# Molekulargenetische und bestäubungsbiologische Untersuchungen der natürlich bestandsbildenden floralen homöotischen *Spe*-Variante des Hirtentäschels (*Capsella bursa-pastoris*)

Dissertation
zur Erlangung des akademischen Grades doctor rerum naturalium
(Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät
der Friedrich-Schiller-Universität Jena

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Geboren am 14. Februar 1979 in Pasewalk

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Meiner Familie

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# 1. Einleitung

## 1.1. Homöosis und ihre entwicklungs- bzw. evolutionsbiologische Bedeutung

Die Entwicklung höherer Organismen unterliegt Steuerungsprozessen, die auf molekularer Ebene in der modernen Entwicklungsbiologie erforscht werden. Innerhalb der Entwicklungsprozesse von der Zygote bis zum komplex aufgebauten höheren Lebewesen werden sowohl morphologische als auch physiologische Differenzierungsprozesse durchlaufen. Dabei werden aus den Stamm- bzw. Meristemzellen verschiedene Gewebe und nachfolgend spezialisierte Gewebe und Organe mit bestimmten Funktionen gebildet, deren Entwicklung auf molekulargenetischer Ebene durch vielfältige Mechanismen reguliert wird. Die charakteristische Ausbildung der Organe an festgelegten Positionen im Organismus folgt einem definierten Bauplan, der genetisch fixiert ist. Aus der Evolution von Entwicklungsprozessen ergeben sich komplexe und diverse Strukturen und ihre Weiterentwicklung (Gilbert *et al.* 1996). Sogenannte Entwicklungskontrollgene steuern diese Prozesse. Sie sind in komplexen Netzwerken hierarchisch organisiert, wobei Gene höherer Ebene nachgeschaltete regulieren (Theißen & Saedler 1995). Durch Veränderungen in nur einem dieser Kontrollgene kann das Zusammenspiel innerhalb diese Netzwerkes so drastisch geändert sein, dass es zu schwerwiegenden Folgen in den Entwicklungsprozessen kommt, die sich später in einer abgewandelten Morphologie eines Individuums äußert (Theißen & Saedler 1995, Theißen 2000, Carroll 2001). Organe können an Positionen im Organismus ausgebildet werden, in denen normalerweise andere Organe entstehen. Dieses Phänomen wird Homöosis genannt und wurde 1894 von Bateson beschrieben und als Begriff geprägt (Bateson 1894).

Homöotische Phänotypen zeichnen sich demnach durch eine veränderte Organidentität aus. Ein anschauliches Beispiel aus der Tierwelt stellt die *antennapedia* Mutante von *Drosophila melanogaster* dar, die Beinglieder anstelle von Antennenfühlern am Kopfsegment ausbildet (Lewis 1994). Gene, die die Identität von Organen festlegen, werden homöotische Gene genannt. Sie kodieren für putative Transkriptionsfaktoren, die an spezifische *cis*-regulatorische Elemente innerhalb der DNA binden und dadurch eine Reihe nachgeschalteter Gene aktivieren, die für die Ausbildung des jeweiligen Organs nötig sind. Innerhalb des Tierreiches zeichnen sich homöotische Gene durch ein hochkonserviertes DNA-Element, die sogenannte Homöobox aus und gehören einer gleichnamigen Genfamilie an (Gehring 1992).

Oftmals versuchen Evolutionsbiologen evolutive Neuerungen durch graduelle, kleinschrittige Veränderungen in den Genen von Populationen zu erklären, wobei sich diese Veränderungen oder die Organismen, die sie tragen durch Anpassung an die Umwelt gegen

die natürliche Selektion behaupten. Diese Erklärungen stehen allerdings bis heute zum Teil im Widerspruch zu fossilen Funden. Diese dokumentieren Diskontinuität innerhalb der Makroevolution, wie z. B. das plötzliche Auftauchen komplexer Blütenstrukturen während der Angiospermenevolution (Theißen 2006).

Mutationen, z. B. eine Punktmutation, in homöotischen Genen können zu drastischen phänotypischen Veränderungen führen. Ob der daraus resultierende Organismus einen Ausgangspunkt für eine neuartige Abstammungslinie darstellen könnte, ist abhängig von seiner Fitness, d. h. der Fähigkeit sich im Rahmen der natürlichen Selektion behaupten zu können (Bateman & DiMichele 2002). Homöotische Mutanten bieten somit eine Grundlage, um Makroevolution zu erklären (Haag & True 2001, Bateman & DiMichele 2002, Ronse de Craene 2003).

### 1.2. Natürlich vorkommende florale homöotische Mutanten

Für die beiden Modellpflanzen *Arabidopsis thaliana* und *Antirrhinum majus* sind zahlreiche homöotische Blütenmutanten beschrieben worden (Meyerowitz *et al*. 1989). Allerdings sind diese oft steril und somit in der Natur nicht überlebensfähig. Natürlich vorkommende florale homöotische Mutanten sind bereits aus der Literatur bekannt (Gottschalk 1971, Ronse de Craene 2003, Hintz *et al*. 2006).

Die *bicalyx* Mutante in *Clarkia concinna* (Onagraceae) zeichnet sich durch zusätzliche sepaloide Organe anstelle von Petalen aus (Ford & Gottlieb 1992). *Bicalyx* wurde bisher nur in einer kleinen Population in Kalifornien angetroffen, wo sie zusammen mit wild-typischen Pflanzen existiert. Über den molekularen Mechanismus dieser Mutation ist momentan noch nichts bekannt.

Eine weitere natürlich verbreitete homöotische Blütenmutante ist von Cubas *et al*. 1999 für *Linaria vulgaris* analysiert worden. In diesem Fall werden anstelle zygomorpher Blüten radiärsymmetrische ausgebildet. Molekularbiologische Analysen einer kleinen Population auf einer schwedischen Insel zeigten, dass es sich um eine Epimutation (Veränderung der DNA-Methylierung) im Orthologen des Gens *CYCLOIDEA* (*CYC*) aus *A. majus* handelt (Cubas *et al*. 1999).

In beiden beschriebenen Fällen ist die genetische Veränderung auf ein rezessives Allel an einem einzelnen (oder an mehreren eng gekoppelten) Locus zurückzuführen (Ford & Gottlieb 1992, Cubas *et al*. 1999). Des Weiteren sind die Verbreitungsgebiete der Mutanten stark limitiert. Bislang wurden sie aber als stabil beobachtet. Die *Linaria*-Mutante vermehrt

sich wahrscheinlich vegetative, so dass die Population vermutlich nur einem Klon entspricht (Theißen 2000).

### 1.3. *Spe* – eine floral homöotische Variante von *Capsella bursa-pastoris*

Eine Besonderheit unter den natürlich verbreiteten floralen homöotischen Mutanten stellt eine Variante von *C. bursa-pastoris* dar. Sie wurde schon vor knapp 200 Jahren in der Literatur erwähnt (Opiz 1821, Trattinnick 1821, Wiegmann 1823). Sie unterscheidet sich vom Wildtypen durch zusätzliche funktionsfähige Stamina, die anstelle der Petalen ausgebildet werden. Aufgrund der modifizierten Blütenmorphologie mit zehn anstatt der gewöhnlichen sechs Stamina als Konsequenz homöotisch transformierter Petalen wurde diese Variante als „dekandrisch“ bezeichnet und ging sogar als neue Art *Capsella apetala* in die Literatur ein (Opiz 1821). Das Phänomen der dekandrischen Blüten wurde von Murbeck (1918) als „staminale Pseudapetalie“ bezeichnete und auch für einige andere Blütenpflanzen beschrieben (weitere Beispiele in Ronse de Craene 2003 und Erläuterungen in Hintz *et al*. 2006, Nutt *et al*. 2006). Aufgrund des beschriebenen modifizierten Blütenbaus wird der dekandrische Phänotyp von *C. bursa-pastoris* als ‚Stamenoid petals’ (*Spe*) bezeichnet und der mutmaßliche mutierte Locus entsprechend als *Spe*-Locus.

Einzigartig für diese homöotische Variante von *Capsella* ist ihr bestandsbildendes Vorkommen (teils in Massenbeständen) an verschiedenen europäischen Standorten (Murbeck 1918, Dahlgren 1919, Gottschalk 1971). Auch über den Vererbungsmodus des dekandrischen Phänotyps wurde gelegentlich berichtet. Systematische Kreuzungsexperimente zeigten intermediäre Organe des zweiten Blütenkreises in der $F_1$-Generation und eine annähernde 1:2:1 Aufspaltung der Blütenphänotypen innerhalb der $F_2$-Generation (Dahlgren 1919). Reichert (1998) beschrieb eine weitere Population, die er über mehrere Jahre (seit 1991) in Weinbergen in Gau-Odernheim/Rheinhessen beobachtete. Er bezeichnete die Bestandsgröße als stabil.

### 1.4. *Capsella* im Allgemeinen und *Spe* im Besonderen

*Capsella* ist eine kleine Gattung innerhalb der bedeutenden Familie der *Brassicaceae* und nahe verwandt mit *Arabidopsis*. Es wird geschätzt, dass sich beide Gattungen vor etwa 10 Millionen Jahren phylogenetisch trennten (Acarkan *et al*. 2000, Koch *et al*. 2003, Koch & Kiefer 2005). Die drei Arten der Gattung *Capsella*, *C. grandiflora*, *C. rubella* und *C. bursa-pastoris*, unterscheiden sich grundsätzlich in Verbreitung, Bestäubungssystem und Ploidiegrad (Hurka *et al*. 2005). *C. bursa-pastoris* ist ein- bis zweijährig, tetraploid und bis

auf die Tropen und Subtropen kosmopolitisch verbreitet. Wie schon erwähnt, ist diese Spezies vorwiegend selbstbestäubend, kann aber auch fremd bestäubt werden, welches durch Auskreuzraten von bis zu 20 % in Untersuchungen nachgewiesen werden konnte (Hurka & Neuffer 1997). Zur Gattung *Capsella* gehören außerdem die diploide, selbstinkompatible *C. grandiflora* und die ebenfalls diploide, aber selbstkompatible *C. rubella*. Letztere war ursprünglich im Mittelmeerraum verbreitet, konnte sich aber im Laufe der Zeit weltweit in Regionen mit mediterranem Klima ausbreiten (Hurka *et al*. 2005). Die verwandtschaftlichen Verhältnisse innerhalb der drei Arten sind etwas umstritten. So wurde zunächst angenommen, dass *C. bursa-pastoris* durch Auto- oder Alloploidisierung der anderen Vertreter hervorgegangen ist (Hurka *et al*. 1989, Hurka & Neuffer 1997). Diese Auffassung wurde aber von Slotte *et al*. 2006 in Frage gestellt, die basierend auf cpDNA- und Kerngen-Sequenzanalysen weder *C. grandiflora* noch *C. rubella* als mutmaßlichen Elter bestimmen konnten. Gemeinsamkeiten zwischen den beiden Genomen von *C. rubella* und *C. bursa-pastoris* werden als Folge von Postpolyploidisierung oder Hybridisierungsereignisse und anschließende Introgression diskutiert (Slotte *et al*. 2006, 2008). Neueste Untersuchungen zur Evolution der Selbstkompatibilität innerhalb der Gattung *Capsella* belegen einen sehr jungen Ursprung von *C. rubella* aus *C. grandiflora* (Foxe *et al*. 2009, Guo *et al*. 2009).

Die Kolonisationsfähigkeit von *C. rubella* und *C. bursa-pastoris* beruht zum einen auf dem Verlust der Selbstinkompatibilität und zum anderen auf der menschlichen Ackerbauaktivität (Neuffer & Meyer-Walf 1996, Neuffer & Hurka 1999, Hurka *et al*. 2003, van Kleunen & Johnson 2007, Guo *et al*. 2009). Samendormanz und endogene Rhythmen spielen bei *C. bursa-pastoris* ebenso eine entscheidende Rolle (Neuffer & Hurka 1988, Hurka & Neuffer 1997, Neuffer & Linde 1999). Durch die Polyploidisierung in *C. bursa-pastoris* konnten sich eine stark ausgeprägte Ökotypdifferenzierung und eine größere genetische Variabilität entfalten und ermöglichen somit eine individuelle Anpassung an verschiedene geographische und daraus resultierende klimatische Bedingungen (Hurka & Neuffer 1991, 1997, Ceplitis *et al*. 2005). Die Ökotypen unterscheiden sich hauptsächlich in Wuchsformen, wie Blattmorphologie und Anzahl der Blätter in der Rosette, Verzweigungsmuster und Wuchshöhe, Blühzeitpunkt oder Samenmorphologie (u. a.: Neuffer & Hurka 1986, Neuffer & Bartelheim 1989, Neuffer 1990, Hurka & Neuffer 1997, Linde *et al*. 2001).

Nicht nur das stabile Vorkommen einer floralen homöotischen Mutante in großen natürlichen Populationen über mehrere Jahrzehnte lässt *Capsella* als ein geeignetes Untersuchungsobjekt für die evolutionäre Bedeutung homöotischer Mutationen bzw. Mutanten erscheinen. Die nahe Verwandtschaft zu *A. thaliana* und die damit verbundene

große Ähnlichkeit der Genome ermöglicht eine weitgehende Übertragung vieler in *A. thaliana* etablierter Methoden und Materialien. Chromosomale Anordnung, Orientierung und Gensequenz zeigen Co-Linearität zwischen den Genomen beider Gattungen, wobei eine Sequenzähnlichkeit von > 90 % auf Ebene der Exons orthologer Gene nachgewiesen werden konnte (Acarkan *et al*. 2000, Boivin *et al*. 2004, Koch & Kiefer 2005).

