the purpose of genomic analysis were immediately placed on ice and subsequently stored at –80°C. Tissues collected for the purpose of RNA isolation and *BnAP3* expression profiling were immediately placed in liquid nitrogen and subsequently stored at –80°C. These tissues included seedling roots, mature stems, young leaves (> 1.0 cm in length), young bracts (> 1.5 cm in length), TSAMs, buds, young gynoecium (unfertilized 0.3 – 0.5 cm in length, collected from unopened buds), and mature gynoecium (fertilized 1.2 – 2.5 cm in length).

4.4.2 Cloning and characterization of *BnAP3*

Using genomic DNA extracted from *Arabidopsis thaliana*, ecotype Columbia (Mclean *et al.*, 1988) as template, a PCR was used to generate a 473 bp product corresponding to positions 2080 – 2552 of the *AP3* gene (accession # AL132971) (Jack *et al.*, 1992). The primers used to generate this product were 5' GTCAACAAGACATACAAA and 5' CAAGAAGATGGAAGGTAATG. A ^{32}P-probe was generated from the 473 bp *AP3* product using an Oligolabelling Kit (Pharmacia Biotech). The probe was used to screen a *Brassica napus* (cv. Westar) cDNA library (Bonham-Smith *et al.*, 1992) with Colony/Plaque Screen™ hybridization transfer membranes (DuPont-NEN), resulting in the isolation of a partial *BnAP3* clone 705 bp in length corresponding to the 3' region of the full length cDNA (pBAP3-1). A 236 bp 5' region of *BnAP3* was isolated using the 5' RACE system, V. 2.0 (GIBCO-BRL) with total mRNA collected from bud tissue of *B. napus* (Verwoerd *et al.*, 1989), and cloned into pBSKS+ (pBAP#9). All procedures were performed as outlined in the manufacturer's instructions. A full-length *BnAP3* clone (accession #AF124814, Appendix A.1) was generated in pBSKS+ by ligating the partial 705 bp fragment of pBAP3-1 to the 5' RACE product at a shared *Bgl*II site.

4.4.3 *BnAP3* RNA expression

Total RNA was isolated from various tissues using the RNeasy Plant Mini Kit (QIAGEN), as outlined in the manufacturer's instructions. *BnAP3* RNA expression was determined by reverse transcriptase-PCR using the OneStep RT-PCR Kit (QIAGEN) and the *BnAP3*-specific primers BAP3-3' (5' TAGTTATTCAAGAAGGTGGAA) and BAP3-5' (5' AAAATGGCGAGAGGGAAGATC). Actin RNA expression was determined as a positive control using the primers BnAcSIL3 (5' GCCAAGATGGATCCTCC) and BnAcPAM5 (5' CCCTGCCATGTATGTTGC). Reactions were performed as outlined in the manufacturer's instructions, using 2.0 μg total RNA from each tissue as template. An annealing temperature of 45°C was used in OneStep RT-PCR to determine actin and *BnAP3* expression in all tissues.

4.4.4 *BnAP3* copy number

The number of *BnAP3* copies in the *B. napus* genome was determined by Southern analysis on genomic DNA (10-15 μg) digested with *Bam*HI, *Eco*RI, and *Hin*DIII. A ^{32}P-probe specific for *BnAP3* was generated from the partial *BnAP3* clone 705 bp in length corresponding to the 3' region of the full length cDNA (MADS box not included). Genomic digests were transferred to GeneScreen Plus nylon membranes (DuPont) and the probe hybridized in QuickHyb solution (Stratagene) as per the manufacturer's instructions.

4.4.5 *AGAMOUS::BnAP3* gene fusion constructs

The *AG* controlling elements (3 kb large intron of the *Arabidopsis* gene) and CaMV 35S minimal promoter necessary to confer the recognized *AG* expression pattern were generously supplied in the pMD992 reporter construct by Dr. L.E. Sieburth (Deyholos and Sieburth, 2000). The β-glucuronidase (GUS) reporter gene was subsequently removed from the pMD992 construct and the *BnAP3* open reading frame cloned in its place using standard molecular biology techniques (Appendix B). The resulting construct, pAG+BAP3 (figure 4.4), was generated within the pCGN1547 binary plant transformation vector (McBride and Summerfelt, 1990).

4.4.6 Plant transformation

The pAG+BAP3 construct was transformed into the *Agrobacterium* strain pC2760 carrying the Ti plasmid LBA4404. Lines of transgenic *Arabidopsis* plants (col.) were established using *Agrobacterium* mediated transformation by a method modified from Clough and Bent (1998). While the above-ground portions of the plants were dipped in *Agrobacterium* (OD_{600} = 0 .8) in 5% sucrose and 0.01% Silwet L-77 (OSi Specialties, Inc., Friendly, WV), a vacuum of 526 – 752 mmHg was drawn and held for 2 min. Transformed *Arabidopsis* seedlings were identified based on their resistance to

kanamycin and by a PCR-based test specific for the pAG+BAP3 construct with the primers BAP3-5' (see earlier) and NOS-3'K (5'GCGGGTACCCGATCTAGTAACATA GATG).

The pAG+BAP3 construct was also used to transform *B. napus* cv. Westar using the pC2760 *Agrobacterium* strain containing the LBA4404 Ti plasmid by the method of Moloney *et al.* (1989). Transformed *B. napus* shoots were identified based on their resistance to kanamycin and subsequent plants were identified as transgenic by a PCR specific for the *NEOMYCINPHOSPHOTRANSFERASE* (*NPT*II) gene using the primers NPTII-1 (5' GAGGCTATTCGGCTATGACTG) and NPTII-2 (5' ATCGGGAGCGGCGATACCGTA). Several putative transformants were randomly selected for further testing by the PCR-based test described above with the primers BAP3-5' and NOS-3'K.

Transgene integration was determined by Southern analysis for the T_1 *Arabidopsis* plants and 16 plants from each of the *B. napus* T_1 lines using the same procedure as that described previously for *BnAP3*. Genomic DNA was digested with *Bam*HI, a restriction site found twice within the construct of the T-DNA (figure 4.1). A 700 bp probe specific to the *npt*II gene, amplified by PCR using the primers NPTII-1 and NPTII-2, was used to determine multiple transgene insertion sites, while avoiding the detection of endogenous genes similar to or contained within the *AG::BnAP3* cassette.

4.4.7 Microscopy and image processing

Tissues were prepared for scanning electron microscopy (SEM) and visualized as described in Pylatuik *et al.* (1998) (Section 2.4.2). Paraffin embedding for light microscopy (LM) was performed as outlined in Jensen (1962). Tissues were processed through an ethanol series (30, 50, 70, 85, 95, 100%), infiltrated with paraplast+ (Oxford labware, St. Louis, USA) at 60°C, ethanol was allowed to evaporate, and the paraplast+ replaced at least 3 times prior to casting. Sections 10 µm in thickness were stained in toluidine blue (O'Brien and M^{c}Cully, 1981) or phloroglucin-HCl (Jensen, 1962). Photographs and slides were digitized using an Epson Perfection 1640SU (Epson

America, Inc., Long Beach, CA) and a CanoScan 2700F (Canon U.S.A. Inc., Lake Success, NY). Brightness, contrast, and gamma levels were adjusted using Adobe Photoshop (Adobe Systems, Mountain View, CA).

4.5 Summary

A cDNA isolated and cloned from *Brassica napus*, *BnAP3*, was found to be homologous to the B class homeotic gene *APETALA3* of *Arabidopsis thaliana*. In order to understand the process of stamen morphogenesis from carpel primordia, expression of *BnAP3* was targeted to whorls 3 and 4 of *Arabidopsis* under the control of *AGAMOUS* *cis*-acting elements. Sepals, petals, and stamens of transformed plants were identical to those of wild-type plants, while the carpels of transformed plants were replaced with four organs that were partially converted to stamens. These fourth-whorl stamen-carpel intermediates often produced microsporangia and pollen. L2 and L3 derived tissues of these organs were converted to those of stamens while L1 derived tissues retained their carpeloid properties. The number, arrangement, and vascular patterning of organs produced in the fourth whorl indicate that organ number and patterning are not random, but are governed by the patterning of the vestigial gynoecium of the transformed flowers.

4.6 Literature cited

Alvarez, J. and Smyth, D.R. (1997) Carpel development genes in *Arabidopsis*. *Flowering Newsletter*, **23**, 12-17.

Alvarez, J. and Smyth, D.R. (1999) *CRABS CLAW* and *SPATULA*, two *Arabidopsis* genes that control carpel development in parallel with *AGAMOUS*. *Development*, **126**, 2377-2386.

Alvarez-Buylla, E.R., Liljegren, S.J., Pelaz, S., Gold, S.E., Burgeff, C., Ditta, G.S., Vergara-Silva, F. and Yanofsky, M.F. (2000) MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cells, roots and trichomes. *Plant J.*, **24**, 457-466.

Baum, S.F., Eshed, Y. and Bowman, J.L. (2001) The *Arabidopsis* nectary is an ABC independent floral organ. *Development* (In Press).

Bonham-Smith, P.C., Oancia, T.L. and Moloney, M.M. (1992) Cytoplasmic ribosomal protein S15a from *Brassica napus*: Molecular cloning and developmental expression in mitotically active tissues. *Plant Mol. Biol.*, **18**, 909-919.

Bowman, J.L., Alvarez, J., Weigel, D., Meyerowitz, E.M. and Smyth, D. R. (1993) Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. *Development*, **119**, 721-743.

Bowman, J.L., Drews, G.N. and Meyerowitz, E.M. (1991a) Expression of the *Arabidopsis* floral homeotic gene *AGAMOUS* is restricted to specific cell types late in flower development. *Plant Cell*, **3**, 749-758.

Bowman, J.L. and Smyth, D.R. (1994) Wild-type flowers: epidermal surface morphology of mature stamens of *Arabidopsis thaliana* (L.) Heynh. var. Columbia. In *Arabidopsis, an atlas of morphology and development.* (Bowman, J.L. ed.) New York: Springer-Verlag, pp. 166.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. (1989) Ge nes dir ecting f lower development in *Arabidopsis*. *Plant Cell*, **1**, 37-52.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. (1991b) Genetic interactions among floral homeotic genes of *Arabidopsis*. *Development*, **112**, 1-20.

Brückner, C. (2000) Clarification of the carpel number in Papaverales, Capparales, and Berberidaceae. *Bot. Rev.*, **66**, 155-307.

Carr, S.M. and Irish, V.F. (1997) Floral homeotic gene expression defines developmental arrest stages in *Brassica oleracea* L. vars. *botrytis* and *italica*. *Planta*, **201**, 179-188.

Clark, S.E., Running, M.P. and Meyerowitz, E.M. (1993) *CLAVATA1*, a regulator of meristem and flower development in *Arabidopsis*. *Development*, **119**, 397-418.

Clark, S.E., Running, M.P. and Meyerowitz, E.M. (1995) *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. *Development*, **121**, 2057-2067.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.*, **16**, 735-743.

Coen, E.S. and Meyerowitz, E.M. (1991) The war of the whorls: Genetic interactions controlling flower development. *Nature*, **353**, 31-37.

Davis, A.R. (1994) Features of the nectary in wild-type and some mutant flowers of *Arabidopsis thaliana* (L.) Heynh. var. Columbia. In *Arabidopsis, an atlas of morphology and development.* (Bowman, J .L. ed.) New York: Springer-Verlag, pp. 172-177.

Davis, A.R., Peterson, R.L. and Shuel, R.W. (1986) Anatomy and vasculature of the floral nectaries of *Brassica napus* (Brassicaceae). *Can. J. Bot.*, **64**, 2508-2516.

Davis, A.R., Pylatuik, J.D., Paradis, J.C. and Low, N.H. (1998) Nectar-carbohydrate production and composition vary in relation to nectary anatomy and location within individual flowers of several species of Brassicaceae. *Planta*, **205**, 305-318.

Deyholos, M.K. and Sieburth, L.E. (2000) Separable whorl-specific expression and negative regulation by enhancer elements within the *AGAMOUS* second intron. *Plant Cell*, **12**, 1799-1810.

Drews, G.N., Bowman, J.L. and Meyerowitz, E.M. (1991) Negative regulation of the *Arabidopsis* homeotic gene *AGAMOUS* by the *APETALA2* product. *Cell*, **65**, 991-1002.

Endress, P.K. (1992) Evolution and floral diversity: The phylogenetic surroundings of *Arabidopsis* and *Antirrhinum*. *Int. J. Plant. Sci.*, **153**, S106-S122.

Erickson, L.R., Straus, N.A. and Beversdorf, W.D. (1983) Restriction patterns reveal origins of chloroplast genomes in *Brassica* amphiploids. *Theor. Appl. Genet.*, **65**, 201-206.

Goto, K., and Meyerowitz, E.M. (1994) Function and regulation of the *Arabidopsis* floral hmeotic gene *PISTILLATA*. *Gen. Dev.*, **8**, 1548-1560.

Gustafson-Brown, C., Savidge, B . a nd Y anofsky, M .F. (1994) Regulation of the *Arabidopsis* floral homeotic gene *APETALA1*. *Cell*, **76**, 131-143.

Hill, J.P. and Lord, E.M. (1989) Floral development in *Arabidopsis thaliana*: A comparison of the wild-type and the homeotic *pistillata* mutant. *Can. J. Bot.*, **67**, 2922-2936.

Hill, T.A., Day, C.D., Zondlo, S.C., Thackeray, A.G. and Irish, V.F. (1998) Discrete spatial and temporal *cis*-acting elements regulate transcription of the *Arabidopsis* floral homeotic gene *APETALA3*. *Development*, **125**, 1711-1721.

Honma, T. and Goto, K. (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature*, **409**, 525-529.

Jack, T., Brockman, L.L. and Meyerowitz, E.M. (1992) The homeotic gene *APETALA3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. *Cell*, **68**, 683-697.

Jack, T., Fox, G.L. and Meyerowitz, E.M. (1994) *Arabidopsis* homeotic gene *APETALA3* ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. *Cell*, **76**, 703-716.

Jenik, P.D. and Irish, V.F. (2000) Regulation of cell proliferation patterns by homeotic genes during *Arabidopsis* floral development. *Development*, **127**, 1267-1276.

Jenik, P.D. and Irish, V.F. (2001) The *Arabidopsis* floral homeotic gene *APETALA3* differentially regulates intercellular signaling required for petal and stamen development. *Development*, **128**, 13-23.

Jensen, W.A. (1962) *Botanical histochemistry: principles and practice.* San Francisco, U.S.A.: W.H. Freeman and Company.

Jofuku, K.D., den Boer, S.G.W., Van Montagu, M. and Okamuro, J.K. (1994) Control of *Arabidopsis* flower and seed development by the homeotic gene *APETALA2. Plant Cell*, **6**, 1211-1225.

Komaki, M.K., Okada, K., Nishino, E. and Shimura, Y. (1988) Isolation and characterisation of novel mutants of *Arabidopsis thaliana* defective in flower development. *Development*, **104**, 195-208.