Bei staminaler Pseudapetalie ist die homöotische Transformation auf nur einen Blütenwirtel beschränkt. Zudem bleiben die Reproduktionsorgane sowohl weiblichen als auch männlichen Geschlechts erhalten. Folglich ist nur mit geringen pleiotropen, negativen Effekten in der Reproduktion zu rechnen, obwohl das Fehlen der Petalen möglicherweise Auswirkungen auf die Attraktion von Pollinatoren hat. Dabei scheint sich der Verlust des Systems der Selbstinkompatibilität bei *C. bursa-pastoris* als weiterer Vorteil zu erweisen, da mittels Selbstbefruchtung drastische Rückgänge in der Samenproduktion infolge mangelhafter Bestäubung vermieden werden können.

Die evolutionsbiologische Bedeutung einer homöotischen Mutante ist mit der detaillierten Beschreibung des Phänotyps und den zugrunde liegenden molekularen Mechanismen nicht vollständig verstanden. Inwieweit die Mutante das Potential besitzt, sich im natürlichen Lebensraum zu etablieren und gegenüber anderen Organismen und Umwelteinflüssen zu behaupten, kann mit Analysen zur Fitness und Bestäubungsbiologie unter (semi-) natürlichen Bedingungen im Freiland untersucht werden (Theißen 2000). Veränderungen in der Blütenarchitektur können für die pflanzliche Fitness von weit reichender Bedeutung sein (Darwin 1877, Waser 1983, Stanton 1986). Korrelationen zwischen Blütenmerkmalen, wie z. B. Anzahl, Größe oder Form, und reproduktiver Fitness konnten bereits gezeigt werden (Gomez *et al*. 2006). Diese können durch potentielle Bestäuber beeinflusst sein oder nicht in Beziehung zu Blütenbesuchern stehen (Conner & Rush 1996, Gomez 2000, Gomez *et al*. 2006). Ebenso wurde gezeigt, dass Mutationen in Genen, die für Farbe und Zellform von Petalen codieren, die Anzahl der Früchte und somit die Fitness beeinflussen (Comba *et al*. 2000). Außerdem können blütenmorphologische Veränderungen das Bestäuberspektrum derartig ändern, dass sympatrische Artbildung gefördert wird (Schemske & Bradshaw 1999). Bestäubungsbiologische Untersuchungen an der vorrangig selbstbestäubenden *A. thaliana* zeigten ein diverses Artenspektrum (Thrips, *Diptera*, Wildbienen u. a.) (Hoffmann *et al*. 2003). Ähnliches wird auch von *C. bursa-pastoris* berichtet (Diplomarbeit Borgwart 1987).

## 1.5. Die Rolle homöotischer Gene bei der Blütenorganogenese

Auch innerhalb des Pflanzenreiches konnten homöotische Veränderungen wesentlich zum Verständnis von Entwicklungs- und Evolutionsprozessen beitragen. Dabei nehmen bei Pflanzen die MADS-Box-Gene die Stellung der Homöobox-Gene aus dem Tierreich ein. MADS-Box-Gene kodieren für putative Transkriptionsfaktoren, die an einer Vielzahl von pflanzlichen Entwicklungsprozessen beteiligt sind (Schwarz-Sommer *et al*. 1990, Theißen & Saedler 1995, Theißen *et al*. 2000, Soltis *et al*. 2006). Allein für *A. thaliana* wurden mehr als 100 verschiedene MADS-Box-Gene gefunden (Riechmann *et al*. 2000, Parenicova *et al*. 2003). Sie spielen in den unterschiedlichsten Bereichen der pflanzlichen Entwicklung eine Schlüsselrolle. So kontrollieren sie nicht nur die Ausbildung der Blüte, sondern regulieren ebenso Prozesse in der Frucht-, Blatt-, Wurzel-, Samen- oder Embryoentwicklung (Rounsley *et al*. 1995, Alvarez-Buylla *et al*. 2000). Ähnlich der tierischen Homöobox besitzen sie ein hoch konserviertes Sequenzelement, dessen Name MADS-Box von einigen der ersten identifizierten Vertretern dieser Genfamilie abgeleitet wurde (Schwarz-Sommer *et al*. 1990): *MINICHROMOSOME MAINTENANCE 1* (*MCM1*, Passmore *et al*. 1988) aus *Saccharomyces cerevisiae*, *AGAMOUS* (*AG*, Yanofsky *et al*. 1990) aus *A. thaliana*, *DEFICIENS A* (*DEF A*, Sommer *et al*. 1990) aus *A. majus* und *SERUM RESPONSE FACTOR* (*SRF*, Norman *et al*. 1988) aus Säugetieren. Die meisten MADS-Domänen-Proteine sind modulartig aus charakteristischen Strukturmotiven aufgebaut und zeichnen sich stets durch den Besitz der hoch konservierten MADS-Domäne aus (Shore & Sharrocks 1995, Theißen *et al*. 2000). MADS-Domänen-Proteine, die dem MIKC-Typ entsprechen, besitzen außerdem eine I-Domäne (intervening), eine Keratin ähnliche K-Domäne und eine variable C-Domäne (Münster *et al*. 1997).

Vergleichende Untersuchungen homöotischer Blütenmutanten in den Modellorganismen *A. thaliana* und *A. majus* trugen entscheidend zum Verständnis der genetischen und molekularen Grundlagen der Blütenentwicklung bei und führten zur Formulierung des ABC-Modells (Haughn & Sommerville 1988, Bowman *et al*. 1991b, Coen & Meyerowitz 1991). In diesem Modell werden die Blütenorganidentitätsgene in die Klassen A, B und C eingeteilt, wobei die Gene der einzelnen Klassen bestimmte Funktionen ausüben. Bei *A. thaliana* werden die Funktionen der Klasse A-Gene *APETALA1* (*AP1*) und *APETALA2* (*AP2*) für die Ausbildung der Sepalen im 1. Wirtel benötigt. Kombinierte Aktivität der Klasse A-Gene und der Klasse B-Gene *APETALA3* (*AP3*) und *PISTILLATA* (*PI*) lässt Petalen im 2. Wirtel entstehen. Ebenso wird die Entwicklung der Stamina im 3. Wirtel durch Kombination zweier Klassen, der Klasse B-Gene und des Klasse C-Gens *AGAMOUS* (*AG*)

realisiert. Die Expression des Klasse C-Gens resultiert in der Ausbildung von Karpellen im 4. Blütenkreis. Zudem wirken Klasse A- und C-Gene antagonistisch und die Terminationsfunktion des Klasse C-Gens verhindert das weitere Wachstum des floralen Meristems (Coen & Meyerowitz 1991). Die grundlegenden Mechanismen des ABC-Modells sind universell für verschiedenste Pflanzengruppen anwendbar (Theißen *et al*. 2000, Erbar 2007, Theißen & Melzer 2007, Soltis *et al*. 2007).

Das ABC-Modell stellt nur eine vereinfachte Form zum Verständnis der Blütenentwicklung dar. Nach der Feststellung, dass ABC-Gene zwar nötig, aber nicht ausreichend sind, um Organidentität zu vermitteln, wurde das Modell schrittweise erweitert. So erwiesen sich die *SEPALLATA* (*SEP*) Gene für die Ausbildung aller Blütenorgane als notwendig (Pelaz *et al*. 2000, Ditta *et al*. 2004). Sie werden als Klasse E-Gene bezeichnet. Außerdem wurden bei Studien mit *Petunia hybrida* die Klasse D-Gene gefunden, die bei der Determination von Ovula eine große Rolle spielen (Angenent *et al*. 1995, Colombo *et al*. 1995, 1997). In *A. thaliana* übernehmen diese Aufgabe die Gene *SEEDSTICK* (*STK*) und *SHATTERPROOF1* und *2* (*SHP1*, *SHP2*), wobei letztere beiden auch wesentlich zur Fruchtöffnung beitragen und *STK* in die Entwicklung der Samenanlagen involviert ist (Liljegren *et al*. 2000, Favaro *et al*. 2003, Pinyopich *et al*. 2003). *SHP1*, *SHP2* and *STK* bilden zusammen mit *AG* eine monophyletische Gruppe innerhalb der MADS-Box-Gene (Becker & Theißen 2003, Zahn *et al*. 2006). Außerdem konnten Überlappungen in ihrer Funktion sowie in Expressionsmustern nachgewiesen werden (Favaro *et al*. 2003, Pinyopich *et al*. 2003). Hinsichtlich ihrer spezifischen Funktion unterscheidet man aber teilweise Klasse C-Gene (Stamina- und Karpellentwicklung) und Klasse D-Gene (Entwicklung der Ovula) (Kramer *et al*. 2004). In ihrer Gesamtheit erweitern Klasse D- und E-Gene das ABC-Modell zum ABC(D)E-Modell (Theißen 2001b).

Alle bisher genannten Blütenorganidentitätsgene gehören zur Familie der MADS-box-Gene (Theißen 2001a, Krizek & Fletcher 2005). Die einzige Ausnahme stellt *AP2* dar, das in die Genfamilie der *AP2/EREBP*-Transkriptionsfaktoren einzuordnen ist (Drews *et al*. 1991, Jofuku *et al*. 1994, Okamuro *et al*. 1997).

Die prinzipielle Aufgabe eines Transkriptionsfaktors ist die Regulation von Genexpression. Diese Faktoren binden an kurze *cis*-regulatorische Sequenzabschnitte (Transkriptionsfaktorbindestellen), die sich meist in nicht Protein codierenden Regionen (insbesondere Promotoren) befinden und relativ konserviert sind. Dabei kann die Transkription von Genen sowohl aktiviert als auch reprimiert werden (Wray *et al*. 2003). Mutationen in *cis*-regulatorischen Abschnitten können zu funktionell signifikanten und

ökologisch relevanten Merkmalsveränderungen beitragen, wie es schon für viele Beispiele sowohl im Tier- als auch Pflanzenreich gezeigt wurde (Wray 2007 und darin enthaltene Zitate). Diesen Sequenzveränderungen wird eine größere evolutionsbiologische Relevanz zugesprochen als Mutationen in codierenden Bereichen, da sich veränderte Phänotypen, verursacht durch Mutationen in *cis*-regulatorischen Bereichen, leichter etablieren lassen (Doebley & Lukens 1998, Wray 2007). Der Grund dafür ist, dass Selektion effizienter angreifen kann, da die Ausprägung oftmals co-dominant ist und pleiotrope Effekte reduziert sind (Stern 2000, Wittkopp *et al*. 2004, Wray 2007). Promotoren sind aus mehreren *cis*-regulatorischen Elementen modulartig aufgebaut, so dass Veränderungen in einem dieser Bereiche meist nur einen Entwicklungsschritt beeinflussen. Hingegen bindet ein Transkriptionsfaktor meist an viele *cis*-Elemente in unterschiedlichen Genen, die an verschiedenen Entwicklungsprozessen beteiligt sein können. Mutationen in den codierenden Bereichen dieser Transkriptionsfaktoren sind daher eine wichtige Ursache für pleiotrope Effekte (Doebley & Lukens 1998).

Zahlreiche Studien über pflanzliche Transkriptionfaktoren zeigen, wie umfangreich und komplex sich die Kontrolle von Genexpression darstellt (Riechmann & Ratcliff 2000, Riechmann 2002, de Folter & Angenent 2006). Beispiele für Gene, die im Netzwerk der Regulation in der Blütenentwicklung von *A. thaliana* eine wichtige Rolle spielen, sind das Meristemidentitätsgen *LEAFY* (*LFY*) (Zusammenfassungen in Ng & Yanofsky 2000, Jack 2004) oder das Organidentitätsgen *AG* (u. a. Jack 2002, Gomez-Mena *et al*. 2005, Liu & Karmarkar 2008).

### 1.6. Charakterisierung von Genen und Genfunktionen

Grundsätzlich gibt es zwei verschiedene Wege die Sequenz und Funktion eines bestimmten Gens über Mutantenanalysen miteinander zu verknüpfen: die „Vorwärtsgenetik“ und die „Rückwärtsgenetik“ (Alonso & Ecker 2006). Ausgehend von einer putativen Gensequenz versucht man bei der reversen Genetik durch Mutagenese dieses Gens und anschließender Phänotypbestimmung auf die Genfunktion zu schließen (vom Gen zum Phänotyp) (Reski 1998). Bei der klassischen „Vorwärtsgenetik“ werden Organismen mit verändertem Phänotyp ausgewählt und nachfolgend das für die Merkmalsveränderung verantwortliche Gen gesucht (vom Phänotyp zum Gen). Oftmals nutzen beide Vorgehensweisen Sequenzinformationen aus Genom-Datenbanken, die von einigen Pflanzen vorliegen, z. B. *A. thaliana*, *Oryza sativa*, *Zea mays*, *etc*. (Alonso & Ecker 2006).

Eine Methode der „Vorwärtsgenetik“ ist die Genkartierung oder Kopplungsanalyse. Die erste genetische Karte zeigte die relative Lage von fünf Merkmalen zueinander auf dem Y-Chromosom von *D. melanogaster* (Sturtevant 1913). Die Merkmale stehen für Gene und werden als Marker bezeichnet. Genetische Karten werden durch Stammbaumanalysen und Kreuzungsexperimente erstellt. Heute sind außer bestimmten Genen verschiedene Sequenzmerkmale als Marker verfügbar (eine Übersicht bei Peters *et al*. 2003). Aufgrund von Sequenzinformationen aus den Genomenprojekten und der vielen Markersysteme kann die Position einer Sequenz präzise bestimmt werden. Das Resultat ist die physikalische Karte.

Für die Charakterisierung eines Gens oder dessen Funktion gibt es zum einen den Ansatz der kartierungsgestützten Klonierung (*map based cloning, positional cloning*) mit dem Ziel, den mutanten Phänotyp auf einer physikalischen Karte zu lokalisieren und anhand der Position das für den Phänotyp verantwortliche Gene zu bestimmen (Peters *et al*. 2003). Zum anderen gibt es den Kandidatengenansatz (*candidate gene approach*), der vorhandene Informationen aus bereits durchgeführten Analysen nutzt, die einen ähnlichen Phänotyp beschreiben (Haag & True 2001). Beide Ansätze setzen das Vorhandensein einer für das untersuchte Merkmal segregierende $F_2$-Population oder andere Kreuzungsversuche (z. B. $F_3$-Population, Rückkreuzung, rekombinante Inzuchtlinie, *etc*.) voraus, um Merkmal und Marker miteinander zu verknüpfen.

## 1.7. Zielsetzung

Hauptaugenmerk dieser Arbeit ist die Charakterisierung des floral homöotischen *Spe*-Phänotyps von *C. bursa-pastoris* sowohl unter molekulargenetischem als auch bestäubungsbiologischem bzw. reproduktivem Gesichtspunkt. Zur Aufklärung molekulargenetischer Zusammenhänge in der Blütenentwicklung haben bisher hauptsächlich Untersuchungen an Mutanten der Modellorganismen *A. thaliana* und *A. majus* beigetragen, die im Labor beispielsweise durch Mutagenese erzeugt wurden. Natürlich vorkommende floral homöotische Mutanten wie die *Spe*-Variante könnten ein geeignetes System darstellen, um die evolutionsbiologische Bedeutung morphologischer Neuheiten, sich in der Natur zu etablieren, zu untersuchen. Deshalb wurden wildtypische und *Spe*-phänotypische *C. bursa-pastoris* Pflanzen als Untersuchungsobjekt in dieser Arbeit ausgewählt.

In dieser Arbeit stand zum einen die molekulargenetische Charakterisierung der floral homöotischen Variante *Spe* von *C. bursa-pastoris* im Mittelpunkt. Dabei sollte der für den *Spe*-Phänotyp verantwortliche Locus durch Co-Segregationsanalysen mit Kandidatengenen identifiziert werden.

Ein weiterer Aspekt war die Frage nach den Auswirkungen drastischer morphologischer Veränderungen, wie die Umwandlung von Petalen in Stamina bei der *Spe*-Variante, auf die Etablierung dieser homöotischen Mutante in der Natur und die Co-Existenz mit dem Wildtyp. Es sollte herausgefunden werden, ob das Artenspektrum und die Besuchshäufigkeit potentieller Bestäuber sowie die Produktion floraler Duftstoffe verändert sind, wenn die Petalen, die oftmals als Lockmittel dienen, durch Stamina ersetzt wurden. Außerdem sollte anhand reproduktiver Merkmale, z. B. Frucht- und Samenanzahl, Keimrate, die individuelle Fitness beider Phänotypen untersucht und miteinander verglichen werden.

## 2. Übersicht zu den Manuskripten

**I** **P. Nutt, J. Ziermann, M. Hintz, B. Neuffer, and G. Theißen (2006): *Capsella* as a model system to study the evolutionary relevance of floral homeotic mutants. *Plant Systematics and Evolution* 259: 217-235.**

Diese Publikation ist ein Übersichtsartikel, in dem *Capsella* als Untersuchungsobjekt aus taxonomischer, ökologischer und genetischer Sicht vorgestellt wird. Es wird die Bedeutung floral homöotischer Mutanten in der Evolution neuer Blütenstrukturen am Beispiel der natürlich bestandsbildenden floralen homöotischen Mutante „*Stamenoid petals*“ (*Spe*) von *C. bursa-pastoris* umfangreich diskutiert und beschrieben, inwieweit diese experimentell analysiert werden kann.

Ich habe Abschnitte des Kapitels „Studying *Spe*“ des Manuskripts verfasst, sowie Abbildung 4 zur Verfügung gestellt. P. Nutt hatte den größten Anteil an der Arbeit. M. Fräger (geb. Hintz) und B. Neuffer korrigierten und ergänzten die Kapitel zur Bedeutung von Homöosis in der Evolution und der Phylogenie von *Capsella*. G. Theißen gab die Idee zu dieser Publikation und korrigierte, vervollständigte und verbesserte das Manuskript in seiner Gesamtheit.

Hiermit bestätige ich den Arbeitsanteil der beteiligten Autoren.

Günter Theißen

**II P. Nutt[1], J. Ziermann[1] and G. Theißen (eingereicht bei *The Plant Cell* am 7. Mai 2008): Ectopic expression and co-segregation of an *AGAMOUS* orthologue in *Stamenoid petals*, a natural homeotic floral variant of *Capsella bursa-pastoris.* ([1] These authors contributed equally to this work)**

Ziel dieser Arbeit war die Charakterisierung der *Spe*-Variante von *C. bursa-pastoris* mit Hilfe morphologischer, genetischer und molekularbiologische Untersuchungen. Neben einer vollständigen und detaillierten Beschreibung der Blütenmorphologie wurde insbesondere der Frage nachgegangen, welche Ursache dem *Spe*-Phänotyp zugrunde liegt, d. h. welcher Locus bzw. Loci und welche molekularen Mechanismen für die Ausprägung verantwortlich sind.

Die Isolierung der genomischen DNA und die Identifizierung der SNPs in den Kandidatengenen wurden von mir durchgeführt. Außerdem habe ich eine segregierende $F_2$ Population erzeugt und diese auf Co-Segregation der SNPs mittels Pyrosequenzierung analysiert und eine Mutation in *CbpAGa* detektiert. Dazugehörige Kapitel, sowie Abb. 6 und Abb. 4 (Suppl.) wurden von mir erstellt. P. Nutt führte die morphologischen und genetischen Untersuchungen durch. Isolierung der cDNA, phylogenetische Rekonstruktion, sowie *Southern* und *in-situ* Hybridisierung der Kandidatengene wurden ebenso von ihr durchgeführt. Sie erstellte die dazugehörigen Kapitel und Abbildungen. Gemeinsam verfassten wir eine vorläufige Diskussion. G. Theißen schrieb die Einleitung und korrigierte, vervollständigte und verbesserte das Manuskript in seiner Gesamtheit.

Hiermit bestätige ich den Arbeitsanteil der beteiligten Autoren.

Günter Theißen

**III** **J. Ziermann, M. Ritz, S. Hameister, C. Abel, M. H. Hoffmann, B. Neuffer and G. Theißen (2009): Floral visitation and reproductive traits of *Stamenoid petals*, a naturally occurring floral homeotic variant of *Capsella bursa-pastoris* (Brassicaeae). *Planta* 230: 1239-1249.**

In dieser Arbeit wird untersucht, ob drastische morphologische Veränderungen, wie die Umwandlung von Petalen in Stamina in der *Spe*-Variante von *C. bursa-pastoris*, bestäubungsbiologische und reproduktive Konsequenzen für diese Pflanzen haben. Dabei soll diskutiert werden, wie sich die floral homöotische *Spe*-Variante in der Natur etablieren und gegenüber dem Wildtyp behaupten kann.

Alle Daten zu den Blütenbesuchern im Botanischen Garten Jena (Arten, Häufigkeit), sowie Daten zu den reproduktiven Merkmalen (Anzahl Blüten, Früchte, Samen) wurden von mir aufgenommen. Ebenso habe ich die Keimversuche durchgeführt und das vorläufige Manuskript verfasst. M. H. Hoffmann hat Blütenbesucher in Halle beobachtet. Blütenduftstoffe wurden von C. Abel analysiert. M. Ritz hat die statistischen Analysen durchgeführt und wesentlich zum Manuskript beigetragen. S. Hameister lieferte Daten vom natürlichen Standort sowie Verbesserungsvorschläge zusammen mit B. Neuffer. G. Theißen korrigierte, ergänzte und verbesserte das Manuskript.

Hiermit bestätige ich den Arbeitsanteil der beteiligten Autoren.

Günter Theißen

## 3. Manuskript I:

P. Nutt, J. Ziermann, M. Hintz, B. Neuffer, and G. Theißen (2006): *Capsella* as a model system to study the evolutionary relevance of floral homeotic mutants. *Plant Systematics and Evolution* 259: 217-235.

Pl. Syst. Evol. 259: 217–235 (2006)
DOI 10.1007/s00606-006-0420-2

**Plant Systematics and Evolution**
Printed in Austria

# *Capsella* as a model system to study the evolutionary relevance of floral homeotic mutants

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Received January 6, 2006; accepted January 9, 2006
Published online: June 19, 2006


**Abstract.** Several lines of evidence suggest that homeotic changes played a considerable role during the evolution of flowers. This, however, is difficult to reconcile with the predominant evolutionary theory which rejects any drastic, saltational change of the phenotype as reasonable mode of evolution due to its assumed negative impact on the fitness of the affected organism. A better understanding of the evolutionary potential of homeotic transitions requires a study of the performance of respective mutant varieties in the wild. Here we introduce "*Stamenoid petals*" (Spe), a variety of *Capsella bursa-pastoris* (shepherd's purse), as a suitable model to study the evolutionary potential of floral homeotic mutants. In the flowers of the *Spe* variety all petals are transformed into stamens, while all other floral organs are unaffected. In contrast to most other homeotic mutants the *Spe* variety occurs on several locations in relatively large and stable populations in the wild. Due to its close relationship to the model plant *Arabidopsis thaliana*, the *Spe* variety of *C. bursa-pastoris* can be rigorously studied, from the molecular genetic basis of the phenotype to its consequences on the fitness in wild habitats. Investigations on *Spe* may thus help to clarify whether homeotic transformations have the potential to contribute to macroevolution.

**Key words:** ABC model, *Capsella bursa-pastoris*, flower development, homeosis, macroevolution, MADS-box genes, stamenoid petals.

## *Capsella* as a model system to study the evolution of flower development

*Capsella* is a small genus within the mustard family (Brassicaceae). According to Hurka and co-workers it comprises only three species, which, however, show remarkable differences in ploidy level, breeding systems and habitat range (Hurka and Neuffer 1997, Zunk et al. 1999, Hurka et al. 2005). Two of the species, *Capsella grandiflora* and *Capsella rubella*, are diploid, while the third one, the well-known weed *Capsella bursa-pastoris* (shepherd's purse) is tetraploid. *C. grandiflora* is obligately outbreeding due to a sporophytic self-incompatibility (SI) system and grows in a very limited habitat in western Greece, Albania and northern Italy. Compared to the other two species it has relatively large, fragrant flowers with showy petals, obviously to attract pollinators. In contrast, *C. rubella* is a completely selfing plant with comparatively small flowers

that grows around the Mediterranean Sea (Hurka et al. 1989). The predominantly selfing *C. bursa-pastoris* is distributed in ruderal habitats all over the world, except in the tropics and subtropics (Hurka and Neuffer 1997). In fact, together with *Polygonum aviculare*, *Stellaria media*, *Poa annua* and *Chenopodium album*, *C. bursa-pastoris* belongs to the most wide-spread flowering plants on our planet (Coquillat 1951, Hurka et al. 2003).

The relationships between the three *Capsella* species have not been resolved yet. It has been suggested that *C. bursa-pastoris* originated by autopolyploidy within *C. grandiflora*, or by allopolyploidy between *C. rubella* and an unknown ancestor (Hurka and Neuffer 1997; Slotte et al., unpubl. data). Some recent molecular data suggest that *C. grandiflora* and *C. rubella* are sister species to the exclusion of *C. bursa-pastoris* (Slotte et al., unpubl. data), suggesting that autopolyploidy in a diploid common ancestor of all three species gave rise to the tetraploid *C. bursa-pastoris* lineage.

Anyway, comparison with outgroup species strongly suggests that concerning reproductive biology *C. grandiflora* represents mostly ancestral and *C. bursa-pastoris* mostly derived character states. For example, the self-compatible (SC) breeding system of *C. rubella* and *C. bursa-pastoris* was evidently caused by breakdown of the self-incompatibility (SI) system still active in *C. grandiflora* (Hurka and Neuffer 1997, Hurka et al. 2005). By enabling reproduction via selfing and thus reproductive assurance under circumstances of poor outcrossing possibilities when mates (or pollinators) are scarce (Shimizu et al. 2004, Shimizu and Purugganan 2005, and references cited therein), this has quite certainly contributed to the colonisation potential of *C. rubella* and *C. bursa-pastoris*. The latter species may have further boosted its colonisation potential and weediness due to polyploidisation followed by a shift to disomic inheritance, as revealed e.g. by the inheritance of allozymes (Hurka et al. 1989, Hurka and Düring 1994), which led to 'fixed heterozygosity' and thus increased the intraspecific genetic diversity. This may have helped to avoid inbreeding depression when outcrossing is rare; outcrossing rates in the field of 0–20 % have been determined and may strongly depend on environmental conditions such as the weather (Hurka and Neuffer 1997). While self-incompatible species may use large petals and floral scent to attract pollinator insects, pollinator attraction may have become less important in the species with SC breeding system, which may explain the reduction of floral size and the loss of floral fragancy in both *C. rubella* and *C. bursa-pastoris*.