Krizek, B.A. and Meyerowitz, E.M. (1996) The *Arabidopsis* homeotic genes *APETALA3* and *PISTILLATA* are sufficient to provide the B class organ identity function. *Development*, **122**, 11-22.

Kunst, L., Kienz, J.E., Martinez-Zapater, L. and Haughn, G.W. (1989) *AP2* gene determines the identity of perianth organs in flowers of *Arabidopsis thaliana. Plant Cell*, **1**, 1195-1208.

Lawrence, M.J. (1951) *Taxonomy of flowering plants.* New York and London: McMillan, pp. 520-524.

McBride, K.E. and Summerfelt, K.R. (1990) Improved binary vectors for *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol.*, **14**, 269-276.

Mclean, M., Baird, W.V., Gerats, A.G. and Meagher, R.B. (1988) Determination of the copy number and linkage relationships among five actin gene subfamilies in *Petunia hybrida. Plant Mol. Biol.*, **11**, 663-672.

Moloney, M.M., Walker, J.M. and Sharma, K.K. (1989) High efficiency transformation o f *Brassica napus* using *Agrobacterium* vectors. *Plant Cell Rep.*, **8**, 238.

Moon, Y.H., Jung, J.Y., Kang, H.G. and An, G. (1999) Identification of a rice *APETALA3* homologue by yeast two-hybrid screening. *Plant Mol. Biol.*, **40**, 167-177.

O'Brien, T.P. and M^{c}Cully, M.E. (1981) *The study of plant structure: principles and selected methods.* Melbourne, Australia: Termarcarphi Pty. Ltd.

Odell, J.T., Nagy, F. and Chua, N.H. (1985) Identification of sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature*, **313**, 810-812.

Polowick, P.L. and Sawhney, V.K. (1988) High temperature induced male and female sterility in canola (*Brassica napus* L.). *Ann. Bot.*, **62**, 83-86.

Pylatuik, J.D., Bonham-Smith, P.C. and Davis, A.R. (1998) The additional stamens of *flo10-1* mutants of *Arabidopsis thaliana* are compromised in production and viability of pollen. *Can. J. Bot.*, **76**, 1733-1742.

Rounsley, S.D., Ditta, G.S. and Yanofsky, M.F. (1995) Diverse roles for MADS box genes in *Arabidopsis* development. *Plant Cell*, **7**, 1259-1269.

Schultz, E.A. and Haughn, G.W. (1993) Genetic analysis of the floral initiation process (FLIP) in *Arabidopsis*. *Development*, **119**, 745-765.

Smyth, D.R., Bowman, J.L. and Meyerowitz, E.M. (1990) Early flower development in *Arabidopsis*. *Plant Cell*, **2**, 755-767.

Theissen, G., Becker, A., Di Rosa, A., Kanno, A., Kim, J.T., Münster, T., Winter, K., and Saedler, H. (2000). A short history of MADS-box genes in plants. *Plant Mol. Biol.*, **42**, 115-149.

Theissen, G. (2001). Development of floral organ identity; stories from the MADS house. *Curr. Opin. Plant Biol.*, **4**, 75-85.

U, N. (1935). Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Jpn. J. Bot.*, **7**, 389-452.

Verwoerd, T.C., Dekker, B.M. and Hoekema, A. (1989) A small scale procedure for the rapid isolation of plant RNAs. *Nucl. Acids Res.*, **17**, 2362.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A. and Meyerowitz, E.M. (1990). The protein encoded by the *Arabidopsis* homeotic gene *AGAMOUS* resembles transcription factors. *Nature*, **346**, 35-39.

Chapter 5. CLONING AND CHARACTERIZATION OF A *SHATTERPROOF1* GENE FROM *BRASSICA NAPUS* SUGGESTS THAT SILIQUE DEHISCENCE IS POSSIBLY REGULATED BY LEAFY

5.1 Introduction

Seed maturation and dispersal is mediated by the fruit of flowering plants. In the Brassicaceae, the fruit is a silicle or silique formed primarily from the ovary of the mature gynoecium. Fruit development in species of this family culminates with dehiscence, a process in which the valves of the fruit abscise from the replum, allowing the seed to be dispersed. Despite the potential agronomic value of controlling seed dispersal in Brassicacean crop plants such as canola or mustard, it was only recently that progress toward understanding the genetic control of seed dehiscence has been made.

The process of abscission, required for fruit dehiscence, has been divided into several stages (Patterson, 2001). The first stage is requirement for the proper ontogeny of the abscission zone. It has been found in *Arabidopsis thaliana* that the closely related MADS-box genes *SHATTERPROOF1* (*SHP1*) and *SHP2* function to control dehiscence zone differentiation and promote lignification in adjacent cells (Liljegren *et al.*, 2000) while another MADS-box gene, *FRUITFULL*, functions to negatively regulate the *SHP* genes (Ferrándiz *et al.*, 2000b). The second stage is the requirement for cells of the abscission zone to respond to abscission signals. These signals may be in the form of plant growth regulators such as ethylene, auxin, or abscisic acid (reviewed in Patterson, 2001). The final stage of abscission is the activation of the process.

Currently, over 45 MADS-box containing genes have been identified in *Arabidopsis* (Alvarez-Buylla *et al.*, 2000). These genes are characterized as transcription factors having a highly conserved DNA-binding/dimerization region called the MADS domain (**M**CM1, **A**GAMOUS, **D**EFICIENS, and **S**RF) which has been identified across different kingdoms (Riechmann and Meyerowitz, 1997). Although the

function of many of these genes has yet to be identified, an overwhelming number are involved in flower development, including the well known A, B, and C-class genes involved in controlling floral organ identity (Coen and Meyerowitz, 1991). Furthermore, analysis of these genes has revealed additional functions besides that of organ identity; therefore the number of processes under the control of this large gene family could be enormous (Riechmann and Meyerowitz, 1997). It is therefore not surprising that MADS-box genes have been implicated in the process of abscission zone formation.

In several MADS-box genes, a large (>1 kb), first or second intron has been identified (Ma *et al.*, 1991). Recently it has been shown that in the *AGAMOUS* (*AG*) MADS-box gene of *Arabidopsis*, the large second intron (approximately 3 kb) contains all of the essential *cis* regulatory elements required to direct proper expression (Deyholos and Sieburth, 2000). One factor known to interact with a motif in the large intron of *AG* is LEAFY (LFY) (Busch *et al.*, 1999), a protein believed to regulate several of the ABC genes controlling floral patterning (Parcy *et al.*, 1998). Because all members of the MADS-box family were likely derived from a common ancestral gene (Alvarez-Buylla *et al.*, 2000), it is only logical to suspect that some regulatory elements may also have been conserved between members. *SHP1* and *SHP2* are the two genes most closely related to *AG* (Rounsley *et al.*, 1995; Alvarez-Buylla *et al.*, 2000). Furthermore, the spatial expression of these genes is restricted primarily to the reproductive organs and is highly active in the nectaries of mature flowers (Yanofsky *et al.*, 1990; Flanagan *et al.*, 1996; Ma *et al.*, 1991; Savidge *et al.*, 1995). These data suggest that there is a high likelihood for the conservation of regulatory elements, possibly located within the large introns of these MADS-box genes.

We have cloned and characterized a MADS-box gene (*BnSHP1*) from the agronomically important species *Brassica napus* (canola) and propose that it is orthologous to the *SHP1* gene of *Arabidopsis*, and likely functions to differentiate abscission zone formation in the valves of siliques. To examine the possibility of conserved regulatory sequences between the *SHP* genes and *AG*, we searched the 5' promoter regions and large introns to identify known regulatory sequences. The LFY

binding site was identified in all genes examined. We have assayed for LFY binding to these regulatory sequences and the results of our investigations are discussed below.

5.2 Results

5.2.1 Isolation and characterization of *BnSHP1*

Screening of the *B. napus* genomic library with the *A. thaliana AG* probe yielded several clones, BAG2-4 – BAG2-7. From the BAG2-5 clone, a gene was sequenced and identified as *B. napus SHATTERPROOF1* or *BnSHP1* (figure 5.1a), which contained high sequence similarity (91%) in its predicted open reading frame (orf) (figure 5.1b) to the *Arabidopsis SHP1* gene. Furthermore, the location of intron splice sites (figure 5.1a and b), confirmed by isolating by RT-PCR, and sequencing the *BnSHP1* transcript, were identical to those of *SHP1*. Within the large (1151 bp) first intron of *BnSHP1*, an inverted repeat corresponding to positions 1188-1194, was identified (figure 5.1c). Based on previous promoter analysis studies with *AP1* (Parcy *et al.*, 1998) and *AG* (Busch *et al.*, 1999), this consensus sequence is considered as a putative LEAFY binding site. Although this site was not found within the large first intron (1298 bp) of the *SHP1* gene of *Arabidopsis*, an inverted repeat very similar to the previously identified LFY binding consensus was found (figure 5.1c). Additionally, the LFY binding consensus was identified in the *SHP2* promoter (figure 5.1c), located 5' to the transcriptional start site (Savidge *et al.*, 1995) corresponding to positions 797-802.

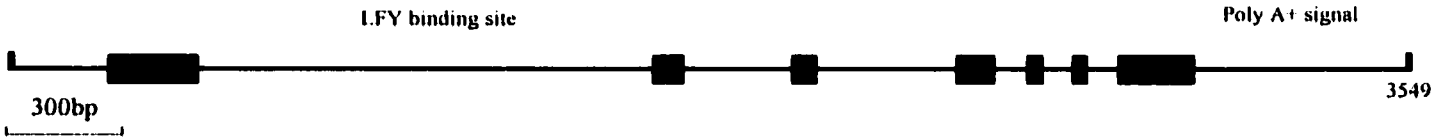
In addition to the LFY binding sites, putative CArG box elements, which are bound by MADS-box proteins (Shore and Sharrocks, 1995), were identified in the introns and/or sequences 5' to the start site of all *SHP* genes. Two putative CArG box elements were identified in *BnSHP1*, one near the middle of first intron and one approximately 200 bp upstream of the start codon (data not shown). Furthermore, putative C ArG b ox e lements were also identified in the first intron and promoter of *SHP1* and *SHP2* respectively. It is possible that these CArG box elements could be bound by the FRUITFULL MADS-box protein, a known regulator of the *SHP* genes (Ferrándiz et al, 2000b).

Figure 5.1a The *BnSHP1* gene. Exons are indicated with black boxes, while introns are indicated with lines. Putative LFY binding site and predicted poly A+ signal are shown.

Figure 5.1b *BnSHP1* open reading frame nucleotide sequence (top line) and predicted amino acid sequence (bottom line). Intron splice sites are indicated with arrowheads.

Figure 5.1c A comparison of the putative LFY binding sites from first intron of *SHP1* and *BnSHP1*, the 5' promoter region of *AP1*, the second intron of *AG* (both *AGI* and *AGII*), and the 5' promoter region of *SHP2*. In order to accommodate the proposed LFY binding site of *SHP1*, a modified consensus has been suggested in place of the previously defined consensus[1] by Busch *et al.* (1999).

a



b

```
1      ATG GAT GAA GGT GGG AGT AGT CAC GAT GCA GAG AGT AGC AAG AAG ATA GGT AGA GGG AAG
1      Met Asp Glu Gly Gly Ser Ser His Asp Ala Glu Ser Ser Lys Lys Ile Gly Arg Gly Lys

61     ATA GAG ATA AAG AGG ATA GAG AAC ACA ACA AAT CGT CAA GTA ACC TTC TGC AAA CGA CGC
21     Ile Glu Ile Lys Arg Ile Glu Asn Thr Thr Asn Arg Gln Val Thr Phe Cys Lys Arg Arg

121    AAT GGT CTT CTC AAG AAA GCT TAT GAG CTC TCT GTC TTG TGT GAT GCT GAA GTT GCC CTC
41     Asn Gly Leu Leu Lys Lys Ala Tyr Glu Leu Ser Val Leu Cys Asp Ala Glu Val Ala Leu

181    GTT ATC TTC TCC ACT CGT GGC CTT CTT TAT GAG TAC GCC AGC AAC AGT GTG AAG GGT ACA
61     Val Ile Phe Ser Thr Arg Gly Leu Leu Tyr Glu Tyr Ala Ser Asn Ser Val Lys Gly Thr

241    ATT GAA AGG TAC AAG AAA GCT TGT TCC GAT GCC GTT AAC CCT CCT ACT GTC ACT GAA GCT
81     Ile Glu Arg Tyr Lys Lys Ala Cys Ser Asp Ala Val Asn Pro Pro Thr Val Thr Glu Ala

301    AAT ACC AAG CAC TAT CAG CAA GAA GCC TCT AAG CTT CGG AGG CAG ATT CGG GAC ATT CAG
101    Asn Thr Lys His Tyr Gln Gln Glu Ala Ser Lys Leu Arg Arg Gln Ile Arg Asp Ile Gln

361    AAT TCG AAC AGG CAT ATT GTT GGA GAA TCA CTT GGT TCA TTG AAC TTC AAG GAA CTC AAA
121    Asn Ser Asn Arg His Ile Val Gly Glu Ser Leu Gly Ser Leu Asn Phe Lys Glu Leu Lys

421    AAC CTA GAA GGA CGG CTT GAA AAA GGA ATC AGC CGC GTC CGA TCC AAG AAG AGT GAA CTT
141    Asn Leu Glu Gly Arg Leu Glu Lys Gly Ile Ser Arg Val Arg Ser Lys Lys Ser Glu Leu

481    TTA GTG GCA GAG ATA GAG TAT ATG CAG AAG AGG GAA ATG GAG TTG CAG CAC GTT AAC ATG
161    Leu Val Ala Glu Ile Glu Tyr Met Gln Lys Arg Glu Met Glu Leu Gln His Val Asn Met

541    TAC CTA AGA GCT AAG ATA GAA CAA GGC GCG AGA TTG AAT CCG GAA CAA CAT GGA TCC GGT
181    Tyr Leu Arg Ala Lys Ile Glu Gln Gly Ala Arg Leu Asn Pro Glu Gln His Gly Ser Gly

601    GTA ATA CAA GGG ACG GCG GTT TAT GAG TCC GGT CTG TCT TCT TCT CAT GAT CAG TCG CAG
201    Val Ile Gln Gly Thr Ala Val Tyr Glu Ser Gly Leu Ser Ser Ser His Asp Gln Ser Gln

661    CAT TAT AAC CGG AAT TAT ATT CCG GTT AAC CTT CTT GAA CCG AAT CAA CAA TTC TCC GGT
221    His Tyr Asn Arg Asn Tyr Ile Pro Val Asn Leu Leu Glu Pro Asn Gln Gln Phe Ser Gly

721    CAA GAC CAA CCT CCT CTT CAA CTT GTT TAA
241    Gln Asp Gln Pro Pro Leu Gln Leu Val ***
```

c

```
          T
GAGCCAATGGGAA      SHP1
   ||| |||
GAGCCAATGGGAA      BnSHP1
   ||| |||
GGACCAGTGGTCC      AP1
   ||| |||
AATCCAATGGTTA      AGI
   ||| ||
TACCCAATGTGTT      AGII
   ||| ||
AGGCCAATGAACA      SHP2
   ||| ||
   CCANTG          consensus[1]
   ||| \\
   CCAN(x)TG       modified
                   consensus
```

Southern analysis of *B. napus* genomic DNA, probed with a fragment of the *BnSHP1* gene which excluded both the MADS-box and K domain, suggests that up to 3 copies of the *BnSHP1* gene are present within the *B. napus* genome (figure 5.2).