Within the small genus *Capsella* we can thus follow up the transition from a diploid, self-incompatible, obligatory outcrossing species with comparatively large and attractive flowers but a quite restricted area of distribution (*C. grandiflora*), to a tetraploid, self-compatible, predominantly selfing species with relatively small flowers but a breathtaking colonisation success almost all around the globe (*C. bursa-pastoris*) (Paetsch et al., unpubl. data). The dramatic difference in 'invasiveness' between *C. grandiflora* and *C. bursa-pastoris* is quite remarkable, and needless to say that the how's and why's of this difference are of great evolutionary and agricultural interest (Hurka et al. 2003).

However, most aspects of the evolutionary transition within *Capsella* are also found in other plant groups, including close relatives of *Capsella*. For example, the shift from outcrossing to self-pollination is one of the most prevalent evolutionary transitions in flowering plants (Stebbins 1950, Barrett 2002, Mable 2005).

Changes from self-incompatible, outcrossing species with complete and conspicuous flowers to self-compatible, selfing species with small or reduced flowers have occurred many times even within the evolution of the Brassicaceae alone. A good example is the genus *Arabidopsis*, including the major plant model *A. thaliana*. Like *C. bursa-pastoris*, *A. thaliana* is a weedy, predominantly selfing species with a reported outcrossing rate of about 1% that prefers disturbed, man-made habitats

(reviewed by Hoffmann et al. 2003). The lineage that led to extant *A. thaliana* separated roughly about 5–6 million years ago from the *Arabidopsis lyrata* lineage, which has still an ancestral SI system and hence is an outcrossing species.

Even more radical evolutionary changes can be observed in *Lepidium*, another genus of Brassicaceae (Bowman 2006). While basal lineages within *Lepidium* are diploids and generally have the canonical Brassicaceae floral ground plan (see below), derived lineages are often self-fertilizing allopolyploids with reduced floral structures and great colonizing potentials. Also in case of *Lepidium* allopolyploidy is assumed to increase the gene pool to avoid inbreeding depression (Lee et al. 2002, Bowman 2006). More than half of the about 200 *Lepidium* species have only two or four rather than six stamens, and in most of these species, petals are rudimentary (Bowman 2006). Thus *Lepidium* species show remarkable simplifications of floral structure beyond simple reduction in floral size.

Obviously, transitions from diploid, self-incompatible, obligatory outcrossing species with comparatively large and attractive flowers, to polyploid, self-compatible, predominantly selfing species with floral reductions, represent a frequent trend in the evolution of Brassicaceae. *Capsella* might thus serve well in comparative studies aiming at understanding parallel and convergent evolution of floral features.

In addition, the genus *Capsella* shows a very rare phenomenon which, nevertheless, could be of great evolutionary importance, i.e. the occurrence of a homeotic variety in quite stable populations in the wild. Several lines of evidence suggest that homeotic changes played a considerable role during the evolution of flowers, but the relevance of homeotic transformations during the origin of morphological novelties has remained a very controversial topic (reviewed in Theißen 2006). How floral homeotic varieties are established in natural populations has remained almost completely unknown so far (Theißen 2000). Due to its close relationship to the model plant *Arabidopsis* numerous experimental tools are available to study the genus *Capsella*, and more are being developed. *Capsella* hence provides an unprecedented opportunity to investigate the origin, performance and evolutionary potential of a floral homeotic mutant in nature.

## Floral homeotic mutants and floral organ identity

To fully understand the scientific relevance of the homeotic *Capsella* variety the meaning of 'homeosis' has first to be clarified. The term was coined by William Bateson in 1894 to describe a type of variation in which 'something has been changed into the likeness of something else'; a well-known example is provided by the *Antennapedia* mutant of the fruit fly *Drosophila melanogaster* which has antennae replaced by leg-like organs (Lewis 1994).

Homeotic mutants are a quite frequent phenomenon in plants, where both vegetative and reproductive organs can be affected (Sattler 1988, Meyerowitz et al. 1989). Especially well-known are floral homeotic mutants, i.e. mutant plants with flowers that have more or less normal floral organs in places where organs of another type are typically found. The analysis of such mutants has been of great help in understanding as to how the different floral organs acquire their specific identity during flower development (Ferrario et al. 2004, Krizek and Fletcher 2005).

The flowers of most Brassicaceae have a well conserved body plan that applies also to the major model plant, *Arabidopsis thaliana*, as well as all *Capsella* species. It comprises four different classes of organs arranged in four whorls, i.e. four sepals in the first, outermost whorl; four petals in the second whorl; six stamens in the third whorl; and two fused carpels in the fourth and innermost whorl (Fig. 1D). In homeotic mutants the identity of floral organs is changed in a systematic way (Coen and Meyerowitz 1991). In *Arabidopsis thaliana* such mutants come in three major

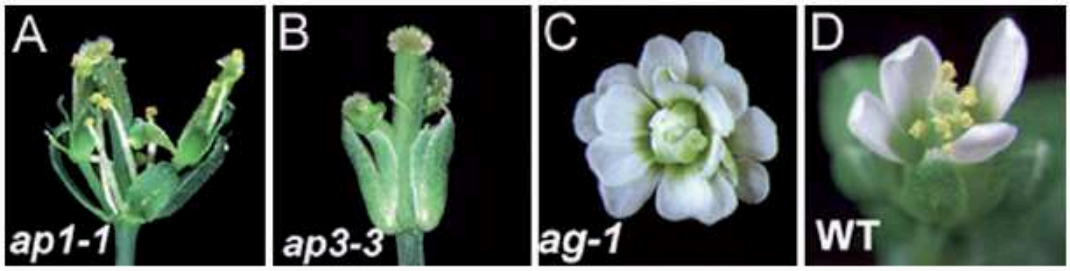


**Fig. 1.** Flower phenotypes of loss-of-function mutants in floral homeotic genes of *Arabidopsis thaliana*. A *ap1-1* is a class A mutant affected in the *APETALA1* (*AP1*) gene that has sepals transformed into carpelloid organs and petals transformed into stamenoid organs. B *ap3-3* is a class B mutant affected in the *APETALA3* (*AP3*) gene that has petals transformed into sepaloid organs and stamens transformed into carpelloid organs. C *ag-1* is a class C mutant in the *AGAMOUS* (*AG*) gene that has stamens transformed into petaloid organs; carpels are replaced by another mutant flower due to the loss of determinate growth of the floral shoot, so that the number of floral organs is significantly increased in the 'filled flowers' of the mutant. D Wild-type flower of *A. thaliana*. Figures from Riechmann and Meyerowitz (1997), with kind permission from Walter de Gruyter GmbH & Co. KG, Berlin, Germany

classes, termed A, B and C. Ideal class A mutants have carpels in the first whorl instead of sepals, and stamens in the second whorl instead of petals (Fig. 1A). Class B mutants have sepals rather than petals in the second whorl, and carpels rather than stamens in the third whorl (Fig. 1B). Class C mutants have petals instead of stamens in the third whorl, and replacement of the carpels in the fourth whorl by sepals. In addition, flower development in these mutants is indeterminate, i.e. there is continued production of mutant floral organs inside the 4th whorl (Fig. 1C). This results in the characteristic phenotype of 'filled flowers' which is well known from many wild and ornamental plants, including *Antirrhinum*, *Rosa* (rose), *Prunus* (e.g. cherry), *Petunia* and *Tulipa* (tulip).

The existence of these classes of mutants suggested that development of the flower is sculpted by homeotic selector genes ('floral organ identity genes') whose expression gives the different floral organs their identity. Such genes can be considered as acting as major developmental switches that activate the entire genetic program for a particular organ, and repress all genes that would interfere with proper organ development. The 'ABC model' was proposed to explain how homeotic genes control floral organ identity (Coen and Meyerowitz 1991). It maintains that organ identity in each whorl is determined by a unique combination of three organ identity gene activities, called A, B and C (Fig. 2E). Expression of A alone specifies sepal formation. The combination A+B specifies the development of petals, and B+C specifies the formation of stamens. Expression of C alone determines the development of carpels. In order to explain the three classes of floral homeotic mutants, the ABC model proposes that the class A and class C genes negatively regulate each other, so that the class A genes become expressed throughout the flower when the class C gene is defect, and *vice versa* (for reviews of the ABC model, see Theißen 2001a, Ferrario et al. 2004, Krizek and Fletcher 2005).

In *Arabidopsis* the class A genes are represented by *APETALA1* (*AP1*) and *APETALA2* (*AP2*), the class B genes by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), and the class C gene by *AGAMOUS* (*AG*). Molecular cloning of these genes revealed that they all encode putative transcription factors (for a review, see Theißen 2001a, Krizek and Fletcher 2005). Thus the products of the ABC genes probably

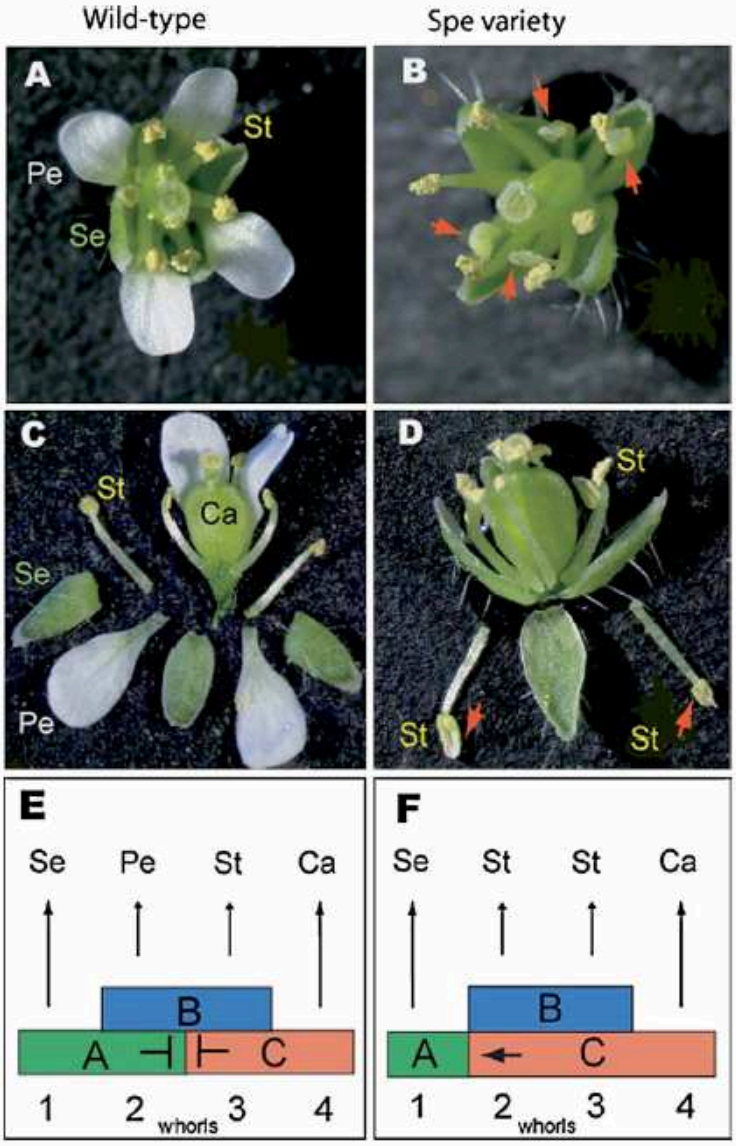


Fig. 2. Structures of flowers of *Capsella bursa-pastoris*, and explanations of organ identities by the ABC model. A, C wild-type flowers, with 4 green, leaf-like sepals in the first, outermost floral whorl, 4 white and showy petals in the second whorl, 6 stamens in the third whorl, and two fused carpels in the fourth, innermost whorl. B, D Flowers of the *Spe* variety, which have the same structure as wild-type flowers, except that petals in the second whorl are transformed into stamens (marked by red arrows). A, B Flower habitus; C, D Partially dissected flowers, to better demonstrate organ identity. E Classical ABC model explaining organ identity in wild-type flowers of *Capsella* and *Arabidopsis*. F Modified ABC model for the *Spe* variety of *C. bursa-pastoris*. Abbreviations: *Ca*, carpels; *Pe*, petals; *Se*, sepals, *St*, stamens

all control the transcription of other genes ('target genes') whose products are directly or indirectly involved in the formation or function of these organs. Except for *AP2* all ABC genes are MADS-box genes encoding MADS-domain transcription factors (for reviews about MADS-box genes in plants, see Becker and Theißen 2003, De Bodt et al. 2003, Riechmann and Meyerowitz 1997, Theißen et al. 2000).