The predicted amino acid sequence (figure 5.3) of BnSHP1 shares 93% identity with that of SHP1. The MADS box of BnSHP1 is 98% similar to that of SHP1, while the K domain of BnSHP1 is 99% similar to that of SHP1 (figure 5.3). The predicted BnSHP1 protein is 248 amino acids in length, one amino acid longer than the predicted protein of SHP1, due to an additional serine residue at position 215 (figure 5.3).

5.2.2 *BnSHP1* expression is restricted to floral tissues

RNA isolated from various tissues and amplified through RT-PCR with primers specific for *BnSHP1* showed that *BnSHP1* RNA is present only in floral tissues. A RT-PCR identified relatively large amounts of *BnSHP1* mRNA (750 bp) in floral buds and gynoecia, both unfertilized and fertilized (figure 5.4). Lesser amounts were observed in apical meristems collected 28 hours following light induction to flowering (TSAMs). The 750 bp *BnSHP1* transcript was not detected in non-floral tissues such as roots, stems, leaves or bracts (figure 5.4).

5.2.3 *In vitro* production of three LFY proteins

In vitro production of LFY protein from the expression vector pGEX-2T in BL21 cells, followed by glutathione S-transferase (GST) isolation and subsequent prothrombin cleavage of the GST moiety, resulted in the isolation of three proteins that were approximately 60, 45, and 40 kDa in size (figure 5.5a). The control experiment, performed with native pGEX-2T vector, resulted in the isolation of a single protein approximately 30 kDa in size, corresponding to the GST protein. A polyclonal LFY antibody localized to all three bands in immunoblots, but did not immunolocalize to the control GST protein (figure 5.5b). Linked transcription:translation *in vitro* using the SD-LFY/pTRI19amp construct also resulted in the production of 3 proteins, equal in

Figure 5.2 Southern analysis of genomic DNA collected from wild-type *B. napus* plants digested with *Nco*I (Nc) and *Not*I (No). A probe specific for the *BnSHP1* gene (excluding the MADS box and K domain) was hybridized to the digested DNA and washed to 0.2X SSPE at 65°C.

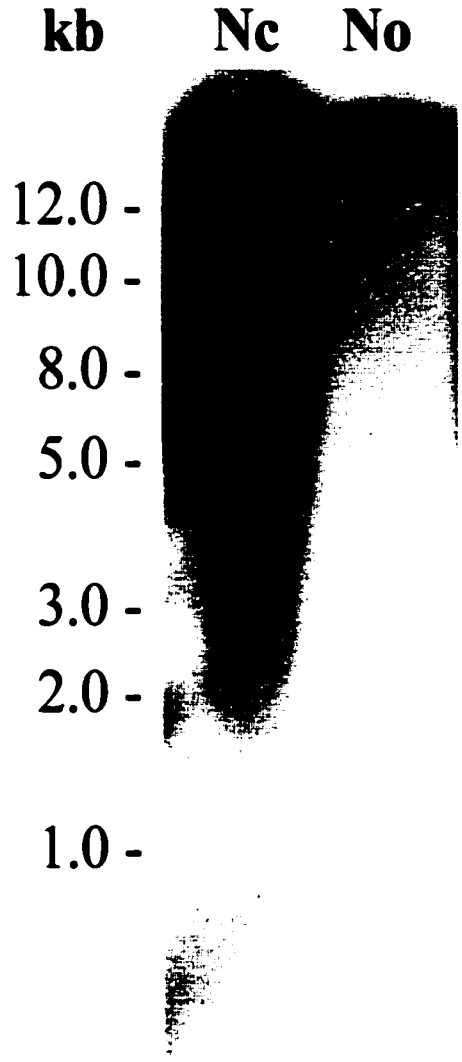

kb
Nc
No
12.0 -
10.0 -
8.0 -
5.0 -
3.0 -
2.0 -
1.0 -

Figure 5.3 Predicted amino acid identities between the BnSHP1 and SHP1 coding regions. BnSHP1 and SHP1 show 91% identity and are predicted to encode proteins of 248 and 247 amino acids, respectively. The MADS-box region is indicated with a solid box that extends from amino acids 17 to 72. The K box region, indicated with a broken box, extends from amino acids 106 to 172. (lower case = consensus)

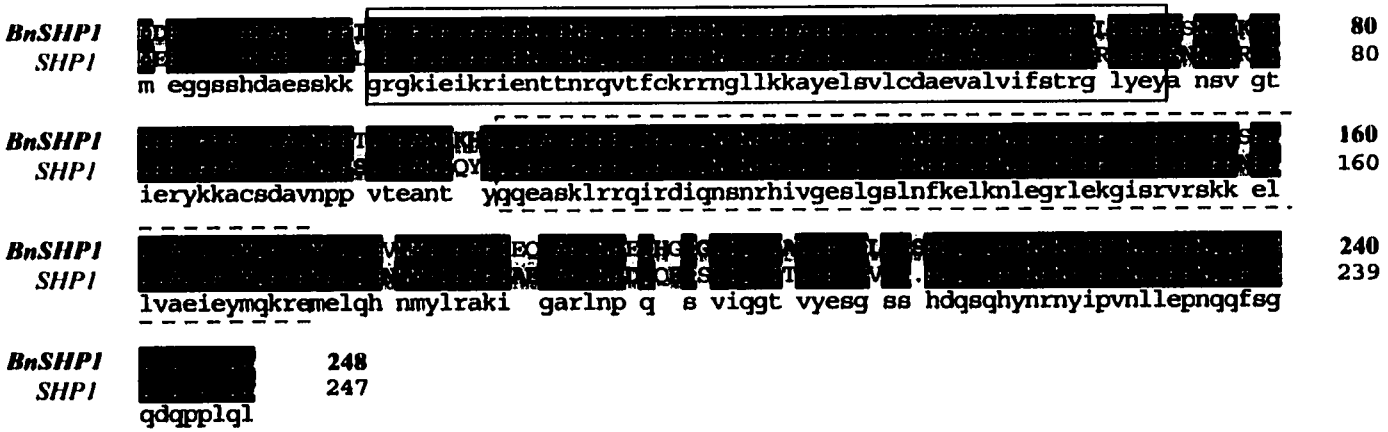

BnSHP1
SHP1
m eggsshdaesskk grgkieikrienttnrgvtfckrrngllkkayelsvlcdaevalvifstrg lyeya nsv gt
80
80
BnSHP1
SHP1
ierykkacsdavnpp vteant yggeasklrrqirdiqnsnrhivgeslgslnfkelknlegrlekgisrvrskk el
160
160
BnSHP1
SHP1
lvaeieymqkremelqh nmylraki garlnp q s viggt vyesg ss hdqsqhynrnyipvnllepnqqfsg
240
239
BnSHP1
SHP1
qdqpplql
248
247

Figure 5.4 Expression profile of *BnSHP1* in tissues collected from *B. napus* as determined using RT-PCR. *BnSHP1* transcripts were not detected in roots, stems, leaves, or bracts. *BnSHP1* transcripts (750 bp in length) were detected in TSAMs (shoot apical meristems collected 28 hours following light induction to flowering), floral buds, and young and mature gynoecia. Actin transcripts (approximately 580 bp in length) served as a constitutive control and were detected in all tissues.

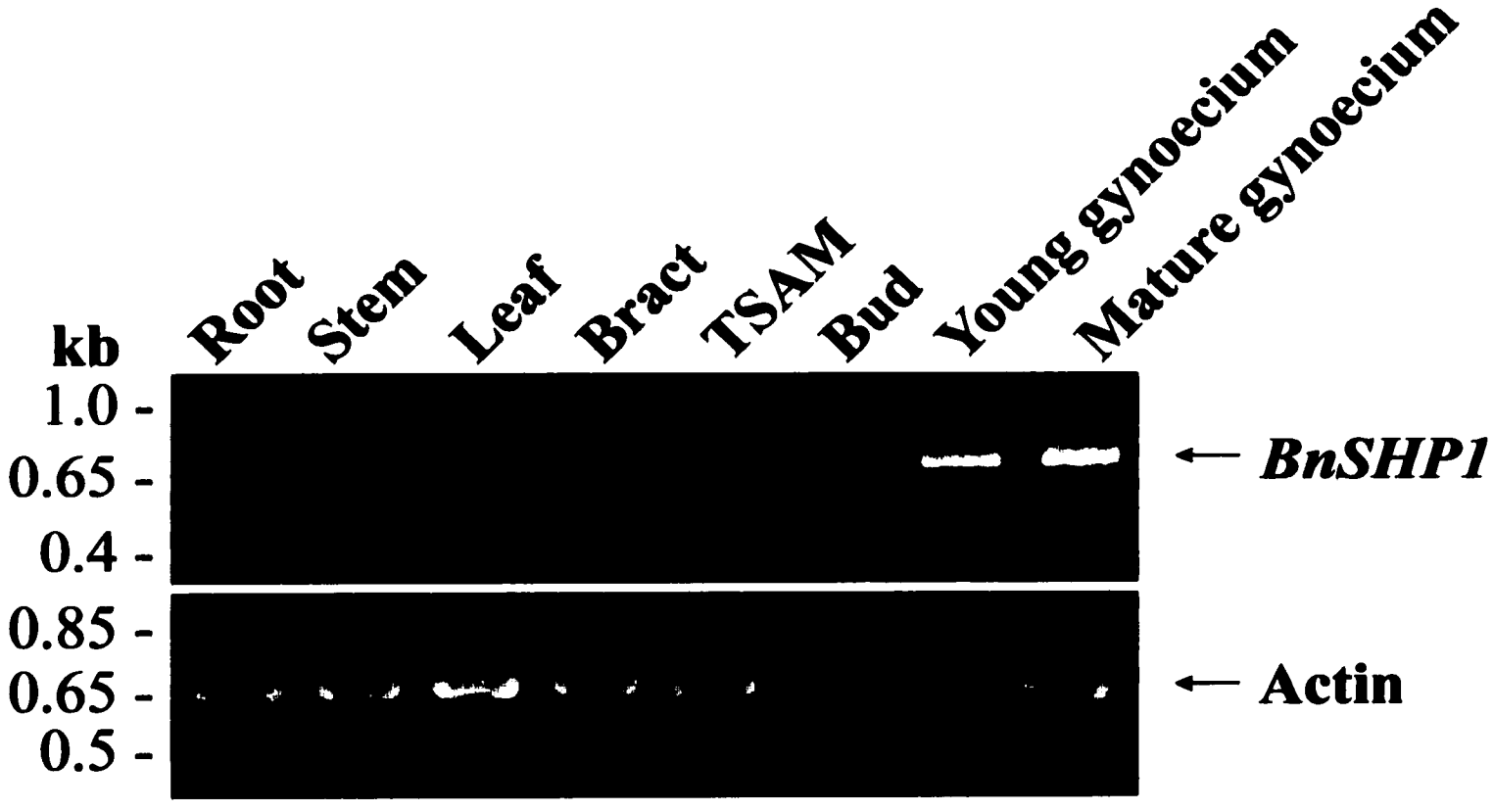
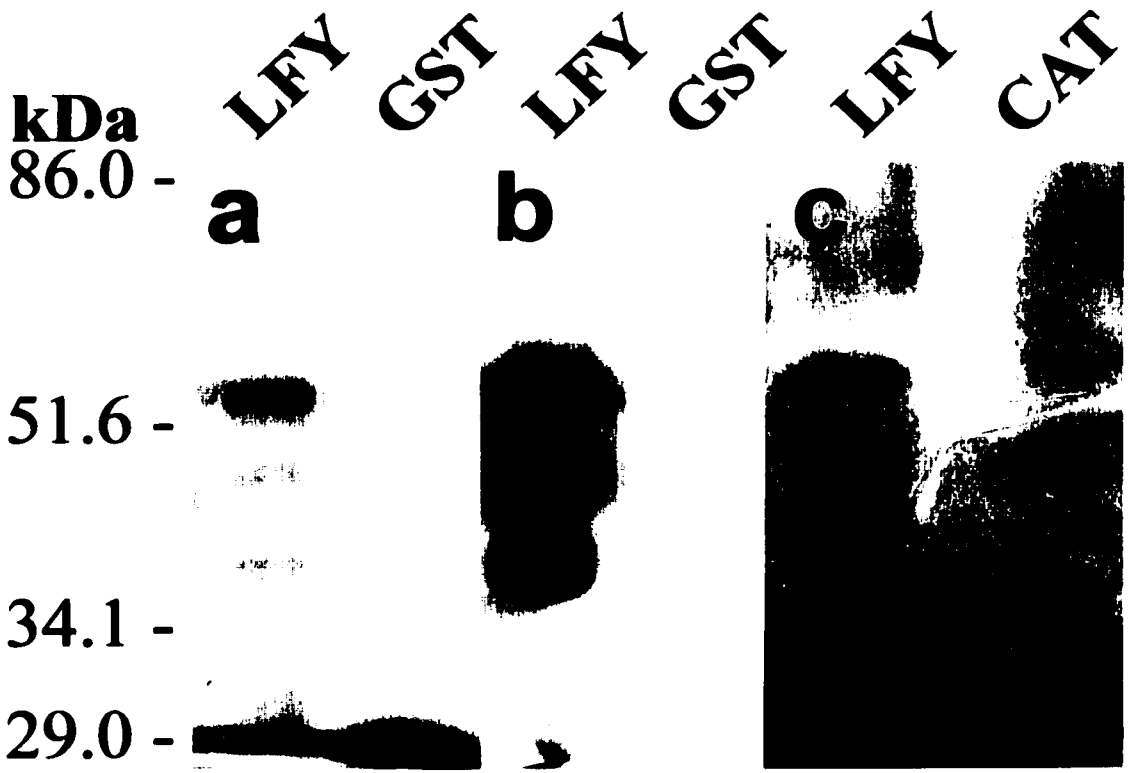

Root
Stem
Leaf
Bract
TSAM
Bud
Young gynoecium
Mature gynoecium
kb
1.0 -
0.65 -
0.4 -
0.85 -
0.65 -
0.5 -
← BnSHP1
← Actin

Figure 5.5 (a) Coomassie blue-stained SDS-page gel. Three proteins were isolated (approximately 40, 45 and 60 kDa in size) after expression in *E. coli* BL21 cells and subsequent GST purification. Unconjugated GST protein migrated at the same rate as a 29 kDa protein marker. (b) Western analysis of (a) with LFY antibody which immunolocalized to all three proteins, but not the GST control. (c) Autoradiograph of labeled protein generated by linked transcription:translation shows 3 proteins identical to those of (a) and (b). The control CAT protein migrates at the same rate as a 29 kDa protein marker.


kDa
LFY
GST
LFY
GST
LFY
CAT
86.0 -
51.6 -
34.1 -
29.0 -
a
b
c

size to those described above (figure 5.5c). We were unable to identify significant LFY production in the inclusion bodies produced in BL21 cells using the GST system.