It soon turned out that the ABC genes are required, but not sufficient for the specification of floral organ identity. Moreover, the ABC model did not provide a molecular mechanism for the interaction of floral homeotic genes during the specification of floral organ identity (Theißen 2001b). These shortcomings of the ABC model could meanwhile be overcome. Identification of class D genes involved in ovule development, and class E genes required for development of sepals, petals, stamens and carpels, led to the extension of the ABC model to the 'ABCDE' model (Angenent and Colombo 1996, Pelaz et al. 2000, Theißen 2001a, Ditta et al. 2004). Transgenic studies in *Arabidopsis* revealed that the ABCDE genes are not only required, but are even sufficient at least to specify petal and stamen identity (Honma and Goto 2001). Moreover, the interaction of floral homeotic genes could be explained at the molecular level by the capacity of floral homeotic proteins to form tetrameric complexes of transcription factors (Egea-Cortines et al. 1999, Honma and Goto 2001), as outlined by the 'floral quartet model' (Theißen 2001a, Theißen and Saedler 2001). On the following, however, it suffices to confine the considerations to the simpler ABC model.

### The evolutionary importance of homeosis

The heuristic value of homeotic mutants for understanding the development (ontogeny) of animals and plants is unquestionable. For example, homeotic mutants have been of great value for gaining a better understanding of how floral organs adopt their specific identity, as outlined above. Whether homeotic transitions represent a reasonable mode of evolution (phylogeny) remained a highly controversial issue, however. According to the still predominant scientific hypothesis, the 'Synthetic Theory' (or 'Modern Synthesis') of evolutionary biology, evolution always proceeds in a gradualistic manner, and hence drastic morphological changes such as homeotic transitions are of no evolutionary relevance (for a review, see Mauricio 2001, Theißen 2006). It is assumed that homeotic transformations require too much change to possibly confer selective advantage and thus always undermine the fitness of the affected organisms in such a serious way that there is strong selection against them (reviewed by Theißen 2006).

Despite all its merits, however, the Synthetic Theory, which is largely based on population genetics, might not be sufficient to explain important aspects of evolution. For example, it may fall short of explaining innovations and constraints, and the evolution of body plans (Riedl 1977, Gilbert et al. 1996, Wagner 2000, Haag and True 2001, Wagner and Müller 2002, Wagner and Laubichler 2004). In parallel to this insight, several lines of evidence have in recent years increased the plausibility of major evolutionary transitions brought about by individual mutations. For example, the analysis of 'Quantitative Trait Loci' (QTL) revealed that novel morphological forms in evolution may result from changes in just a few genes of large effect. Instructive examples are provided by the domestication of maize (*Zea mays*. ssp. *mays*) from teosinte (*Zea mays* ssp. *parviglumis*), and by species differences in monkeyflowers (*Mimulus* species) pollinated by bumble-bees (*Mimulus lewisii*) or hummingbirds (*Mimulus cardinalis*) (reviewed by Mauricio 2001).

The evolutionary relevance of major loci of large effect is difficult to reconcile with the view of the Synthetic Theory that morphological changes are based on mutations at many gene loci, each of small effect. Other major shortcomings of the Synthetic Theory, e.g. in explaining evolutionary novelties and constraints, led to the reintegration of developmental biology

into evolutionary biology, giving rise to 'evolutionary developmental biology' ('evo-devo'). According to the rationale of evo-devo novel morphological forms in evolution often result from changes in developmental control genes (for details, see Gould 1977, Gilbert et al. 1996, Theißen et al. 2000, Carroll 2001, Arthur 2002).

Due to their potential to bring about drastic yet coordinate changes in the adult phenotype by modifying development, homeotic genes are especially attractive study objects of evo-devo projects. Some homeotic phenotypes (e.g. actinomorphic rather than zygomorphic flowers, four-winged rather than two-winged insects), resemble differences in character states between major organismic lineages. There is a long debate going on as to whether the genes underlying such 'phylomimicking mutants' define loci that play an important role in character changes during macroevolution (Kellogg 2000, Haag and True 2001). It is clear that changes in the expression domains of floral homeotic genes in mutant or transgenic plants can bring about homeotic transformations of floral organs. For example, the expression of class C genes in the whorls of the perianth leads to a transformation of sepals into carpelloid organs and of petals into stamenoid organs (Bradley et al. 1993). Similarly, the ectopic expression of class B genes in the 1st and 4th floral whorls of *Arabidopsis* leads to a transformation of sepals into petaloid organs and of carpels into stamenoid organs (Krizek and Meyerowitz 1996). Now there is evidence that such changes do not only underlay transgenic and mutant plants of negligible evolutionary potential, but also natural morphological diversity generated during macroevolution, and hence are suitable models for evolutionary processes. Tulips (*Tulipa gesneriana*) and other lily-like plants (Liliaceae), for example, have flowers displaying organ identities quite similar to the ones of higher eudicots, but first whorl organs are typically petaloid like second whorl organs rather than sepaloid. This suggests that a homeotic transition in the first floral whorl from sepaloid to petaloid organ identity, or vice versa, occurred during the evolution of flowering plants. Petaloid organ identity requires the function of class B floral homeotic genes (Fig. 2E). It did not come as a big surprise, therefore, that when putative class B genes were investigated in tulip, they were found to be expressed not only in the petaloid tepals of the second floral whorl, but also in the organs of similar identity in the first whorl (Kanno et al. 2003). Similar examples are provided by many flowers of the basal eudicot family Ranunculaceae, which have distinctly different petaloid organs in the first two whorls. Expression studies suggested that petaloidy of 1st whorl organs is due to a shift of class B gene expression towards the 1st floral whorl (Kramer et al. 2003).

These findings support the view that shifts in the boundaries of floral homeotic gene expression that brought about floral homeotic changes contributed to the diversity of floral architecture. They add to a growing stream of reasoning fuelled by evolutionary and cladistic analyses of morphological characters, all indicating that homeosis played a significant role in plant evolution (Sattler 1988; Iltis 2000; Kellogg 2000; Baum and Donoghue 2002; Rudall and Bateman 2002, 2003; Rutishauser and Moline 2005; for a recent review see Theißen 2006). Ronse De Craene (2003), for example, outlined several lines of evidence that the petals of the Rosaceae (comprising well-known cultivated plants such as rose, strawberry, apple, almond, apricot and peach) were derived from stamens.

An especially intriguing candidate case is provided by the enigmatic *Lacandonia schismatica*, which has 'inside-out flowers' with stamens in the centre of the flowers surrounded by carpels (Ambrose et al. 2006). This is arguably one of the most dramatic deviations from the typical floral ground plan known (usually, stamens surround the carpels in the centre of the flower), suggesting that a homeotic change affecting the reproductive organs occurred relatively recently during evolution in the lineage that led to *Lacandonia*. One should note, however, that the interpretation of the

reproductive units of *Lacandonia* is still controversial; what appears to be flowers has alternatively been viewed as pseudanthia (Rudall 2003), so that the evolutionary developmental biology of *Lacandonia* needs further clarification. If the reproductive structures of *Lacandonia* are indeed true flowers, however, their origin very likely involved a considerable change in the ABC system of floral organ identity specification.

Models of how evolutionary variation of the ABC system of floral organ identity specification could explain floral diversification during evolution have been provided e.g. by Bowman (1997), Albert et al. (1998), Theißen et al. (2000) and Kramer et al. (2003). Consequences of homeotic transitions in evolution for our understanding of organ homology have been discussed by Theißen (2005).

Theoretically, homeotic changes could occur in a gradual mode during evolution (Sattler 1988). However, given that full conversions in organ identity usually take place in a mutant individual just by the mutation of a single homeotic gene, a saltational mode of character change appears more plausible, at least from a genetic point of view (Theißen 2006). This, however, would be difficult to reconcile with the Synthetic Theory, maintaining that all kinds of evolution are gradual and based on changes in allele frequency at many loci. And it would have a quite dramatic consequence: homeotic mutants should represent important steps during macroevolutionary transitions.

## It's the ecology, stupid! – Floral homeotic mutants in the wild

Investigations following the evo-devo rationale and using the toolkit of developmental genetics have provided detailed information about as to how floral homeotic mutants can be generated in the first place. To establish novel structures in evolution, however, it does not suffice to generate a morphological feature by mutation in an individual organism; rather, the mutant allele must also go to fixation in a population in the wild. Unfortunately, evo-devo tells us little about the performance of homeotic mutants in natural environments – a crucial aspect for the evolutionary relevance of homeotic mutants (Theißen 2000). Non-gradual modes of evolution may not be generally accepted unless a sufficient fitness of respective mutants has been documented in natural habitats. To clarify that point the population dynamics of homeotic mutants has to be studied in extensive field work (Theißen 2000, 2006, Bateman and DiMichele 2002, Vergara-Silva 2003, Dietrich 2003).

Quite a large number of floral homeotic variants have been described in the literature (see e.g. Masters 1869, Darwin 1876, Murbeck 1918, Gottschalk 1971, Meyerowitz et al. 1989, Ronse De Crane 2003), but often heritability of the deviant phenotype – a requirement for any evolutionary relevance – remained unknown. And clearly, not even every mutant will do. Most of the 'classical' floral homeotic mutants, e.g. the ones that gave rise to the ABC model, have a dramatically reduced fitness, as is obvious already under laboratory conditions. The most extreme case is represented by class C loss-of-function mutants such as *agamous* of *A. thaliana*, which completely lack reproductive organs (Fig. 1C). Since *Arabidopsis* is not capable to reproduce vegetatively, such a mutant would soon become extinct in the wild. Class B null mutants (Fig. 1B), lacking stamens, are at least completely male sterile, but even class A mutants (Fig. 1A), although they have more rather than less reproductive organs in their flowers, may be hampered in their reproductive fitness.

A better way to identify floral homeotic mutants with evolutionary potential might be to watch out for such plants that appear in populations in the wild, thus revealing at least some minimum kind of competitiveness under natural growth conditions. Very few candidates that meet this criterium have been described, however. One is *bicalyx*, a recessive variety of *Clarkia concinna* (Onagraceae) in which the usually pink and showy trilobed petals are transformed into sepaloid organs

due to a mutation at a single locus (Ford and Gottlieb 1992). The *bicalyx* variety occurs only in a small population north of San Francisco (Point Reyes, California, USA) accompanied by a majority (70%–80%) of wild type plants. Although the observation time of the population was just four years, it was reported to be stable, most probably due to the predominantly selfing mode of propagation (Ford and Gottlieb 1992).

Another case is a peloric variety of *Linaria vulgaris* (common toad-flax) that has actinomorphic rather than zygomorphic flowers and exists on a small island near Stockholm/Sweden (Cubas et al. 1999). Like the homeotic *Clarkia* also the *Linaria* is mutated in a single, recessive locus. However, while the *bicalyx* gene has not been molecularly characterized so far, it turned out that the *Linaria* variety is affected in a *CYCLOIDEA*-like gene, but by epimutation (methylation of DNA that leads to transcriptionally silencing) rather than change in the DNA sequence (Cubas et al. 1999). Both the *Clarkia* and *Linaria* varieties have a very limited range of distribution, and their fitness and competitiveness in the field has not been tested yet, but is questionable – the *Linaria* epimutant, for example, may only propagate vegetatively (Theißen 2000). The peloric variety of *Linaria* has been described for more than 200 years already, but whether its long-lasting existence on the island near Stockholm is due to true persistence of an ancestral mutant, or due to frequent reappearance after fast dieing out, has not been determined so far (Theißen 2000). Taking together, the evolutionary potential of the wild floral homeotic varieties reported so far is doubtful (Theißen 2006).

### A floral homeotic variety of shepherd's purse

In contrast to the geographically very limited cases outlined above, a floral homeotic variety of shepherd's purse (*Capsella bursa-pastoris*) has been described for almost 200 years from different locations throughout Europe, and has been documented to exist in populations of remarkable size for a considerable number of years. This homeotic variety was termed '*dekandrisch*' ('decandric') (Opiz 1821), referring to the fact that its flowers have ten (greek: 'deka') rather than the usual six stamens of the wild-type (compare Fig. 2B, D with Fig. 2A, C). This is so because the four petals are transformed into stamens, hence the flowers of the decandric variety lack petals (Fig. 2B, D). Opiz (1821) considered the decandric variety a new species and named it '*Capsella apetala*'. Almost simultaneously decandric *C. bursa-pastoris* plants were found in Vienna/Austria (Trattinnick 1821) and in Braunschweig/Germany (Wiegmann 1823). Opiz (1821), Schlechtendahl (1823) and De Candolle (1827, cited after Dahlgren 1919) propagated *C. bursa-pastoris* plants via seeds and observed that the decandric phenotype is heritable.