5.2.4 Electromobility shift assays

Using LFY protein, either generated from the GST gene fusion system or from the linked transcription:translation system (dialyzed or non-dialyzed), we were unable to elicit an electromobility shift using either the *BnSHP1* or *AGI* LFY binding site. The *in vitro* generated LFY protein did not bind to either the 149 bp double stranded sequence, generated by PCR from *BnSHP1*, or the 22 bp synthetic double stranded oligonucleotide sequence. Furthermore, the positive control, a 29 bp synthetic double stranded oligonucleotide from *AG* containing a sequence previously shown to be bound by LFY, was also unable to elicit a mobility shift.

5.3 Discussion

5.3.1 *BnSHP1* is a member of the MADS-box gene family and is orthologous to the *SHATTERPROOF1* gene of *Arabidopsis*

BnSHP1 has high sequence similarity to other members of the MADS-box gene family, particularly *SHP1* (formerly called *AGL1*; Ma et al., 1991). *BnSHP1* contains both a MADS box and a K domain, each of which are highly conserved with those of *SHP1* (98% and 99% identity, respectively). Furthermore, the locations of intron splice sites within the *BnSHP1* open reading frame are identical to those of the *SHP1* gene (figure 5.1a) and the approximate sizes of each of the six introns from each gene are similar (data not shown).

BnSHP1 expression pattern, as determined by RT-PCR, was very similar to that of *SHP1* and *SHP2* (Ma *et al.*, 1991). The detection of *BnSHP1* in gynoecia of *B. napus* is consistent with the role of *SHP1* controlling cell differentiation and lignification in the dehiscence zone and adjacent cells at the valve margins in *Arabidopsis* (Liljegren *et al.*, 2000). The strongest *BnSHP1* signal produced by RT-PCR was also observed in the

mature gynoecium of *B. napus* flowers, corresponding to the observation that *SHP1* mRNA levels are highest in mature gynoecia of *Arabidopsis*, as seen by *in situ* hybridization (Flanagan *et al.*, 1996), The presence of a signal in apical meristems collected 28 hours following light induction to flowering (TSAMs), suggests that *BnSHP1* is expressed very early in flower development. This result contrasts the finding that in *Arabidopsis*, *SHP1* is not expressed until stage 7 in flower development, at the tip of the growing gynoecial tube (Flanagan *et al.*, 1996). Furthermore, stage 7 of flower development does not occur until approximately 5.25 days following floral initiation (Smyth *et al.*, 1990). One explanation for this observation is that during the process of tissue collection, apical meristems were not synchronized and as such, some had undergone the transition to flowering prior to light induction. Alternatively, *BnSHP1* might be expressed at low levels during early flower development and due to the higher sensitivity of RT-PCR vs. *in situ* hybridization, it was detected in our experiments. Collectively, this evidence suggests that *BnSHP1* is orthologous to *SHP1* of *Arabidopsis* and is probably involved in controlling dehiscence zone formation in the siliques of *B. napus*.

5.3.2 Highly conserved sequences within the first intron of *BnSHP1* and *SHP1* suggest the presence of essential regulatory elements

Important regulatory elements controlling gene expression have been identified in the introns of the genes they regulate. A recent example of this phenomenon is the *AGAMOUS* (*AG*) gene from *Arabidopsis*, the second intron (approximately 3.0 kb) of which contains elements responsible for both the temporal and spatial regulation of its expression (Sieburth and Meyerowitz, 1997; Deyholos and Sieburth, 2000). Furthermore, these regulatory elements within the *Arabidopsis AG* intron can direct similar expression patterns in other plant species (Pylatuik *et al.*, submitted) suggesting that, in addition to conserved exon sequences which encode the actual protein, essential regulatory sequences within the introns can also be conserved among species.

Typically, the introns of plants are small, with an average length of 200-400 bp (Carels and Bernardi, 2000); comparatively, the *BnSHP1* first intron, like the second

intron of *AG*, is rather large (1151 bp). A comparison of the first intron of *BnSHP1* with that of the *Arabidopsis SHP1* showed that the two sequences were 49% similar at the nucleotide level. However, regions with much higher identity were observed, including a 110 bp region (corresponding to positions 1093-1192 in *BnSHP1*) with 85% identity between the intron sequences of the two species. Within this conserved 110 bp region of *BnSHP1*, an inverted repeat identical to the LFY binding site (Busch *et al.*, 1999) was identified, while in *SHP1*, a similar consensus sequence was also identified (figure 5.1c). Because this region of intron has been highly conserved in the *SHP1* gene of *Arabidopsis* and *B. napus*, and it contains a known binding site for a potential upstream regulator, we propose that this sequence serves as an essential regulatory element for the expression of *SHP1*.

5.3.3 LFY: a possible regulator of *SHATTERPROOF* genes

LFY has previously been identified as a direct upstream activator of MADS box genes such as *APETALA1* and *AGAMOUS* (Parcy *et al.*, 1998; Busch *et al.*, 1999). AGAMOUS (Flanagan *et al.*, 1996) and FRUITFULL (Ferrándiz *et al.*, 2000b) have been shown to regulate *SHP1* and *SHP2* expression. It is therefore possible that LFY could also regulate the expression of the *SHATTERPROOF* genes. Unfortunately, all morphological studies of the *lfy* mutant to date have failed to address in detail any consequences to the gynoecium; therefore, the effects of *lfy* on the development of valve dehiscence zones is difficult to assess. Recently, FRUITFULL was found to be a negative regulator of *SHP1* and *SHP2* (Ferrándiz *et al.*, 2000b). Furthermore, LFY expression, as well as the relative level of LFY activity, was shown to be positively regulated by FRUITFULL (Ferrándiz *et al.*, 2000a). These results, along with the identification of a putative LFY binding site in the *SHP1* gene and the promoter of *SHP2* raises the possibility that LFY might play a direct role, perhaps mediated by FRUITFULL, in the down-regulation of the *SHATTERPROOF* genes.

5.3.4 Full-length LFY protein does not bind to a consensus LFY binding sequence

The orf of pIL8 encodes a full length LFY protein that is 420 amino acids in length. From this orf, we successfully generated LFY protein in either *E. coli* BL21 cells or by linked transcription:translation *in vitro*. Previously, bacterial expression of LFY used an orf with a large C-terminal deletion of unconserved amino acids corresponding to positions 391-421 of the predicted protein (Parcy *et al.*, 1998). The fact that we were able to generate full-length LFY protein is contrary to the finding that the 30 amino acid deletion described above was necessary to allow expression in bacteria (Parcy *et al.*, 1998). However, in addition to producing the complete LFY protein, predicted to be approximately 60 kDa in size (Doris Wagner, personal communication), two additional proteins approximately 45 and 40 kDa, were produced (figure 5.5a-c). These additional proteins were identified as derived from the *LFY* orf, because the LFY antibody was able to immunolocalize to all three proteins. Similarly, when expressed in aspen (*Populus*), the full-length LFY orf also produced three proteins similar in size to the *in vitro* synthesized proteins described above (Rottmann *et al.*, 2000).

We were unable to demonstrate LFY binding using the *AG*-LFY or *BnSHP1*-LFY binding sites, with the combined three LFY proteins. There are two possibilities that could explain the lack of LFY binding. First, full length LFY, although expressed in BL21 cells, was improperly translated, resulting in its inability to bind the LFY consensus sequence. An artifact of this improper translation could be the production of two smaller truncated proteins approximately 45 and 40 kDa in size. However, when this construct is expressed in aspen, three LFY proteins were also produced and the construct reliably produced a predictable phenotypic effect (Rottmann *et al.*, 2000). This raises the second possibility that; only the LFY protein containing the C-terminal 30 amino acid deletion is capable of binding the consensus sequence, even though it remains functionally equivalent *in vivo* to the full-length. It is therefore possible that the truncated version of LFY can interact with the putative binding site, while full length LFY is inhibited from binding, perhaps requiring the cooperative action of some other cofactor. The hypothesis of an additional factor 'X', involved in the activation of *AG*,

has previously been suggested (Parcy *et al.*, 1998). These authors observed that ectopically expressed LFY required a strong VP16 activation domain from the viral transcription factor VP16 to make *AG* activation independent of factor 'X'. Furthermore, other examples have been identified where partial proteins were found to be more active than their full-length counterparts (Yang *et al.*, 2000).

5.4 Experimental procedures

5.4.1 Plant material

Seeds of *Brassica napus* cv. Westar were donated by Dr. W. Keller, Plant Biotechnology Institute, National Research Council, Saskatoon. Plants used for tissue collection were grown in the Phytotron Facility (College of Agriculture, University of Saskatchewan) on Terra-lite Redi-earth® (W.R. Grace & Co. Canada Ltd., Ajax, Ont.) with a 16 hour/8 hour light/dark cycle (230 mmol photons $\cdot m^{-2} \cdot s^{-1}$) and a 23°C/18°C day/night temperature cycle. Plants grown for the collection of transitional shoot apical meristems (TSAMs - apical meristems harvested 28 hours following light induction to flowering) were cultivated as described in Bonham-Smith *et al.* (1992). Tissues harvested from *B. napus* plants for the purpose of genomic analysis were immediately placed on ice and subsequently stored at –80°C. Tissues collected for the purpose of RNA isolation and *BnSHP1* expression profiling were immediately placed in liquid nitrogen and subsequently stored at –80°C. These tissues included seedling roots, mature stems, young leaves (> 1.0 cm in length), young bracts (> 1.5 cm in length), TSAMs, buds, young gynoecia (unfertilized, 0.3 – 0.5 cm in length), collected from unopened buds, and mature gynoecia (fertilized, 1.2 – 2.5 cm in length).

5.4.2 Cloning and characterization of *BnSHP1*

Using genomic DNA extracted from *Arabidopsis thaliana*, ecotype Columbia (Mclean *et al.*, 1988) as template, a PCR was used to generate a 228 bp product corresponding to the second exon of the *AG* gene and consisting largely of the MADS

box (Yanofsky *et al.*, 1990). The primers used to generate this product were 5' ATCACGGCGTACCAATCGGAGCTA and 5' GTTAGAGTACTCATAGAGACG ACCACG. A ^{32}P-probe was generated from the 228 bp *AG* product using an Oligolabelling Kit (Pharmacia Biotech) as per the manufacturer's instructions. The probe was used to screen a *Brassica napus* (cv. Bridger) genomic library in λEMBL3 (Clonetech) with Colony/Plaque Screen™ hybridization transfer membranes (DuPont-NEN), resulting in the isolation of a genomic fragment approximately 20 kb in length called BAG2-5. A 1.2 kb *NcoI*/*BglI* fragment of BAG2-5, to which the *AG* probe hybridized to, was sub-cloned into the vector pMECA (Thomson and Parrott, 1998). Sequencing of the sub-clone showed a high level of identity to the *Arabidopsis SHP1* gene. Further sequencing of an additional 2.1 kb of BAG2-5 downstream of the subclone identified the complete sequence of a *SHP1* ortholog called *BnSHP1* (accession #AF226865, Appendix A.2). The predicted 750 bp open reading frame (accession #AY036062, Appendix A.3) was confirmed by RT-PCR using the primers 5' TCGGTGGTTTATTCATTTGGTG and 5' TTAAACAAGTTGAAGAGGAGGTTGG.

5.4.3 *BnSHP1* RNA expression

Total RNA was isolated from various tissues using the RNeasy Plant Mini Kit (QIAGEN), as outlined in the manufacturer's instructions. *BnSHP1* RNA expression was determined by reverse transcriptase-PCR using the OneStep RT-PCR Kit (QIAGEN) with the *BnSHP1*-specific primers described above. Actin RNA expression was determined as a positive control using the primers 5' GCCAAGATGGATCCTCC and 5' CCCTGCCATGTATGTTGC. Reactions were performed as outlined in the manufacturer's instructions, using 2.0 μg total RNA from each tissue as template with an annealing temperature of 45°C.

5.4.4 *BnSHP1* copy number

The number of *BnSHP1* copies in the *B. napus* genome was determined by Southern analysis on genomic DNA digested with *Nco*I and *Not*I. A 777 bp ^{32}P-probe

specific for *BnSHP1* was generated, corresponding to regions 1174-1950 of the *BnSHP1* gene. Genomic digests were transferred to GeneScreen Plus nylon membranes (DuPont) and the probe hybridized in QuickHyb solution (Stratagene) as per the manufacturer's instructions.

5.4.5 Synthesis of LEAFY protein

LEAFY protein was generated from the *LFY* open-reading frame (orf) of plasmid pIL8, generously provided by Dr. Detlef Weigel, Salk Institute. First, the *LFY* orf was cloned in frame into the expression vector pGEX-2T, transformed into *E. coli* BL21 cells, and LFY protein was expressed and isolated using the GST Gene Fusion System (Pharmacia Biotech) as described in the manufacturer's instructions. From the induced BL21 cells, LFY protein was also isolated from inclusion bodies, as described in Lui (2000). Second, using PCR, the *LFY* orf was generated with an upstream Shine-Dalgarno (SD) sequence with the primers 5' GGCGGATCC*AGGAGG*ATATTCAT GGACCCTGAA (italicized bases denote the SD) and 5' GCGAATTCCCTAGAAAC GCAAGTCGTCGC. The product, SD-LFY, was cloned in frame into the vector pTRI19amp at the *Bam*HI/*Eco*RI restriction sites to yield the SD-LFYorf plasmid. LFY protein was generated from the SD-LFYorf plasmid using the PROTEINscript^{TM}-PRO (Ambion) linked transcription:translation kit as described in the manufacturer's instructions. To purify and renature the LFY protein from components of the linked transcription/translation kit, the product was dialyzed over 2 days in 6 changes of 20 mM Tris pH 7.5, 150 mM NaCl, 0.25 mM EDTA, 1 mM dithiothreitol (DTT), and 20 mM $MgCl_2$ at 4 °C.

5.4.6 SDS-PAGE and Western analysis

Protein products generated from the GST gene fusion and linked transcription:translation systems were analysed by SDS-polyacrylamide gel electrophoresis using a 5% stacking/10% resolving gel as described in Sambrook *et al.* (1989). The identity of GST-purified protein was confirmed by immunochemical

detection with a polyclonal antibody to LFY, used at a 1:2000 dilution, supplied by Dr. Doris Wagner, California Institute of Technology (Wagner *et al.*, 1999). An anti-rabbit IgG-HPR secondary antibody (Santa Cruz Biotechnology) was immunolocalized to the LFY antibody and the conjugated horse radish peroxidase activity visualized using Western Blot Chemiluminescent Reagent + (NEN Life Science).