Because decandric flowers exhibit additional stamens replacing petals, Murbeck (1918) termed the phenomenon '*Staminale Pseudapetalie*' ('Stamenoid pseudo-apetaly'). He analysed plants of different provenience (Sweden, Germany, Prague/Czech Republic and South Dakota/USA) and found flowers with petals transformed into almost normal stamens which, at least in some Swedish individuals, produced pollen. However, Murbeck (1918) also identified quite a number of individuals with flowers displaying changes other than ideal homeotic transformations, including the development of rudimentary organs or stamens that deviate from their normal form and do not produce pollen, lack of organs, and incomplete transformations from petals to stamens. Stamenoid pseudo-apetaly was also reported for many other flowering plants, such as the monocots meadow saffron (*Colchicum*), tulip (*Tulipa*), hyacinth (*Hyacinthus*), daffodil (*Narcissus*), *Iris*, *Crocus* and different orchids as well as many eudicots ranging from basal taxa such as *Ranunculus* and *Papaver* to a diverse range of higher eudicots (Murbeck 1918, Ronse De Craene 2003). Murbeck (1918) already considered the frequent occurrence of stamenoid pseudo-apetaly as of evolutionary relevance,

assumed to reflect the origin of the perianth by conversion of stamens into petals. The interpretation of floral homeotic mutations as 'atavistic' changes that reproduce ancestral character states is highly controversial, however (e.g. Meyerowitz et al. 1989).

Dahlgren (1919) crossed various wild-types with decandric *C. bursa-pastoris* plants and found that in the $F_1$ generation the organs of the second floral whorl showed characteristics intermediate between petals and stamens. In the $F_2$ generation an approximately 1:2:1 segregation of individuals with stamenoid, intermediate and wild-type (petal) organs, respectively, in the second floral whorl appeared, suggesting that stamenoidy of 2nd whorl organs is co-dominant (or incompletely penetrant) to petaloidy and inherited by a single locus (or two or more closely linked loci). However, since a clear phenotypic distinction between intermediate and stamenoid second whorl organs was not always possible, Dahlgren (1919) counted them together and therefore reported a 1:3 segregation of wild-type and '*apetala*' plants.

In his seminal book on the importance of gene mutations for the evolution of plants Gottschalk (1971) dedicated a whole paragraph to the decandric variety of *Capsella*. Because of its sometimes massive occurrence in the wild he argued that the mutant variety must have a selective advantage compared to the wild type (a questionable argument, however, see below and Theißen 2006).

Besides brief mentioning in the literature (Meyerowitz et al. 1989) the decandric *C. bursa-pastoris* fell into oblivion for quite a while, until it was re-discovered in 1991 by Reichert (1998) on field paths in vine yards in Gau-Odernheim (Rheinhessen/Germany). Although not referring to the previous reports his description of the phenotype resembles those by Opiz (1821), Murbeck (1918) and Dahlgren (1919) in great detail. Reichert (1998) observed the population for several years and found the number and distribution of decandric plants to be quite stable, even though they are growing mixed with and embedded in populations of wild-type plants. The author noticed the peculiarity of a homeotic variant growing in the wild in a stable population of many plants rather than just appearing as a single mutant individual.

## Studying *Spe*

As has been outlined above the decandric variety of *Capsella bursa-pastoris* is not the only floral homeotic mutant that has been found in the wild. Here we argue, however, that it has unprecedented features that make it an especially suitable system to study the evolutionary potential of floral homeotic mutants.

The relatively frequent and long-lasting presence in the wild guarantees that the fitness of the homeotic variety is at least not drastically hampered compared to that of the wild-type. Moreover, what appears important from an experimental point of view, *Capsella* is quite closely related to the major model plant *Arabidopsis*; both lineages may have diverged from one another about 10–14 million years ago (Koch and Kiefer 2005). This means that in contrast to many other floral homeotic mutants in the wild, the floral homeotic variety of *C. bursa-pastoris* can be rigorously studied, from the molecular genetic basis of the phenotype to its consequences on the ecology in the field, due to the fact that suitable experimental tools are available. Respective studies are underway and may help to answer the question as to whether non-gradualistic changes at the phenotypic level, such as homeotic transformations, have the potential to contribute to macroevolution (Theißen 2006).

We have identified additional decandric populations of *C. bursa-pastoris* throughout Europe (P.N. and B.N., unpublished data). Current work in our laboratories focuses on the populations from Gau-Odernheim (Reichert 1998) and an additional population found in the area of the Desenberg near Warburg (Westphalia/Germany). A careful analysis of the phenotype and mode of inheritance

corroborated many of the findings reported in the literature (Opiz 1821, Trattinnick 1821, Murbeck 1918, Dahlgren 1919, Gottschalk 1971, Reichert 1998), suggesting – but by no means conclusively demonstrating – that the same gene(s) or even allele(s) are involved. Transformation of petals into stamens was complete or almost so in most of the mutant plants being investigated from both Gau-Odernheim (Fig. 2B, D) and Warburg. The pollen produced by the extra stamens proved to be viable and functional in fertilization (P.N., unpublished data).

Segregation of the mutant phenotype in the $F_2$ generation of crosses between mutant and wild-type plants varies, depending on the crosses being analysed, between stamenoid vs. petaloid 2nd whorl organs in a ratio of about 3:1, and stamenoid vs. intermediate vs. wild type second whorl organs in a ratio of about 1:2:1 (P.N., unpublished data). This suggests that the mutant phenotype of the homeotic populations in Gau-Odernheim and Warburg is caused by (a) (co-)dominant mutant allele(s) at a single locus, like for the varieties investigated before (Dahlgren 1919). Tests for allelism of the mutant loci in the Gau-Oderheim and Warburg populations are underway. Due to its phenotype and dominant mode of inheritance we have termed the locus/loci mutated in the decandric *C. bursa-pastoris* varieties "*Stamenoid petals*" (*Spe*).

Shull (1914) described a mutant with altered fruit shape in *Capsella bursa-pastoris* segregating in a Mendelian ratio of 1:15, suggesting that recessive alleles of two unlinked genes are responsible for the mutant phenotype. Shull (1914) concluded that capsule form in *C. bursa-pastoris* is determined by duplicate genes. In retrospect his observation can be explained with *Capsella bursa-pastoris* being a tetraploid plant. Our and the previous findings on the 3:1 (or 1:2.1) segregation of the *Spe* phenotype are not in conflict with these observations, considering that *Spe* is (co-) dominant rather than recessive, and that *C. bursa-pastoris*, despite being tetraploid, switched to a disomic mode of inheritance. We assume that the mutation conferring the *Spe* phenotype is just in one of the two genomes of *C. bursa-pastoris*.

According to the ABC model development of stamen identity requires class C floral homeotic gene activity, suggesting that in the *Spe* varieties class C gene expression is ectopically shifted towards the 2nd floral whorl, and class A gene activity is suppressed there (Fig. 2F). Ectopic class C gene function might be provided by an orthologue of the canonical class C gene of *Arabidopsis thaliana*, *AG*, or by any one of its closely related paralogues, *SHP1*, *SHP2* and *STK*, which share high sequence similarities with *AG* (Becker and Theißen 2003). Except *STK*, all these *AG*-type genes produce stamenoid organs in the 2nd whorl upon ectopic expression in *A. thaliana* (Favaro et al. 2003, and literature cited therein). In certain genetic backgrounds, e.g. *apetala2 agamous*, the *SHP*/*STK* genes are sufficient and required for carpel formation (Pinyopich et al. 2003). Due to the tetraploidy of the *C. bursa-pastoris* genome each of these loci might well be represented by two different (yet highly similar and related) genes in shepherd's purse, resulting in eight hot candidate genes for providing ectopic class C gene activity in the *Spe* variety. To test the modified ABC model for *Spe* (Fig. 2F), mRNA expression patterns of orthologues of class A, B and C floral homeotic genes in *Spe* and wild-type flowers of *C. bursa-pastoris* are currently being determined.

Scenarios not involving the ectopic expression of an *AG*-like gene cannot be excluded, but appear unlikely at the moment. This does not necessarily mean, however, that an *AG*-like gene is the *Spe* locus itself. In principle, ectopic expression of a class C gene could be brought about by a change in a *cis*-regulatory element of an *AG*-like gene itself, but also by a *trans*-acting factor functioning 'upstream' of the *AG*-like gene (and not necessarily directly binding to the *AG*-like gene itself). As we have learned recently, such an upstream-factor is not necessarily a protein, but could also be a regulatory RNA. For example, among the negative

regulators of *AG* in the 1st and 2nd floral whorl is the non-MADS class A protein AP2, whose translation is inhibited by the microRNA miR172 in whorls 3 and 4 (reviewed by Steimer et al. 2004). It is thus conceivable that extension of the expression domain of miR172 from whorls 3 and 4 towards whorl 2 leads to an inhibition of AP2 synthesis and hence to an ectopic expression of *AG* in whorl 2.

Remarkably, the many screens for flower developmental genes in *A. thaliana* did so far not come up with a *Spe*-like mutant phenotype, at least to the best of our knowledge. Single-mutant flowers with fully stamenoid second whorl organs have typically also carpelloid first whorl organs (see, for example, Fig. 1A), suggesting differences in the regulation of floral organ identity between *Capsella* and *Arabidopsis* that deserve further studies.

Nevertheless, that *cis*- as well as *trans*-regulatory changes leading to ectopic expression of class C gene activity can bring about a *Spe* phenotype is even demonstrable in *A. thaliana*. Transformation of petals into stamens was realized in transgenic plants that express the class C gene *AG* under control of the promoter of the class B gene *AP3*, driving gene expression in the 3rd floral whorl – where *AG* is expressed anyway -, but also ectopically in the 2nd whorl (Fig. 3A, Jack et al. 1997). Even though an illegitimate recombination between a class C coding region and a class B gene regulatory region appears an extremely unlikely scenario for the origin of the *Spe* mutant locus in *C. bursa-pastoris*, the transgenic scenario nicely illustrates the feasibility of the modified ABC model depicted in Fig. 2F. It thus appears not unlikely that e.g. a mutation in a class C gene in *C. bursa-pastoris* destroyed a *cis*-regulatory element that is required to keep expression of the gene out of the 2nd floral whorl, especially since such a mutation could easily be dominant or co-dominant. An alternative scenario would be the addition of a 2nd whorl-specific enhancer to an *AG*-type gene, either by illegitimate recombination (as already mentioned above), or mutation. Even though this scenario appears less likely than the destruction of a negative regulatory element, the existence of 2nd whorl-specific regulatory sequences e.g. in the class B gene *AP3* (Hill et al. 1998) makes the respective event at least conceivable.

In contrast, loss-of-function mutations in trans-regulators of *AG* are more likely recessive rather than dominant. A *Spe* phenocopy in *A. thaliana* is even only known from a double mutant affected in two loci, *RABBIT EARS* (*RBE*) and *ROXY1* (Xing et al. 2005). Since a *rbe roxy1* double recessive mutant phenotype is unlikely to be established in natural populations except under circumstances of strong positive selection, we currently consider *Spe* being an *AG*-type gene a much more likely scenario, even though one certainly cannot exclude that floral gene regulation in *Capsella* is different from that in *Arabidopsis*.

Cloning of the *Spe* locus itself is, therefore, another important task for the future. We are currently applying a combined candidate and map based cloning approach which is facili-

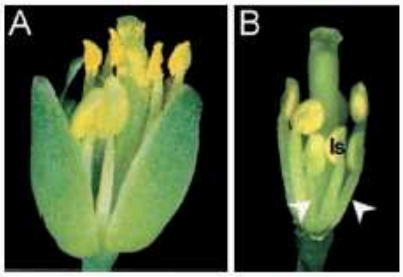


Fig. 3. Flowers of *Arabidopsis thaliana* with a *Spe* phenotype. A Flower of a transgenic plant in which the class C floral homeotic gene *AGAMOUS* is expressed under the control of the promoter of the class B gene *APETALA3* (AP3::*AG*), leading to the ectopic expression of the class C gene in the second floral whorl and hence the transformation of petals into stamens. B Flower of a *roxy1–3 rbe-2* double mutant; sepals were removed; white arrowheads point to extra stamens that develop in the second whorl. A is from Jack et al. (1997) with kind permission from Blackwell Publishing, Oxford, UK; B is from Xing et al. (2005) with kind permission from The Company of Biologists Ltd., Cambridge, UK. Abbreviation: *ls* lateral stamen

tated by the close relationship between *Capsella* and *Arabidopsis*. Comparative analysis revealed that the genome organization is still largely conserved between *Arabidopsis* and *Capsella* (Acarkan et al. 2000, Koch and Kiefer 2005). The genome sequence of *A. thaliana* has already been determined (Arabidopsis Genome Initiative, 2000), and sequencing of the *A. lyrata* and *C. rubella* genomes has been initiated (Joint Genome Institute, United States Department of Energy). All these findings and developments provide knowledge and tools facilitating gene mapping and cloning in *Capsella*.

Candidate genes for *Spe* currently being considered are all the *AG*-type genes mentioned above (orthologues of *AG*, *SHP1*, *SHP2*, *STK*), but also some *trans*-acting regulators of *AG* that show stamenoid petals upon mutation, such as *APETALA2* (*AP2*) (Drews et al. 1991, Deyholos und Sieburth 2000, Bomblies et al. 1999), *LEUNIG* (*LUG*, Liu und Meyerowitz 1995, Liu et al. 2000, Conner and Liu 2000), *SEUSS* (*SEU*, Franks et al. 2002), *CURLY LEAF* (*CLF*, Goodrich et al. 1997) and the already mentioned *RBE* and *ROXY1*. These putatively *trans*-acting candidates are considered with lower priority, however, because mutant alleles are usually recessive and mutant plants often display considerable pleiotropic effects beyond stamenoidy of petals, sometimes even outside of the flower. For example, Fray et al. (1997) described a double mutant of rape seed (*Brassica napus*) possibly affected in a *CLF* orthologue; its flowers very much resemble the *Spe* phenotype, but its vegetative leaves show a strong curly leaf phenotype known well from the original *clf* mutant of *Arabidopsis* (Goodrich et al. 1997). Restriction of the mutant effect to floral organs is characteristic for the *Spe* phenotype, however (Fig. 2B, D).