5.4.7 Electromobility shift assays

Using the primers 5' CTGAAATCAAAGTGGTACGAGC and 5' CAGAACAAGGTCCATGCATA, a PCR was used to amplify and label (by the incorporation of ^{32}P-dCTP into the reaction) a 149 bp fragment corresponding to bases 1167-1315 of the *BnSHP1* gene which contained the putative consensus LFY binding site (Busch *et al.*, 1999). A second, more specific *BnSHP1* probe, also containing the putative binding site, was generated from complementary oligonucleotide sequences corresponding to bases 1182-1203, synthesized with 5' guanine (G) overhangs. These oligonucleotides were annealed in 50 mM NaCl by boiling 5 minutes followed by cooling gradually to room temperature over several hours. A probe was generated by end-filling 1.0 µg of the DNA at the 5' G overhangs with ^{32}P-dCTP in 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 100 mM DTT, and 50 µg/ml BSA (Fraction V; Sigma) for one hour at room temperature. Labeling reactions were cleaned using the Qiaquick Nucleotide Removal Kit (Qiagen) as described in the manufacturer's instructions. Positive control probes were generated and labeled as described above, from oligonucleotides corresponding to bases 46872-46900 of the BAC clone F13C5, containing a sequence within the *AG* second intron which had previously been shown to interact with LFY (Busch *et al.*, 1999).

Binding reactions between LFY and the DNA probes were performed as described in Parcy *et al.* (1998) and van der Fits *et al.* (2000). Reactions were separated on a non-denaturing 4% acrylamide gel and visualized by autoradiography.

5.5 Summary

We describe here the cloning and characterization of a *Brassica napus SHATTERPROOF1* (*BnSHP1*) gene. Based on sequence similarity and expression pattern, we conclude that *BnSHP1* is a MADS-box class of transcription factor, orthologous to the *SHATTERPROOF1* gene of *Arabidopsis thaliana* which is required for proper dehiscence zone formation in mature siliques. *BnSHP1* shares 91% identity with its *Arabidopsis* counterpart, and its MADS box and K domain are 98% and 99% similar, respectively. *BnSHP1* is expressed only in flowers, particularly in gynoecia. Furthermore, sequence analysis of the large first intron of *BnSHP1*, as well as the regulatory regions of both *SHP1* and *SHP2* of *Arabidopsis*, has identified a conserved LEAFY binding site within these genes, suggesting that they may be regulated, in part, by the LEAFY protein.

5.6 Literature cited

Alvarez-Buylla, E.R., Liljegren, S.J., Pelaz, S., Gold, S.E., Burgeff, C., Ditta, G.S., Vergara-Silva, F. and Yanofsky, M.F. (2000) MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cells, roots and trichomes. *Plant J.*, **24**, 457-466.

Bonham-Smith, P.C., Oancia, T.L. and Moloney, M.M. (1992) Cytoplasmic ribosomal protein S15a from *Brassica napus*: Molecular cloning and developmental expression in mitotically active tissues. *Plant Mol. Biol.* **18**, 909-919.

Busch, M.A., Bomblies, K. and Weigel, D. (1999) Activation of a floral homeotic gene in *Arabidopsis*. *Science*, **285**, 585-587.

Carels, N. and Bernardi, G. (2000) Two classes of genes in plants. *Genet.*, **154**, 1819-1825.

Coen, E.S. and Meyerowitz, E.M. (1991) The war of the whorls: Genetic interactions controlling flower development. *Nature*, **353**, 31-37.

Deyholos, M.K. and Sieburth, L.E. (2000). Separable whorl-specific expression and negative regulation by enhancer elements within the *AGAMOUS* second intron. *Plant Cell* **12**, 1799-1810.

Ferrándiz, C., Gu, Q., Martienssen, R. and Yanofsky, M.F. (2000a) Redundant regulation of meristem identity and plant architecture by *FRUITFULL, APETALA1,* and *CAULIFLOWER. Development*, **127**, 725-734.

Ferrándiz, C., Liljegren, S.J. and Yanofsky, M.F. (2000b) Negative regulation of the *SHATTERPROOF* genes by FRUITFULL during *Arabidopsis* fruit development. *Science*, **289**, 436-438.

Flanagan, C.A., Hu, Y. and Ma, H. (1996) Specific expression of the *AGL1* MADS-box gene suggests regulatory functions in *Arabidopsis* gynoecium and ovule development. *Plant J.*, **10**, 343-353.

Liljegren, S.J., Ditta, G.S., Eshed, Y., Savidge, B., Bowman, J.L. and Yanofsky, M. F. (2000) *SHATTERPROOF* MADS-box genes control seed dispersal in *Arabidopsis*. *Nature*, **404**, 766-770.

Lui, H. (2000) Characterization of ICK_2 - a cyclin dependent kinase inhibitor in *Arabidopsis thaliana*. MSc. Thesis, University of Saskatchewan, Saskatoon.

Ma, H., Yanofsky, M.F. and Meyerowitz, E.M. (1991) *AGL1-AGL6*, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. *Genes Dev.*, **5**, 484-495.

Mclean, M., Baird, W.V., Gerats, A.G. and Meagher, R.B. (1988) Determination of the copy number and linkage relationships among five actin gene subfamilies in *Petunia hybrida. Plant Mol. Biol.*, **11**, 663-672.

Parcy, F., Nilsson, O., Busch, M.A., Lee, I. and Weigel, D. (1998) A genetic framework for floral patterning. *Nature*, **395**, 561-566.

Patterson, S.E. (2001) Cutting loose: abscission and dehiscence in *Arabidopsis*. *Plant Physiol.*, **126**, 494-500.

Riechmann, J.L. and Meyerowitz, E.M. (1997) MADS domain proteins in plant development. *Biol. Chem.*, **378**, 1079-1101.

Rottmann, W.H., Meilan, R., Sheppard, L.A., Brunner, A.M., Skinner, J.S., Ma, C., Cheng, S., Jouanin, L., Pilate, G. and Strauss, S .H. (2000) Diverse effects of overexpression of *LEAFY* and *PTLF*, a poplar (*Populus*) homolog of *LEAFY/FLORICAULA*, in transgenic poplar and *Arabidopsis*. *Plant J.*, **22**, 235-245.

Rounsley, S.D., Ditta, G.S. and Yanofsky, M.F. (1995) Diverse roles for MADS box genes in *Arabidopsis* development. *Plant Cell*, **7**, 1259-1269.

Sambrook, J., Frisch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual, 2nd ed.* Plainview, U.S.A.: Cold Spring Harbor Press. pp. 18.47-.59.

Savidge, B., Rounsley, S.D. and Yanofsky, M.F. (1995) Temporal relationship between the transcription of two *Arabidopsis* MADS box genes and the floral organ identity genes. *Plant Cell*, **7**, 721-733.

Shore, P. and Sharrocks, A.D. (1995) The MADS-box family of transcription factors. *Eur. J. Biochem.*, **229**, 1-13.

Sieburth, L.E. and Meyerowitz, E.M. (1997) Molecular dissection of the *AGAMOUS* control region shows that *cis* elements for spatial regulation are located intragenically. *Plant Cell*, **9**, 355-365.

Smyth, D.R., Bowman, J.L. and Meyerowitz, E.M. (1990) Early flower development in *Arabidopsis*. *Plant Cell*, **2**, 755-767.

Thomson, M.J. and Parrott, W.A. (1998) pMECA: A Cloning plasmid with 44 unique restriction sites that allows selection of recombinants based on colony size. *Biotechniques*, **24**, 922-928.

van der Fits, L., Zhang, H., Menke, F.L.H., Deneka, M. and Memelink, J. (2000) A *Catharanthus roseus* BPF-1 homolgue interacts with an elicitor-responsive region of the secondary metabolite biosynthetic gene *Str* and is induced by elicitor via a JA-independent signal transduction pathway. *Plant Mol. Biol.*, **44**, 675-685.

Wagner, D., Sablowski, R. W. M. and Meyerowitz, E. M. (1999) T ranscriptional activation of APETALA1 by LEAFY. *Science*, **285**, 582-584.

Yang, H., Wu, Y., Tang, R., Liu, D., Liu, Y. and Cashmore, A.R. (2000) The C t ermini o f *Ar abidopsis* c ryptochromes m ediate a constitutive light response. *Cell*, **103**, 815-827.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A. and Meyerowitz, E.M. (1990) The protein encoded by the *Arabidopsis* homeotic gene *AGAMOUS* resembles transcription factors. *Nature*, **346**, 35-39.

Chapter 6. GENERAL DISCUSSION AND FUTURE PROSPECTS

6.1 General discussion

Although reasonable progress has been made over the past decade in our understanding of the genetic mechanisms controlling flower development in *Arabidopsis*, very little is known about this process in related, economically important *Brassica napus*. Based on the burgeoning research effort underway, one can safely assume that the genetic control of flower development is largely conserved in distantly related plant species. However, the further testing of current models within established systems such as *Arabidopsis*, as well as in other related species such as *B. napus*, can only serve to improve and strengthen our understanding of development. Furthermore, our understanding of development provides opportunities to explore novel solutions to practical problems, such as hybrid seed production, as well as providing new evidence to address long-standing questions such as gynoecium ontogeny.

I began my research by assuming that the ABC model of flower development could be adopted from *Arabidopsis* and applied to *B. napus*. In keeping with the objective of determining the feasibility of creating female-sterile plants to be used as pollen donors, it was important to assess the production and viability of pollen produced by a female-sterile mutant, in this case, *flo10-1* (Chapter 2). Based on my assumption, the results of this initial assessment would serve as a useful indicator as to the viability and production of pollen in *B. napus*, should female-sterility be created in this species. I found that the quantity and viability of pollen produced by the lateral and median stamens of mutant plants was not compromised, when compared with that from stamens of wild-type plants. The flowers of *flo10-1* plants are female-sterile due to the proliferation of additional stamens at the expense of the gynoecium. I found that although the size of anthers and pollen from additional stamens was comparable to that of the medians and laterals, pollen production and viability were reduced. Despite these

shortcomings in the additional stamens, these results demonstrate that female-sterile mutants have the potential to serve as efficient pollen donors.

From close examination of *flo10-1* flowers, it was apparent that the arrangement and patterning of additional stamens was not random. There was a tendency for additional stamens to preferentially occur in the median plane of flowers, corroborating evidence of sided bias previously seen with other floral mutants (Bowman, 1994; Kunst et al., 1989). Although the question as to what factors play a role in contributing to this bilateral symmetry remains unanswered, I hypothesised that it might be related to higher cell number in the median versus the lateral plane. Furthermore, the results of my work with the *flo10-1* mutant also suggest, for the first time, that the *FLO10* gene could be responsible for the natural variation in stamen number often seen in wild-type populations of *Arabidopsis* (Müller, 1961; Running and Meyerowitz, 1996; Pylatuik et al., 1998).

In Chapter 3 I tested whether *cis* regulatory elements from the homeotic class C gene of *Arabidopsis* called *AGAMOUS* (*AG*) could direct a similar expression pattern in *B. napus*. In order to test this hypothesis, the *AG cis* regulatory elements, located within the second intron of the *AG* gene, were fused to a GUS reporter gene and the construct was transformed into *B. napus* plants. As hypothesized, the *cis* elements directed expression primarily to the reproductive organs in *B. napus*, which demonstrated for the first time, that the function of *AGAMOUS* regulatory elements, in addition to its open reading frame (Mandel et al., 1992; Rigola *et al.*, 2001; Rutledge *et al.*, 1998; Tandre *et al.*, 1995), are conserved across species. The results of this experiment showed that I could successfully use the regulatory elements of the *Arabidopsis AG* gene to direct a similar expression pattern in the closely related species, *B. napus*. Additionally, these results strengthen the idea that many elements of the ABC model, both functional and regulatory, are conserved between species.

Although the general domain of *Arabidopsis AG* expression was conserved in *B. napus*, I also observed subtle differences in tissue specificity between the two species. Primarily, the regulatory elements of the *Arabidopsis AG* gene did not direct expression in the ovules of *B. napus*. This result shows that despite the vast similarities, subtle

regulatory differences can exist between the floral homeotic genes, even in closely related species.

In Chapter 4, I described the cloning and characterization of a *B. napus* class B gene homologous to *APETALA3* (*AP3*) of *Arabidopsis* called *BnAP3*, thus partly fulfilling my objective to identify genes involved in floral development from *B. napus*. The ABC model of flower development predicts that if *BnAP3* (or *AP3*) is expressed under the control of the *AG* regulatory elements, the identity of the gynoecium will be changed to stamens, with no consequence to the sepals, petals, or stamens of the flower. In developing and evaluating the *AG::BnAP3* lines of *Arabidopsis* and *B. napus*, I not only tested this prediction of the ABC model, which had previously never been examined, but also fulfilled a primary objective of my thesis, which was to examine the feasibility of creating female-sterile flowers based on the application of this model. The results of this experiment strengthened the integrity of the ABC model in that staminoid organs arose in place of the gynoecium in both *Arabidopsis* and *B. napus*. Furthermore, our results suggested that this was a feasible approach to creating female-sterile flowers.

By producing staminoid organs at the expense of the carpels, the introduction of the *AG::BnAP3* construct into plants only partially fulfilled the predictions of the ABC model of flower development. It was unclear however, why these organs still retained carpeloid properties. Furthermore, it was unclear why the results of the experiment were far less dramatic in *B. napus* compared with that of *Arabidopsis*.

Using scanning electron microscopy and light microscopy, it was concluded that tissues of these organs derived from the L2 and L3 histogenic layers were staminoid, while tissues derived from the L1 histogenic layer were carpeloid. Therefore, these organs were not completely converted to stamens because they were effectively periclinal chimeras of stamen and carpel tissue. I propose that this phenotype was the result of differential *BnAP3* expression, directed by the *AG* regulatory elements. This theory has recently been verified by the work of Jenik and Irish (2001) who generated *ap3/AP3/AP3* chimeras in *Arabidopsis*, which resulted in the same phenotype in third whorl organs.

In *AG::BnAP3* lines of *B. napus*, phenotypes similar to that seen in *Arabidopsis* were observed however, they did not occur with as high a frequency. I reasoned that

these minimal effects could be attributed to the low dosage of *BnAP3* levels produced under the control of the *AG* regulatory elements, compared to the large allotetraploid genome of *B. napus* (Wendel, 2000).

Finally, from close examination of *AG::BnAP3 Arabidopsis* flowers, it was apparent that the number and arrangement of staminoid organs, like the additional stamens described in Chapter 2, were not random. The most regular feature of these transgenic flowers was that in place of the gynoecium, they simultaneously produced four organs, two of which arose in the median axis and two in the lateral axis. I have argued that these organs may have been produced from four defined growth points within the fourth whorl, probably corresponding to four predetermined growth points of the wild-type gynoecium. The question of how many organs comprise the Brassicaceaen gynoecium is a long-standing one and despite this research, the question still remains. However, the Brassicaceae gynoecium has, for convenience's sake, been regarded over the last several years as being comprised of two organs (Bowman, 1994). These results should serve, if nothing else, as a reminder to keep open questions such as gynoecium ontogeny, and not disregard the significance of experimental results for the sake of streamlining interpretation.