The origin and performance of *Spe* plants outside of the greenhouse is investigated both in ordered field plots in Botanical Gardens in Jena and Halle (Saale), and in their 'natural' environment in Gau-Odernheim and Warburg (which are typically man-made or disturbed habitats such as vineyards, however). Isozyme studies (Hurka et al. 1989, Hurka and Düring 1994) and AFLP-fingerprinting are being used to determine whether all *Spe* plants are monophyletic, even when growing at different locations, or whether the different *Spe* populations originated independently in the different habitats from wild-type plants.

Although *C. bursa-pastoris* is predominantly self-pollinating, even low rates of outcrossing could help to avoid inbreeding depression and hence might be of considerable importance for plant fitness and evolutionary potential. In case of the also predominantly selfing *A. thaliana* flower visits by potential pollinators such as solitary bees, dipterans and thrips have been observed in the field (Mitchell-Olds 2001, Hoffmann et al. 2003). In principle, the *Spe* variety could attract less, more, or the same number of floral visitors; moreover, the spectrum of floral visitors could be changed, for example because *Spe* flowers produce more pollen (preferred by beetles) than wild-type flowers, while the latter might be more attractive to bees and flies due to the presence of petals. To find out, we designed field plots in the Botanical Gardens of Jena (Fig. 4A) and Halle (Saale). Magnifying glasses were used to inspect individual flowers to identify small insects, such as thrips. Larger insects were caught by net or only counted by visual inspection, especially solitary bees (Figs. 4B, C), because many of them are endangered species. Especially species from the genus *Andrena* (Westrich 1990) were detected on both *Spe* and wild-type flowers of *C. bursa-pastoris* (J.Z., unpublished preliminary data). Two of these species are oligolectic, which means they are specialized to plant species belonging to the *Brassicaceae* family. So far we did not observe a dramatic change in the spectrum or number of floral visitors comparing *Spe* with wild-type plants (J.Z., unpublished preliminary data). Moreover, the numbers of fruits and seeds produced under our experimental conditions appears to be very similar for wild-type and *Spe* plants (J.Z., unpublished preliminary data). We thus did not find any evidence so far that the *Spe*

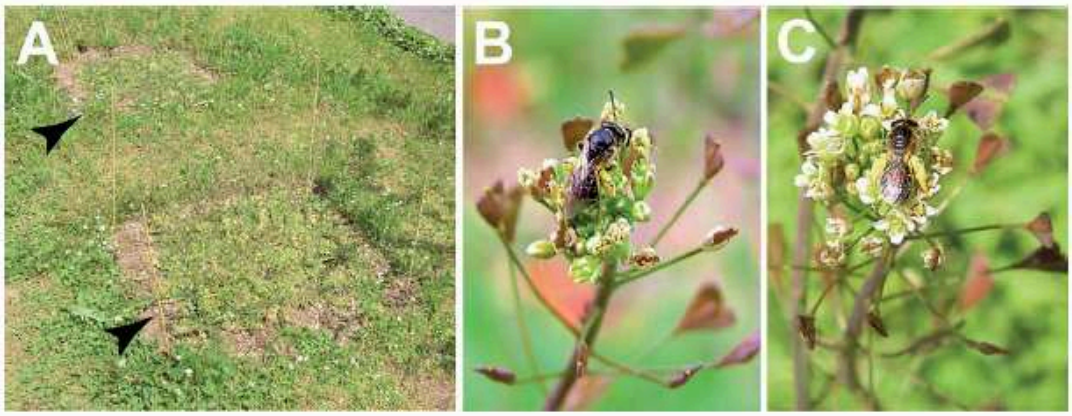


**Fig. 4.** Outdoor investigations of *Capsella bursa-pastoris* in the Botanical Garden of Jena. **A** Field plots (5 × 5 plants) of the *Spe* mutant (upper arrow) and the wild-type (lower arrow). **B, C** Solitary bees visiting the inflorescences of a *Spe* variety (**B**) and a wild-type plant (**C**), respectively

variety is handicapped in its reproductive fitness, which is in line with its persistence in wild habitats for many years. Also because *Capsella bursa-pastoris* is mainly selfing rather than outcrossing these findings do not come as a big surprise.

Loss of perianth organs and increase in pollen production (e.g. by increasing stamen number) is a typical syndrome during evolution of wind-pollination, so represents the *Spe* variety a first step towards the evolution of wind-pollination within *Capsella*? This may sound absurd, but the consequences of the *Spe* mutation on outcrossing mediated by both floral visitors and wind will be determined in the botanical garden of Osnabrück/Germany. Moreover, the development of *Spe* populations in their natural habitats will be monitored as long as possible. Taken together, these endeavours will help us to learn more about the evolutionary potential of the *Spe* variety, and, hopefully, of floral homeotic mutants in general.

## Summary and outlook

Comprising just three species *Capsella* is only a very small genus. However, with shepherd's purse it includes one of the most widely distributed flowering plants on our planet, and shows a number of interesting phenomena of parallel and convergent evolution affecting reproductive traits. In addition, with the *Spe* variety *C. bursa-pastoris* shows a very rare yet for the evolution of life on earth potentially very important phenomenon, namely the occurrence of a homeotic variety in quite stable populations in the wild. There is considerable circumstantial evidence that homeotic changes played a considerable role during the evolution of flowers, but the relevance of homeotic transformations during the origin of morphological novelties has remained a very controversial topic. Thus the *Spe* variety may help us to better understand one of the arguably most enigmatic aspects of life on earth, i.e. the origin of complex evolutionary novelties. Specifically, a detailed study of the *Spe* variety may not only tell us more about the developmental genetic mechanisms that generate novel form in the first place – something we know about quite a lot already -, but also whether and how drastic morphological variants are established in natural populations. Reconciling population genetics and macroevolution appears to be a considerable challenge for the future, and *Capsella* could be of great help here. Once *Spe* has been

molecularly cloned, for example, it could be tested whether the locus is under purifying or positive selection. Another important question of future research will be to find out as to whether epimutations (e.g. methylation of DNA) serve as transitional steps in a 'trial phase' of mutant phenotypes during the establishment of morphological novelties (Theißen 2000).

So *Capsella* might be only a small genus, but it has great potential in evolutionary biology. Choosing it as a favourite model system for his investigations on the biogeography and phylogeny of plants about three decades ago was certainly a wise decision of Herbert Hurka. However, with the experimental tools currently becoming available it could well be that the best time of *Capsella* in evolutionary biology is yet to come.

This article is dedicated to Prof. Herbert Hurka on occasion of his 65th birthday, to honor his long-standing contributions to research on *Capsella*. The authors are grateful to Klaus Mummenhoff and Marcus Koch for their invitation to contribute to this volume. Many thanks to John Bowman for a number of valuable suggestions that helped to improve the manuscript. Many thanks also to Matthias Hoffmann (Halle Botanical Garden) and Conny Bartholmes, Steffen Hameister und Frank Buschermöhle (from our own laboratories) for discussions in the frame of our project on the *Capsella* variety *Spe*. Work on *Spe* in the authors' laboratories is supported by grants TH 417/4-1 and NE 314/7-1 from the Deutsche Forschungsgemeinschaft (DFG).

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## 4. Manuskript II:

P. Nutt[1], J. Ziermann[1] and G. Theißen (eingereicht bei *The Plant Cell* am 7. Mai 2008): Ectopic expression and co-segregation of an *AGAMOUS* orthologue in *Stamenoid petals*, a natural homeotic floral variant of *Capsella bursa-pastoris.*

([1] These authors contributed equally to this work)

# Ectopic expression and co-segregation of an *AGAMOUS* orthologue in *Stamenoid petals*, a natural floral homeotic variant of *Capsella bursa-pastoris*

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## ABSTRACT

We report the combined genetic and molecular analysis of a floral homeotic variant that occurs in natural populations in the wild. We demonstrate that the organs in the 2nd floral whorl of *Capsella bursa-pastoris* (shepherd's purse) variant "*Stamenoid petals*" (*Spe*) develop into functional stamens rather than petals, while the identity of all other floral organs and the timing of organ formation is the same as in wild-type flowers. Genetic analyses revealed that *Spe* is a co-dominant allele of a single locus, and that two populations in Germany with a *Spe* phenotype are affected at the same locus. In an $F_2$ mapping population, the *Spe* phenotype co-segregates with the *CbpAGa* locus, but not with any of the seven other members of the clade of *AGAMOUS*-like genes in the genome of the tetraploid *C. bursa-pastori*s. *In situ* hybridization analyses showed that the *AGAMOUS* co-orthologues of *C. bursa-pastoris* are ectopically expressed in primordia of 2nd whorl organs of *Spe* flowers, thus explaining why these organs develop into stamens rather than petals. As the only major sequence difference between wild-type and mutant alleles, we identified a deletion of 22 base pairs in a highly conserved region of the 2nd intron of *CbpAGa*. Our findings strongly suggest that *Spe* is a mutant allele of *CbpAGa* in which a previously unrecognized negative *cis*-regulatory element of *AGAMOUS* genes, which keeps class C homeotic gene expression out of the 2nd floral whorl, has been deleted.

## INTRODUCTION

Homeotic mutants show a type of variation in which "something has been changed into the likeness of something else" (Lewis, 1994). They are frequent in plants, affecting both vegetative and reproductive organs (Sattler, 1988; Meyerowitz et al., 1989). Floral homeotic mutants have more or less normal floral organs in places where organs of another type are typically found. The model plant *Arabidopsis thaliana* (thale cress) has flowers that consist of four different types of organs, i.e. sepals, petals, stamens and carpels, which are arranged in four whorls. In *A. thaliana* homeotic mutants have been categorized into three classes termed A, B and C (Coen and Meyerowitz, 1991). Ideal class A mutants have carpels instead of sepals in the 1st floral whorl, and stamens rather than petals in the 2nd whorl. Class B mutants contain sepals rather than petals in the 2nd and carpels rather than stamens in the 3rd whorl. And class C mutants have flowers in which reproductive organs (stamens and carpels) are replaced by perianth organs (petals and sepals, respectively), and in which the determinacy of floral growth is lost, resulting in an increased number of floral organs (Meyerowitz et al., 1989).

The defined classes of floral homeotic mutants have been explained by the ABC model (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994; Krizek and Fletcher, 2005). It proposes three different floral homeotic functions to explain how the different floral organs adopt their identities during development. Corresponding to the mutant classes these functions are termed A, B and C, with A specifying sepals in the 1st floral whorl, A+B petals in the 2nd, B+C stamens in the 3rd and C carpels in the 4th whorl. To account for class A and C mutant phenotypes it was proposed that the A function and the C function work antagonistically, so that the A function is expressed throughout the flower in a C loss-of-function mutant, and *vice versa* (Coen and Meyerowitz, 1991).

In *Arabidopsis* the class A genes are represented by *APETALA1* (*AP1*) and *APETALA2* (*AP2*), the class B genes by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), and the (single) class C gene by *AGAMOUS* (*AG*). All these genes encode putative transcription factors (reviewed in Krizek and Fletcher, 2005). Except for *AP2* all ABC genes are MADS-box genes encoding MIKC-type MADS-domain transcription factors (reviewed in Becker and Theißen, 2003).

The molecular mechanism by which class A, class B and class C proteins interact involves another class (E) of MIKC-type transcription factors termed SEPALLATA (or AGL2-like) proteins (Pelaz et al., 2000; Honma and Goto, 2001; Ditta et al., 2004). According to the floral

quartet model (Theißen and Saedler, 2001) the proteins encoded by the class ABC genes bind to the class E proteins in a combinatorial way to constitute multimeric regulatory complexes that specifically recognize *cis*-regulatory elements ('CArG-boxes') of their target genes. Hence it goes without saying that the study of floral homeotic mutants has told us a great deal about the development of floral organ identity.

In contrast, whether floral homeotic mutants and genes play a role in evolution is a highly contentious topic. On the one hand, there is considerable circumstantial evidence that they do; on the other hand this is difficult to reconcile with the predominant evolutionary theory which rejects any drastic change of the phenotype as reasonable mode of evolution due to its assumed negative impact on the fitness of the affected organism. Anyway, structural diversity of flowers, such as differences between the flowers of *Arabidopsis*, columbine, tulip and orchids, has been explained to quite some extend by modifications of the ABC system of floral organ identity specification, especially by changes in the spatiotemporal expression domains of the ABC genes which lead to homeosis (Kanno et al., 2003; Kramer et al., 2003; Mondragón-Palomino and Theißen, 2008). Beyond that, there is further evidence provided by evolutionary analyses of morphological characters of both extant and extinct plants that homeosis played a significant role in plant evolution (Sattler, 1988; Kellogg, 2000; Baum and Donoghue, 2002; Rudall and Bateman, 2002, 2003; Ronse De Craene, 2003; Theißen, 2006).