If we compare the general approach, described in Chapter 4, taken to achieve female-sterility, with those that generate male-sterility, there are a few fundamental differences. Much of the current research effort associated with generating male-sterility is based on the fundamental concept of disabling, or ablating, pollen or anthers by selectively expressing a toxic agent in these organs (Arnison, 1997). As a result, anthers either fail to make or shed pollen. Conversely, our approach to generating female-sterile plants alters the floral ground plan by manipulating the plants' endogenous genes and shifts its resources from producing both male and female organs, to producing just male organs. One advantage to this approach is that the plants' resources are not exhausted on producing organs that will never be used. Also advantageous, particularly to an industry that is increasingly scrutinized by the public, is that this approach to female-sterility can be achieved without the introduction of foreign genes. Recognizing the advantages to this approach o f s elective s terilization, I a lso

tried, although unsuccessfully, to generate male-sterile lines of *B. napus* (Appendix C), based on the ABC model of flower development.

In Chapter 5, I described the cloning and characterization of a *B. napus* gene orthologous to *SHATTERPROOF1* (*SHP1*) of *Arabidopsis* called *BnSHP1*. The identification of *BnSHP1*, along with *BnAP3* discussed in Chapter 4, fulfilled my objective to identify genes involved in floral development from *B. napus*. Careful analysis of possible regulatory regions within *BnSHP1*, *SHP1*, and *SHP2*, identified conserved LFY binding sites and CArG-box elements within the intron and 5' sequences of these genes. These results suggest that these genes, essential for the differentiation of valve dehiscence zones, are possibly regulated by the LFY protein (Busch et al., 1999), and MADS-box genes such as *FRUITFULL* (Ferrándiz et al., 2000). Examination of the intron sequences of *BnSHP1* from *B. napus* and *SHP1* from *Arabidopsis*, show highly conserved regions, suggesting that like *AG*, the regulatory sequences of other flowering genes have been conserved across these species.

Based on the results discussed in this thesis, I have concluded that it is feasible to create female-sterile plants in *Arabidopsis* and *B. napus*, by linking the class B activity of *BnAP3* to the class C controlling elements of *AG*. This work has further tested and verified the integrity of the ABC model, while offering new insights into other aspects of flower development such as patterning and gynoecium ontogeny. Lastly, this work has resulted in the identification of genes controlling flower development in *B. napus*, and has shown that although *Arabidopsis* and *B. napus* are very closely related, some aspects of gene function, such as regulation, have started to diverge between the two species. Much can be learned by studying a model system such as *Arabidopsis* however, despite the many commonalities between plants, model systems can only extend so far before differences in gene regulation, protein function, genome size, and ploidy level (Wendel, 2000) force the examination of a single species for its own merit.

6.2 Future prospects

The results of this study have raised many questions regarding transgenic approaches to altering floral morphology in *B. napus*, the conservation of regulatory

elements across closely related species, the function of genes involved in flower development, and the ontogeny of the Brassicaceaen gynoecium.

It is clear from this work, that a particular transgenic approach will not always achieve the same result in *B. napus* as it does in *Arabidopsis*. When using molecular-based approaches for altering development, polyploids such as *B. napus* pose unique problems which could be related to the presence of multiple gene copies, gene silencing, dosage effect, or dosage compensation (Wendel, 2000). Therefore, to achieve completely female-sterile lines of *B. napus*, future strategies could examine the use of stronger promoters to overcome the possibility of multi-copy or dosage-dependent problems, or the use of homologous genes from other species (e.g. use *AP3* in place of *BnAP3*) to overcome possible problems associated with gene silencing or dosage compensation. Furthermore, Krizek and Meyerowitz (1996) found that the conversion of the gynoecium to stamens was more pronounced when the second class B gene, *PISTILLATA* (*PI*), was ectopically expressed in combination with *AP3*. Therefore another possible strategy could be to introduce both *AP3* and *PI* into the fourth whorl under the regulation of *AG*.

The results discussed here suggest that the *AG cis* elements of the second intron direct expression only in tissues derived from the L2 and L3 histogenic layers of fourth whorl organs. To further test this proposal, the existing *AG::BnAP3 Arabidopsis* lines could be crossed with *ap3* mutants, and to observe the effects in the third whorl organs as well as organs of the fourth and subsequent whorls.

From this research, new insight has been gained with respect to the conservation and divergence of regulatory elements among close and distantly related species. Are the regulatory elements of *AG* an exception, or is there also significant conservation among regulatory sequences, both 5' and intronic, of other flowering genes? The results obtained from sequence analysis of the newly identified *BnSHP1* and *SHP1*, suggests that many regulatory elements may be conserved.

Finally, this work has identified new genes associated with flower development. In the *B. napus BnSHP1* gene, a putative LEAFY binding site was identified which was also found in the *SHP1* and *SHP2* genes of *Arabidopsis*. Although full-length LEAFY protein has never been shown to bind this sequence, truncated versions of LFY have

been shown to bind (Parcy et al., 1998; Busch et al., 1999). An interaction between the putative LFY binding sites of the *SHATTERPROOF* genes and the truncated LFY protein would verify the proposed LFY binding consensus, while also suggesting that LFY is an upstream regulator of these genes. If LFY is a regulator of the *SHATTERPROOF* genes, are *lfy* mutants themselves compromised (or exaggerated) with respect to valve dehiscence zone formation?

With further improvements, complete female-sterility can be generated in *B. napus*, and this novel approach might also be tested in other species. As more developmental genes are identified, and their regulatory components characterized, our understanding of floral development and how it differs between species will be enhanced.

6.3 Literature cited

Arnison, P.G. (1997) Hybrid seed – new systems for the next century. In *PBI Bulletin-January*. Saskatoon, National Research Council.

Bowman, J.L. (1994) *Arabidopsis, An atlas of morphology and development.* New York: Springer-Verlag.

Busch, M. A., Bomblies, K. and Weigel, D. (1999) Activation of a floral homeotic gene in *Arabidopsis*. *Science*, **285**, 585-587.

Ferrándiz, C., Liljegren, S. J. and Yanofsky, M. F. (2000) Negative regulation of the *SHATTERPROOF* genes by FRUITFULL during *Arabidopsis* fruit development. *Science*, **289**, 436-438.

Jenik, P.D. and Irish, V.F. (2001) The *Arabidopsis* floral homeotic gene *APETALA3* differentially regulates intercellular signaling required for petal and stamen development. *Development*, **128**, 13-23.

Krizek, B.A. and Meyerowitz, E.M. (1996) The *Arabidopsis* homeotic genes *APETALA3* and *PISTILLATA* are sufficient to provide the B class organ identity function. *Development*, **122**, 11-22.

Kunst, L., Kienz, J.E., Martinez-Zapater, L. and Haughn, G.W. (1989) *AP2* gene determines the identity of perianth organs in flowers of *Arabidopsis thaliana*. *Plant Cell*, **1**, 1195-1208.

Mandel, M.A., Bowman, J.L., Kempin, S.A., Ma, H., Meyerowitz, E.M. and Yanofsky, M.F. (1992) Manipulation of flower structure in transgenic tobacco. *Cell*, **71**, 133-143.

Müller, A. (1961) Zur Charakterisierung der Blüten und Infloreszenzen von *Arabidopsis thaliana* (L.) Heynh. *Kulturpflanze*, **9**, 364-393.

Parcy, F., Nilsson, O., Busch, M. A., Lee, I. and Weigel, D. (1998) A genetic framework for floral patterning. *Nature*, **395**, 561-566.

Pylatuik, J.D., Bonham-Smith, P.C. and Davis, A.R. (1998) The additional stamens of *flo10-1* mutants of *Arabidopsis thaliana* are compromised in production and viability of pollen. *Can. J. Bot.*, **76**, 1733-1742.

Rigola, D., Pè, M.E., Mizzi, L., Ciampolini, F. and Sari-Gorla, M. (2001) *CaMADS1*, an *AGAMOUS* homologue from hazelnut, produces floral homeotic conversion when expressed in *Arabidopsis*. *Sex. Plant Reprod.*, **13**, 185-191.

Running, M.P. and Meyerowitz, E.M. (1996) Mutations in the *PERIANTHA* gene of *Arabidopsis* specifically alter floral organ number and initiation pattern. *Development*, **122**, 1261-1269.

Rutledge, R., Regan, S., Nicolas, O., Fobert, P., Côté, C., Bosnich, W., Kauffeldt, C., Sunohara, G., Séguin, A. and Stewart, D. (1998) Characterization of an *AGAMOUS* homologue from the conifer black spruce (*Picea mariana*) that produces floral homeotic conversions when expressed in *Arabidopsis*. *Plant J.*, **15**, 625-634.

Tandre, K., Albert, V.A., Sundas, A. and Engström, P. (1995) Conifer homologues to genes that control flower development in angiosperms. *Plant Mol. Biol.*, **27**, 69-78.

Wendel, J.F. (2000) Genome evolution in polyploids. *Plant Mol. Biol.*, **42**, 225-249.

APPENDIX A: GENBANK SUBMISSIONS

A.1 *BnAP3* cDNA (#AF124814)

```
LOCUS       AF124814      941 bp    mRNA            PLN       21-MAY-2001
DEFINITION  Brassica napus APETALA3 (BnAP3) mRNA, complete cds.
ACCESSION   AF124814
VERSION     AF124814.1  GI:6841081
KEYWORDS    .
SOURCE      rape.
  ORGANISM  Brassica napus
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots;
            Rosidae; eurosids II; Brassicales; Brassicaceae; Brassica.
REFERENCE   1  (bases 1 to 941)
  AUTHORS   Pylatuik,J.D., Davis,A.R. and Bonham-Smith,P.C.
  TITLE     Molecular cloning and characterization of a Brassica napus APETALA3
            cDNA
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 941)
  AUTHORS   Pylatuik,J.D., Davis,A.R. and Bonham-Smith,P.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-JAN-1999) Biology, University of Saskatchewan, 112
            Science Place, Saskatoon, SK S7N 5E2, Canada
FEATURES             Location/Qualifiers
     source          1..941
                     /organism="Brassica napus"
                     /db_xref="taxon:3708"
     gene            1..941
                     /gene="BnAP3"
     CDS             37..711
                     /gene="BnAP3"
                     /note="MADS box transcription factor"
                     /codon_start=1
                     /product="APETALA3"
                     /protein_id="AAF28894.1"
                     /db_xref="GI:6841082"
                     /translation="MARGKIQIKRIENQTNRQVTYSKRRNGLFKKAHELTVLCDARVS
                     IIMFSSSNKLHEFISPNTTTKEIIDLYQTVSDVDVWSAHYERMQETKRKLLETNRNLR
                     TQIKQRLGECLDELDIQELRSLEEEMENTFKLVRERKFKSLGNQIETTKKKNKSQQDI
                     QKNLIHELELRAEDPHYGLVDNGGDYDSVLGYQLRFHQNHHHHYPNHALHAASASDII
                     TFHLLE"
BASE COUNT      315 a    182 c    191 g    253 t
ORIGIN
        1 taacaaaata tcaaaaacta aatagagaga ggaaaaatgg cgagagggaa gatccagatc
       61 aagaggatag agaaccagac aaaccgacaa gtgacctatt ccaagagaag aaatggtttg
      121 ttcaagaaag ctcacgagct cacggttttg tgtgacgcta gggtttcgat tatcatgttc
      181 tctagttcca acaagcttca tgagtttatc agccctaaca ccacaacgaa ggagatcata
      241 gatctgtacc aaacagtttc tgatgttgat gtttggagcg ctcactatga gagaatgcaa
      301 gaaaccaaga ggaaattgtt ggagacgaac agaaatctcc ggactcagat taaacagagg
      361 ctaggtgagt gtttagacga gcttgatatt caggagctgc gtagtcttga ggaagaaatg
      421 gaaaacactt tcaaactcgt tcgcgagcgc aagtttaaat cacttgggaa ccaaatcgag
      481 accaccaaga aaaagaacaa gagtcaacaa gacatacaaa agaatctcat acatgagctg
      541 gaactaagag cagaagatcc tcattatgga ctagtagaca atggaggaga ctacgatteg
      601 gttcttggat atcaacttcg tttccatcag aaccatcacc accattaccc caaccatgcc
      661 cttcatgcag catctgcctc tgatatcatt accttccacc ttcttgaata actaagggtt
      721 tgaaggttgc tgatattgtc attatctatc tatttaataa aataaatact taaaagcgta
      781 aggctggtct taccgaaacc ttaaacggtc atgtttctta attatgtatt ttcttaaacc
      841 ttaactatgg attgtgtgat cagtagcttg ctgctttaac ttcttgcgga ttgaatgtgt
      901 ttcaacacta cgtatgatat tatatttgac gtttcctact t
//
```

A.2 *BnSHP1* gene (#AF226865)

```
LOCUS       AF226865     3549 bp    DNA             PLN       11-JUN-2001
DEFINITION  Brassica napus SHATTERPROOF1 (BnSHP1) gene, complete cds.
ACCESSION   AF226865
VERSION     AF226865.1  GI:12655900
KEYWORDS    .
SOURCE      rape.
  ORGANISM  Brassica napus
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots;
            Rosidae; eurosids II; Brassicales; Brassicaceae; Brassica.
REFERENCE   1  (bases 1 to 3549)
  AUTHORS   Pylatuik,J.D., Davis,A.R. and Bonham-Smith,P.C.
  TITLE     Molecular cloning and characterization of a B. napus SHP1 gene
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 3549)
  AUTHORS   Pylatuik,J.D., Davis,A.R. and Bonham-Smith,P.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-JAN-2000) Biology, University of Saskatchewan, 112
            Science Place, Saskatoon, Sk S7N 5E2, Canada
FEATURES             Location/Qualifiers
     source          1..3549
                     /organism="Brassica napus"
                     /cultivar="Bridger"
                     /db_xref="taxon:3708"
     mRNA            join(<1..473,1625..1706,1980..2041,2395..2494,
                     2576..2617,2690..2731,2808..>3158)
                     /gene="BnSHP1"
                     /product="SHATTERPROOF1"
     gene            <1..>3158
                     /gene="BnSHP1"
     exon            <1..473
                     /gene="BnSHP1"
                     /number=1
     CDS             join(247..473,1625..1706,1980..2041,2395..2494,2576..2617,
                     2690..2731,2808..3002)
                     /gene="BnSHP1"
                     /note="MADS-domain protein"
                     /codon_start=1
                     /product="SHATTERPROOF1"
                     /protein_id="AAK00646.1"
                     /db_xref="GI:12655901"
                     /translation="MDEGGSSHDAESSKKIGRGKIEIKRIENTTNRQVTFCKRRNGLL
                     KKAYELSVLCDAEVALVIFSTRGLLYEYASNSVKGTIERYKKACSDAVNPPTVTEANT
                     KHYQQEASKLRRQIRDIQNSNRHIVGESLGSLNFKELKNLEGRLEKGISRVRSKKSEL
                     LVAEIEYMQKREMELQHVNMYLRAKIEQGARLNPEQHGSGVIQGTAVYESGLSSSHDQ
                     SQHYNRNYIPVNLLEPNQQFSGQDQPPLQLV"
     exon            1625..1706
                     /gene="BnSHP1"
                     /number=2
     exon            1980..2041
                     /gene="BnSHP1"
                     /number=3
     exon            2395..2494
                     /gene="BnSHP1"
                     /number=4
     exon            2576..2617
                     /gene="BnSHP1"
                     /number=5
     exon            2690..2731
```

```
                     /gene="BnSHP1"
                     /number=6
     exon            2808..>3158
                     /gene="BnSHP1"
                     /number=7
     polyA_signal    3153..3158
                     /gene="BnSHP1"
BASE COUNT     1102 a    635 c    593 g   1219 t
ORIGIN
        1 attatacagt tgaaatcaac cctaaaatcc caaatttgga gttcaaagtt tttttatttg
       61 tttgaattac aatctcagct gattgatttt ttagtgacca aatcatttga tatatttatt
      121 taatttttgcc tctcttgatc tgcaaaaata tttgatcata aacttgaata gcatcgctct
      181 ctagttcaat atctctccca cttctttttcg gtggtttatt catttggtga cgatatcaca
      241 gaagcaatgg atgaaggtgg gagtagtcac gatgcagaga gtagcaagaa gataggtaga
      301 gggaagatag agataaagag gatagagaac acaacaaatc gtcaagtaac ctttctgcaaa
      361 cgacgcaatg gtcttctcaa gaaagcttat gagctctctg tctttgtgtga tgctgaagtt
      421 gccctcgtta tcttctccac tcgtggcctt ctttatgagt acgccagcaa caggtatgct
      481 tctcctaccc acaccttgat ctagctttct tgattaattt actactacaa tcctagttaa
      541 tatgagccaa gattagggtt ttgtttaaat tacaatcctg aatttttctat ttttatatata
      601 aaaattagat ctcaataggg ctaccattgt ctctctagat ctgtgtatat ccaaataatg
      661 aagacggaag aaagctgtct tgtcttctca acttctcgtt agttctgatct ttgttagttt
      721 cactcttttt ctgcagatca ctagaacctg tttcatgtca tgtcagcttc tataaaatgc
      781 ttttatcttg acgacccata ctatgtcttt ctttaaatat tattagggtt tcgtcagtaa
      841 aaaaaaactg ggtagtacgc aatagcatgc atatatgtaa atatgcaaga cttatgtaac
      901 cctcctgtct tgtgaaactt gggacatagc ctaatgatgg ttgtcactat gacactatgg
      961 atccccttta attttttttcc taacccaaga aaacaaatgc cgaccgataa aactttagtt
     1021 atatataaaa tatataacat ctatctggag ttcgtatgtt gagaatatat atatgtgact
     1081 atttaaaatc taggcccttt aaggatgtaa aatatgtgta ttccattaat atattaatga
     1141 gagggagata actcagagag aagtgtctga aatcaaagtg gtacgagcca atgggaatct
     1201 atagcactct gagctccatt tatatgtgct gttgtatttg aaaaaaaaca gtttaatcatc
     1261 cttaaagcat actttgatga cattaaacca tataatatgc atggaccttg ttctgtattc
     1321 ctcctcaaac cgtaagtaat taccagtttg aatccatata ttaattaatt gctgcatcag
     1381 ccatttttaa atatgtacat tgaaaaagta gtttactcga gcacaatgtg tgtcattgaa
     1441 gttttctcctc gtagtggtca aaaaactggt ccaaaccttca aagccatcac attccttgtc
     1501 gattttaagg ttttgccccc aaaataaaca ttccaaaacc ttaatcaaga aatgtcgtcc
     1561 caattatctc tgttttaaga gtatattaat taaattaaat ataatggttt ctttaacttt
     1621 ctagtgtgaa gggtacaatt gaaaggtaca agaaagcttg ttccgatgcc gttaaccctc
     1681 ctactgtcac tgaagctaat accaaggtac cattcttgta tagttttttt tttactagcc
     1741 ctctcttttt tcttattttt atgatcaatt attaacgttt agaaagtgaa atctttttaa
     1801 aatgtgtata tatatgtggt ttcttgtttc tatgatgatc aattatgtat tcgtgtcaaa
     1861 agaacattac taacaaaatt cttaacattt acacccaaaa gtaaaaacat tattaacaaa
     1921 aagagtggat tcctgaaatg cattgagacg gttgtatttg tatgcatgga acccttcagc
     1981 actatcagca agaagcctct aagcttcgga ggcagattcg ggacattcag aattcgaaca
     2041 ggtaagtaac tatagctctt ctgaggtttc ttgttttgat cactactttc ctattatata
     2101 gctgatcatt tcgattagtt taactgaaaa aattacagaa cctgagtcac gtaagttata
     2161 attcattcaa aatcgttcat tccaaataat tttttttctt ttttggtagg attgttaggt
     2221 tggttaactt acttggaatt gcttgaatct ctgcttggtt ttgtgatata tggtatatgg
     2281 aaccataaat aaaaacttgg gtttaatttt cgtgtttttt tgccaaatag tttactttta
     2341 gttacgtttg aacgagtgca aatgtttatt aatgttcatg tttatgaatt gaaggcatat
     2401 tgttggagaa tcacttggtt cattgaactt caaggaactc aaaaacctag aaggacggct
     2461 tgaaaaagga atcagccgcg tccgatccaa gaaggtacgt actgataaac ctatacgtct
     2521 atgtctctct atagtttata tatagtttcc tcgctcttat atgaatcttt tccagagtga
     2581 acttttagtg gcagagatag agtatatgca gaagagggta agtaacgttt cttcccaatc
     2641 tttcatcgtt cttttacatg ggttttgagt tttgccataa accatgtagg aaatggagtt
     2701 gcagcacgtt aacatgtacc taagagctaa ggttagtcac gtcttcatcc tctaaccgag
     2761 ataatgaacg tgtatcacaa ccaaactttg atgttcggtt tgtgcagata gaacaaggcg
     2821 cgagattgaa tccggaacaa catggatccg gtgtaataca agggacggcg gtttatgagt
     2881 ccggtctgtc ttcttctcat gatcagtcgc agcattataa ccggaattat attccggtta
     2941 accttcttga accgaatcaa caattctccg gtcaagacca acctcctctt caacttgttt
     3001 aagcttaatc atgattaaaa cttctttctc cccctccccc ctccaaacgt ttttcagaga
     3061 gagacaaaga gtaaattaca tttatgcgac attcttattc atagttaagg ttccaataat
     3121 gataaaaaca aaaatcttgt tcctattaca aaaataaaac ttacaaacat ttattatgtc
     3181 atgaatatta tgatatgtag ctataattaa acaataatac attattttag taagctactt
```

```
3241 gggataataa tgaaaaaaca agctacatat tcataaaatc tagattactc ttcagtatat
3301 agacatgaga gaaccatgaa gcagaggctg aaggctaaac cttcttccct ccgttctctc
3361 atacagattt gctaactttt tcttttatct tcttttcatc agttcatgat tttacatttt
3421 ctaacgaaga tgtttgatga atttacgtat ctataccagt aagatcttac cttagggtta
3481 ctaatctcta gccacggttt tgctggaccg ctaaaatgta gaaccgcagc agatttcaga
3541 atctcttca
//
```

A.3 *BnSHP1* cDNA (#AY036062)

```
LOCUS       AY036062      789 bp    mRNA            PLN       18-JUN-2001
DEFINITION  Brassica napus SHATTERPROOF1 (BnSHP1) mRNA, complete cds.
ACCESSION   AY036062
VERSION     AY036062
KEYWORDS    .
SOURCE      rape.
  ORGANISM  Brassica napus
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots;
            Rosidae; eurosids II; Brassicales; Brassicaceae; Brassica.
REFERENCE   1  (bases 1 to 789)
  AUTHORS   Pylatuik,J.D., Davis,A.R. and Bonham-Smith,P.C.
  TITLE     Isolation of the coding region of BnSHP by RT-PCR
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 789)
  AUTHORS   Pylatuik,J.D., Davis,A.R. and Bonham-Smith,P.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-MAY-2001) Biology, University of Saskatchewan, 112
            Science Place, Saskatoon, SK S7N 5E2, Canada
FEATURES             Location/Qualifiers
     source          1..789
                     /organism="Brassica napus"
                     /db_xref="taxon:3708"
     gene            1..789
                     /gene="BnSHP1"
                     /note="genomic sequence found in GenBank Accession Number
                     AF226865"
     5'UTR           1..39
                     /gene="BnSHP1"
     CDS             40..789
                     /gene="BnSHP1"
                     /note="putative transcription factor"
                     /codon_start=1
                     /product="SHATTERPROOF1"
                     /protein_id="AAK62033"
                     /translation="MDEGGSSHDAESSKKIGRGKIEIKRIENTTNRQVTFCKRRNGLL
                     KKAYELSVLCDAEVALVIFSTRGLLYEYASNSVKGTIERYKKACSDAVNPPTVTEANT
                     KHYQQEASKLRRQIRDIQNSNRHIVGESLGSLNFKELKNLEGRLEKGISRVRSKKSEL
                     LVAEIEYMQKREMELQHVNMYLRAKIEQGARLNPEQHGSGVIQGTAVYESGLSSSHDQ
                     SQHYNRNYIPVNLLEPNQQFSGQDQPPLQLV"
BASE COUNT      245 a    159 c    193 g    192 t
ORIGIN
        1 tcggtggttt attcatttgg tgacgatatc acagaagcaa tggatgaagg tgggagtagt
       61 cacgatgcag agagtagcaa gaagataggt agagggaaga tagagataaa gaggatagag
      121 aacacaacaa atcgtcaagt aaccttctgc aaacgacgca atggtcttct caagaaagct
      181 tatgagctct ctgtcttgtg tgatgctgaa gttgccctcg ttatcttctc cactcgtggc
      241 cttctttatg agtacgccag caacagtgtg aagggtacaa ttgaaaggta caagaaagct
      301 tgttccgatg ccgttaaccc tcctactgtc actgaagcta ataccaagca ctatcagcaa
      361 gaagcctcta agcttcggag gcagattcgg gacattcaga attcgaacag gcatattgtt
      421 ggagaatcac ttggttcatt gaacttcaag gaactcaaaa acctagaagg acggcttgaa
      481 aaaggaatca gccgcgtccg atccaagaag agtgaacttt tagtggcaga gatagagtat
      541 atgcagaaga gggaaatgga gttgcagcac gttaacatgt acctaagagc taagatagaa
      601 caaggcgcga gattgaatcc ggaacagcat ggatccggtg taatacaagg gacggcggtt
      661 tatgagtccg gtctgtcttc ttctcatgat cagtcgcagc attataaccg gaattatatt
      721 ccggttaacc ttcttgaacc gaatcaacaa ttctccggtc aagaccaacc tcctcttcaa
      781 cttgtttaa
//
```

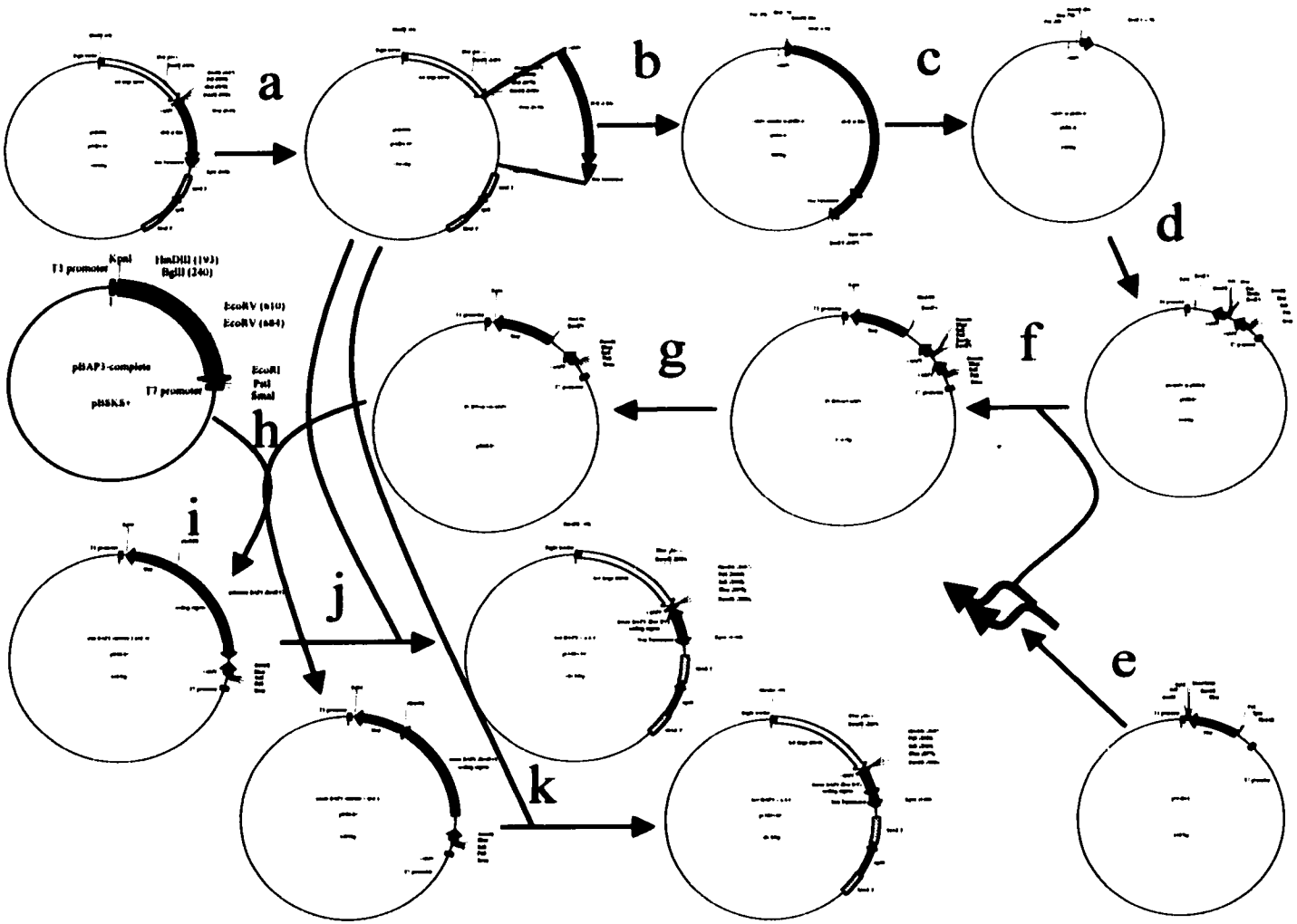
A.4 *BnAGL6* genomic sequence (#AY036063)

```
LOCUS       AY036063     2733 bp    DNA             PLN       27-JUN-2001
DEFINITION  Brassica napus genomic sequence.
ACCESSION   AY036063
VERSION     AY036063
KEYWORDS    .
SOURCE      rape.
  ORGANISM  Brassica napus
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots;
            Rosidae; eurosids II; Brassicales; Brassicaceae; Brassica.
REFERENCE   1  (bases 1 to 2733)
  AUTHORS   Pylatuik,J.D., Davis,A.R. and Bonham-Smith,P.C.
  TITLE     Partial gene sequence with identity to AGL6
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2733)
  AUTHORS   Pylatuik,J.D., Davis,A.R. and Bonham-Smith,P.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-MAY-2001) Biology, University of Saskatchewan, 112
            Science Place, Saskatoon, SK S7N 5E2, Canada
FEATURES             Location/Qualifiers
     source          1..2733
                     /organism="Brassica napus"
                     /db_xref="taxon:3708"
     misc_feature    1..2733
                     /note="similar to Arabidopsis thaliana AGL6 gene"
BASE COUNT      910 a    473 c    534 g    916 t
ORIGIN
        1 agctcagtat caagaaaaag gagagagaaa tatgggaaga gggagagtgg agatgaagct
       61 aatagagaac aaaatcaata gacaagtgac cttctcaaaa ggaagaaacg gtttgatgaa
      121 gaacagctta ttaagctttc tgttctatgc gatgctgaag ttgctctcat cgtctttctt
      181 agccagcgga aagctctacg agttcggcag tgtcgggtat ctttgtgctc cttcgtagct
      241 cttaataata ttcaataaag ttaggtttct tagagcatgt ttattagtga aacccattag
      301 tgtctcttag gtaggttgta atgatttttt aattattaaa attatgataa gagactttgc
      361 taagagacaa ttaattatag gggtttattg gtggtatctt aattaaggtt cttaacaata
      421 aaaaaagatt aaatttattt ttaaacataa tcacatttta gttgttcatg cgcttgtcta
      481 tcacgaattc ttgttcgaac acccatctgg ttggcgatat tagtagggat atctgtagaa
      541 taggtgagaa caacatgtga acttccgctc ccttctcctt gttggaaatc tgagacatca
      601 tattgagtgt attcatctcg ttcgtcttca actatcatat tatggagtat gatgcatgct
      661 ctcataatct ttccaatctt gactttagcc caacaaagtg ccggattttt aacaatggca
      721 aatcgagctt gcaaaactcc gaaagcccgc tcgacatctt tttggacagc ttcttgacgt
      781 tgcgcaaata aaactacttt aggcccttgt ggaattaaaa tggattggat aaaagttgcc
      841 cattttggat aaataccatc ggtgagatag taagccaaat gatactctct tccgttgacc
      901 gagaaattca cttgcggggc tcgaccattt attatgtcat caaaaaccgg tgagcgatca
      961 agaacattaa tatcatttag aggtacctgg aggtcccaaa tctaaaaggg aaaaaccaga
     1021 tctgacgaag cccttcagtc ctaaacgccg accctcttca ccgtcgtcac gccgtgagtc
     1081 tcgctgttgc tccatggaaa gccgtgacct tctccatcga aacgaaaaca ggtaacgttt
     1141 tgtcagccac agaacaccga gcatctcgac aaaggattga gcaaaggtga agagatcaaa
     1201 agaaaaaaag atcaggaaaa ggaatggagc ctccggcacc gacaaaaaat gccggaccgg
     1261 agctagattt attttcagaa gcggtggcga gttaagagaa aaaaggggct agggtttttt
     1321 tgacaaagga tggagcgtct caaacacgga tattagatta ttcaccatat ctttgacaaa
     1381 aaaaaagaag attattcacc atattaatct aacaacaaat atattaacaa ggatttaaat
     1441 ttgatgtcaa aaaacaagag tttacataaa tataaaacat acatttcgtg tacgatacac
     1501 attattcctt tgatctacaa aaaaaatagc gatttttgag gagagaggag ctcagtatca
     1561 agaaaaagga gagagaaata tgggaagagg gagagtggag atgaagctaa tagagaacaa
     1621 aatcaataga caagtgacct tctcaaaagg aagaaacagt ttgatgaaga aagcttatga
     1681 gcttttctgtt ctatgcgatg ctgaagttgc tctcatcgtc ttctctagcc gcggaaaact
     1741 ctacgagttc ggcagtgtcg ggtatctttg tgctccttcg gagctcttaa taatattcaa
     1801 taaagttagg tttcttagag catgtttatt agtgaaaccc attagtgtct tttaggtagg
     1861 ttgtaatgat tttttaatta ttaaaattat gataagagac tttgctaaga gacaattaat
```

1921 tataggggtt tattggtggt atcttaatta aggttcttaa caataaaaaa aaaattattt
1981 ttaaacataa aattttaaaa gattaaattt atttattaaa taaaacaaag tcaaacttaa
2041 catttaaac atatatttta taatagaaac ataaaaacat aaaaataaat attacggaaa
2101 taaacgataa taatttcaag cggcatcaaa gctaagttgt tgtcttgatc acttccgaat
2161 ttacgccata tatgttcaac caaatcactt tttagttgtt catgcgcttg tctatcacga
2221 attcttgttc gaacacccat ctggttggcg atattagtag ggatatctgt agaataggtg
2281 agaacaacat gtgaacttcc gctcccttct ccttgttaga aatctaagac atcatattga
2341 gtgtattcat ctcgttcgtc ttctactatc atataatgga gtatgatgca tgctctcata
2401 atctttccaa tcttgacttt atcccaacaa agtgtcggat ttttaacaag gcaaatcgag
2461 cttgcaaaac tccgaaagcc cgctcgacat ttttttggac agcttcttga cgttgcgcaa
2521 ataaaactgc tttcggccct tgtggaattg aaatggattg gataaaagtt gcccattttg
2581 gattaatacc atcggtgaga tagtaagcca aatgatactc tcttccgttg accgaaaaat
2641 ttacttgcgg ggctcgacca tttattatgt catcaaaagc cggtgagcga tcaagaacat
2701 taatatcatt taaggtacct ggaggtccac aaa

APPENDIX B: *AG::BnAP3* AND *AG::antiBnAP3* CASSETTE CONSTRUCTION

Figure B.1 Cloning strategy used to generate the AG+BAP3 and AG-BAP3 constructs. (a) The pMD992 construct was digested with *PstI*/*KpnI* to yield a CaMV *35S* minimal promoter:*GUS:NOS* terminator cassette which was then ligated (b) into the pMECA vector. The *GUS:NOS* portion of the cassette was removed by digestion with *NcoI* and *KpnI* (c), followed by subsequent end-filling and ligating to yield the isolated CaMV 35S minimal promoter. The *35S* minimal promoter was ligated into pBSKS+ at the *PstI*/*Eco*RV sites (d), accidentally yielding a tandem repeat of the promoter. The *NOS* terminator, amplified by PCR with *KpnI*/*Hin*DIII sites (e), was ligated downstream of the promoters (f). One of the CaMV *35S* minimal promoters was then removed by digestion with *Eco*RV (g), and the *BnAP3* open reading frame, isolated by PCR with *Pfu* polymerase (h), was cloned in both the sense and antisense orientations (i). The new *35S* minimal promoter:*BnAP3:NOS* terminator and *35S* minimal promoter:*antiBnAP3:NOS* terminator constructs were ligated back into the pMD992 vector at the *KpnI*/*PstI* sites to yield the AG+BAP3 (j) and AG-BAP3 (k) constructs. All constructs were confirmed by sequencing.


a
b
c
d
e
f
g
h
i
j
k
T3 promoter
KpnI
HindIII (193)
BglII (240)
EcoRV (610)
EcoRV (684)
pBAP3-complete
T7 promoter
pBSKS+
EcoRI
PstI
SmaI

APPENDIX C: *AG::antiBnAP3* RESULTS IN *B. NAPUS*

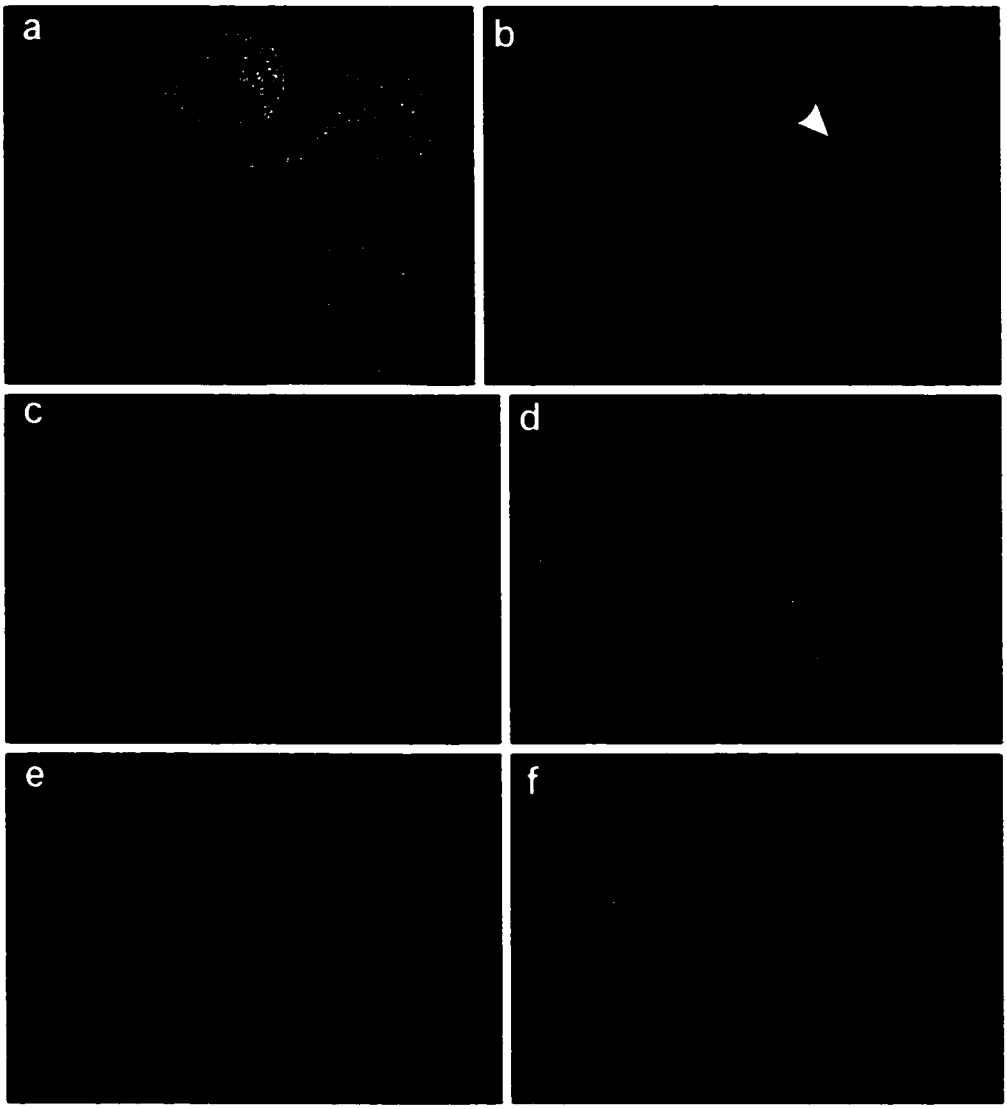
C.1 Summary of the *AG::antiBnAP3* line in *B. napus*

In this experiment, I hypothesized that by expressing an antisense construct of *BnAP3* under the regulation of the *AGAMOUS* (*AG*) promoter, endogenous levels of *BnAP3* could be reduced or eliminated within whorls 3 and 4 of *B. napus*. The predicted outcome of this experiment would be the conversion of stamens in whorl 3, to carpels, with little or no effect on the organs in whorls 1, 2 and 4.

B. napus was transformed by the method described in Section 3.4.2. with the pAG-BAP3 construct (antisense *BAP3* driven by the *AG* promoter) illustrated in Appendix B. Nineteen plants were identified that were resistant to kanamycin. From these 19 T_0 plants, 12 were confirmed to have inserts as indicated by a PCR specific for the *NEOMYCINPHOSPHOTRANSFERASE* (*NPTII*) open reading frame, as described in Section 3.4.2. The T_0 plants did not show any unusual phenotypes and it was speculated that homozygosity would be required for the transgene to produce novel phenotypes. Sixteen T_1 generation plants were grown from seed from each of the 12 T_0 lines for a total of 192 plants. From each of these plants, tissue was collected, DNA extracted, and Southern analysis performed as described in Section 4.4.4 to determine the transgene insertion events that occurred for the *AG::antiBAP3* construct in each plant. For each plant, the phenotype was assessed 3 times per week for two weeks. The most common phenotypic abnormalities, shown in figure C.1, included decreased stamen (a), petal, or sepal number, abnormal or missing gynoecia (b), and chimeric floral organs (c-f). However, there was no correlation found between these phenotypic abnormalities and transgene insertion events (data not shown).

Although the observation of reduced stamen number would appear to conform to the predicted outcome of this experiment, many of the phenotypic abnormalities such as organ chimeras and reduced gynoecia do not. In a similar manner to that discussed in Section 4.3.2, the minimal effects seen in *AG::antiBnAP3* plants could be attributed to a low level of *antiBnAP3* transcripts compared to the large allotetraploid genome of *B. napus*. However, the aforementioned observations do not corroborate the ABC model of flower development described in Section 3.1 for *Arabidopsis*. Further work is required before any conclusions can be drawn from this experiment.

Figure C.1 Flowers of *AG::antiBnAP3 B. napus* plants. Out of the 192 *AG::antiBAP3* transgenic plants examined, 662 flowers (approximately 3% of total) showed abnormalities. 490 (74%) of the abnormal flowers were missing stamens, similar to the flower shown in (a). In these 490 flowers, 53% of the missing stamens were from the median positions, 12% from the lateral positions and in 35% of the cases, it could not be determined from which position the stamens were missing. (b) 152 (23%) of the aberrant flowers were missing a gynoecium, while another 118 (18%) had the wild type gynoecium (left) replaced with an abnormal or filamentous structure (right) that was often tipped with stigmatic papillae (arrowhead). (c-f) Wild-type organs are shown on the left and right of each photograph while aberrant organs chimeric for each of the two wild-type organs are shown in the centre. (c) In 26 flowers, stamen-carpel chimeras were identified and (d) in 21 flowers, stamen-petal chimeras were identified. More rarely, stamen-sepal (e) and sepal-petal (f) chimeras were found. Additionally, one to two missing sepals were observed in 98 flowers (15%) and one or more missing petals were observed in 275 flowers (42%) (photographs not shown). Often, a single flower would contain more than one type of abnormality.


a
b
c
d
e
f