Like all other mutants, homeotic mutants originate as rare individuals in populations of wild-type organisms. To establish new evolutionary lineages homeotic mutants thus need to survive many years under conditions of natural selection until the mutant homeotic locus went to fixation and possibly modifying mutations have fine-tuned and optimized the new 'body design'. How does this work? Unfortunately, the mechanisms which explain how homeotic mutants originate tell us little about the performance of homeotic mutants in natural ecosystems (Theißen, 2000). Thus to determine the evolutionary potential of floral homeotic mutants, their population dynamics has to be studied in extensive field work (Theißen, 2000, 2006; Bateman and DiMichele, 2002).

There is little doubt that most floral homeotic mutants have their fitness so strongly reduced that their long-term survival in nature is almost certainly hampered (Nutt et al., 2006). Thus probably only a small fraction of homeotic mutants will have sufficient fitness under natural growth conditions to establish new evolutionary lineages. A reasonable method to identify such mutants might be to look for populations of floral homeotic variants in the wild. However, up to now only few of such populations have been described in the literature. One case is *bicalyx*, a variant of *Clarkia concinna* (Onagraceae) in which the petals are

transformed into sepaloid organs (Ford and Gottlieb, 1992); another example is a peloric version of common toad-flax (*Linaria vulgaris*) with actinomorphic rather than zygomorphic flowers (Cubas et al., 1999). Both are mutated at a single, recessive locus. It turned out that the toadflax variant is affected in a *CYCLOIDEA*-like gene by methylation of DNA (epimutation), but for the *bicalyx* variant no molecular data has been reported so far (Cubas et al., 1999).

Both the *Clarkia* and *Linaria* variants described have a very limited range of distribution, and their fitness in the wild is probably significantly lower than that of the wild-types, so that their evolutionary potential is questionable at best (Theißen, 2000). To develop a more comprehensive understanding of the evolutionary importance of floral homeotic mutants - and homeotic variants in general – model systems are required that can be studied at different levels of biological complexity, ranging from the molecular mechanisms that bring about the variant phenotype to the ecology in the field (Nutt et al., 2006; Hintz et al., 2006; Bateman and Rudall, 2006). Studying the molecular developmental genetics of a system efficiently requires tools that are only available for so called 'model organisms'. Populations of natural homeotic variants have not been reported for the predominant flowering plant model system *A. thaliana* so far. Therefore, we started a research program on *Stamenoid petals* (*Spe*), a remarkable variant of *Capsella bursa-pastoris* (shepherd's purse), which is a close relative of *A. thaliana*. In the flowers of the *Spe* variant the four petals appear to be transformed into stamen-like organs, while all other floral organs seem to be unaffected (Nutt et al., 2006; Hintz et al., 2006).

As outlined in detail elsewhere, the *Spe* variant has been described at different places in Europe for almost 200 years now. One quite large and stable population on field paths in vine yards in Gau-Odernheim (Rheinhessen/Germany) has been monitored for about 20 years (Nutt et al., 2006). The long-lasting existence of the *Spe* variant in the wild indicates that its fitness cannot be dramatically different from that of the wild-type with which it co-exists.

Like *A. thaliana*, *C. bursa-pastoris* is self-compatible and easy to cultivate and propagate. Albeit longer than that of *A. thaliana*, the life cycle of *C. bursa-pastoris* allows the analysis of three to four generations per year. And even though *C. bursa-pastoris* is a tetraploid plant, it shows disomic inheritance (Hurka et al., 1989), which makes crossing experiments easier to interpret. Because of the close relationship between the genus *Capsella* and *A. thaliana* numerous experimental tools can be adapted quite easily to study the *Spe* variant. We have shown already that *C. bursa-pastoris* is amenable to genetic transformation by the 'floral dip' method (Bartholmes et al., 2008), which facilitates the analysis of gene function. Moreover,

the order, orientation and sequence of genes are very similar in *Arabidopsis* and *Capsella*, with more than 90% sequence identity within exons (Boivin et al., 2004; Koch and Kieffer, 2005). This allows the identification of genes within *Capsella* with the help of the *Arabidopsis* genome. We are thus confident that the *Spe* variant of *C. bursa-pastoris* represents an excellent system to study all aspects of the developmental and evolutionary biology of a floral homeotic mutant.

Here we provide a detailed report about flower development in the *Spe* variant compared to that of the wild-type, involving two populations. We investigated the mode of inheritance employing crosses between wild-type and *Spe* plants, and the molecular mechanism by which the *Spe* mutant phenotype is brought about. Based on the ABC model we hypothesize that in the *Spe* mutants ectopic expression of a class C gene, or a closely related gene, is extended from the 3rd and 4th whorl towards the 2nd whorl and thereby suppressing class A genes in this whorl (Nutt et al., 2006). Most obvious candidate genes for this ectopic expression are an orthologue of the *Arabidopsis* class C gene *AGAMOUS* or one of its closely related paralogues *SHATTERPROOF1* (*SHP1*), *SHP2*, and *SEEDSTICK* (*STK*) (Becker and Theißen, 2003). For all of these genes, except *STK*, it has been shown that their ectopic expression in *A. thaliana* leads to the formation of stamen-like organs in the 2nd whorl (Pinyopich et al., 2003). To test our hypothesis we investigated mRNA expression patterns of the orthologues of class A, B and C floral homeotic genes, and their closely related paralogues, in wild-type and *Spe* flowers of *C. bursa-pastoris*. Finally, we report identification of a candidate mutation in an allele of a putative class C floral homeotic gene that co-segregates with the mutant phenotype.

Our findings represent a valuable basis for a rigorous and comprehensive analysis of the evolutionary importance of homeosis in an experimentally tractable model system.

## RESULTS

### Structure, development and function of *Spe* inflorescences, flowers and floral organs

Wild-type plants of *Capsella bursa-pastoris* develop flowers that are very similar to those of *Arabidopsis thaliana*, including four sepals in the 1st floral whorl, four white petals in the 2nd, six stamens in the 3rd and two fused carpels in the 4th whorl (Figure 1A, C). Individual flowers of *C. bursa-pastoris* are rather inconspicuous, but at anthesis many flowers of an

inflorescence are grouped together in a corymboid structure that may serve as a recognition unit for floral visitors (Figure 1E).

In the flowers of both *Spe* variants investigated in this study, 1947-*Spe* and 1948-*Spe*, the organs of the 2nd flower whorl are transformed into stamen-like organs, thus raising the overall number of stamens or stamen-like organs per flower from six (in the wild-type) to ten, while no petals develop anymore (Figure 1B, D). The floral organs of all other whorls appear unchanged. Consequently, the inflorescences of *Spe* plants (Figure 1F) appear yellow-greenish and thus may be less attractive for visually oriented visitors than wild-type inflorescences (Figure 1E).

Since *Spe* flowers differ from wild-type flowers of *C. bursa-pastoris* mainly (if not exclusively) in the homeotic transformation of the organs in the 2nd floral whorl, these stamen-like organs were analysed in more detail. Wild-type stamens of *C. bursa-pastoris* typically consist of a long filament topped by the anther, which contains two thecae with two pollen sacs separated by the connective (Figure 1G). 3rd whorl stamens of *Spe* variants are indistinguishable from those of wild-type plants. The ectopic stamens in the 2nd whorl have the same general structure as wild-type stamens, but their filaments are shorter and their anthers are more variable in size, ranging from slightly smaller to slightly bigger than those of typical 3rd whorl organs (Figure 1H, I). Scanning Electron Microscopy (SEM) revealed that the epidermis structure of the anther of 2nd whorl stamens is very similar to that of *Spe* 3rd whorl stamens as well as stamens of wild-type plants (Figure 1J-L). In all cases cell margins are lobed and the moderately bulged surface is ridged by cuticular waxes as it was shown previously for *Arabidopsis thaliana* anthers (Smyth et al. 1990). Also, filament surfaces showed identically long rectangular cell shapes in the two *Spe* variants as well as in wild-type stamens. In both *Spe* lines the 2nd whorl stamens contain approximately half the amount or even less pollen than 3rd whorl stamens of *Spe* variants or wild-type plants.

Detailed analysis also revealed different peculiarities in the two *Spe* lines. In the variant 1947-*Spe* some anthers, in addition to having short filaments, are longer and wider than the usual ones. In these anthers the yellow colour appears brighter and the pollen sacs contain even less pollen compared to other *Spe* stamens (Figure 1M, N). Very few of the anthers show incompletely developed and empty pollen sacs and form a short petaloid blade at their tip (Figure 1O). The surface structure of those white coloured areas consists of smaller, isodiametric and dome shaped cells similar to the epidermis of wild-type petals (Figure 1P, Q), indicating some remnants of petaloidy. In the variant *Spe*-1948 the anthers do not show petaloid residuals, but they are sometimes smaller than 3rd whorl stamens, and they

contain less pollen (Figure 1I). Very few of these anthers seem to be less properly developed, with occasionally only one pollen sac per theca. Here, laceration sometimes fails so that pollen remains kept inside of the anther (Figure 1R). However, the epidermis surface of these smaller anthers was indistinguishable from those of 3rd whorl stamens in SEM analysis (Figure 1K, L).

In summary, with few exceptions the 2nd whorl organs of *Spe* plants resemble perfect stamens.

To determine whether 2nd whorl stamens of *Spe* plants do not only resemble stamens morphologically, but also functionally, we tested whether these organs produce viable pollen and whether this pollen is fertile. Pollen of variants 1947-*Spe* (Figure 1T) and 1948-*Spe* (data not shown) were treated as described by Alexander (1969) and showed the same pink to purple staining as pollen of wild-type plants (Figure 1S), indicating that the ectopic stamens of *Spe* plants produce viable pollen like the ordinary stamens of wild-type plants do.

The fertility of the pollen of ectopic stamens was tested by pollination of emasculated flowers of wild-type and *Spe* plants with the pollen of 2nd whorl stamens of both *Spe* lines (Supplemental Table 1 online). Except for two experiments, where fertilization failed, this yielded about the same number of seeds per fruit (12.8-24.5) as in positive control experiments in which pollen of 3rd whorl *Spe* stamens was used, or in which flowers of *Spe* and wild-type plants were simply left untreated for selfing as positive controls for fertilisation success (Supplemental Table 1). Seed set in all these experiments was an order of magnitude higher compared to negative control experiments in which stamens in flowers of both *Spe* lines were completely removed to provide an estimate for pollen-contamination during the preparation process (Supplemental Table 1). These data demonstrate that ectopic 2nd whorl stamens produce functional pollen like 3rd whorl stamens do.

Taken together, our data show that the *Spe* lines represent natural homeotic floral variants in which 2nd whorl organs develop into functional stamens.

We next investigated whether *Spe* flowers show not only homeotic, but also heterochronic changes. As a prerequisite for respective studies we established an overview about the stages of wild-type flower development in *C. bursa-pastoris* based on landmark events (Supplemental Table 2 online) similar to the more detailed one provided by Smyth et al. (1990) for the close relative *A. thaliana*. It turned out that the development of *C. bursa-pastoris* wild-type flowers (Figure 2A, C) very much resembles that of *A. thaliana* flowers. The only major deviation is that petals develop more slowly when compared to carpels, so

that the carpel tube is closed at the tip and starts to develop stigmatic papillae before petal tips reach level with the tips of lateral stamens (Figure 2C).

When the landmark events of flower development of the *Spe* variants 1947-*Spe* and 1948-*Spe* were compared to that of the wild-type, no significant deviations were observed (Figure 2B). There is also no change in the number or arrangement of floral primordia produced by the inflorescence meristem, or of floral organ primordia generated by the floral meristem. Also in *Spe* flowers organ development follows exactly the defined chronology observed for wild-type flowers (Figure 2A-D). Most importantly, the transformed 2nd whorl organs of both *Spe* variants show the same retardation of organ outgrowth as the petal organs of wild-type plants do (Figures 2D). This indicates that even though the 2nd whorl organs of *Spe* flowers are homeotically transformed, their development reveals no heterochronic changes, but follows the timing of wild-type 2nd whorl organs. Since in *Spe* flowers the development of the 2nd whorl stamens is delayed compared to the 3rd whorl stamens, the completion of outgrowth and the subsequent opening of the thecae and the pollen release occur later in these organs. Also late organ development seems not to be changed compared to normal petal development in terms of chronology. Carpel and fruit development shows no obvious difference between wild-type and *Spe* lines. Furthermore, the alternating 2nd whorl organ position and the assignment of abaxial and adaxial sides of the transformed organs are not affected (Figure 2).

**A co-dominant allele of a single genetic locus is responsible for the *Spe* phenotype**

To determine the transmission of the *Spe* phenotype to progeny 1947-*Spe* and 1948-*Spe* plants were selfed for six or four generations, respectively. Like the parental plants all offspring developed inflorescences in which all flowers showed a full *Spe* phenotype as described (e.g., Figure 1B), while 1947-wt plants never developed *Spe* flowers when selfed for several generations. Our observations indicate that the *Spe* phenotype is stably inherited with perfect penetrance and full expressivity over several generations, suggesting that it is based on a true mutation rather than an epigenetic change. Moreover, the fact that no wild-type plants occurred after selfing of *Spe* plants indicates that the parental plants were homozygous for all loci relevant for the *Spe* phenotype.

To identify the mode of phenotype expression both *Spe* lines were crossed with wild-type plants in reciprocal directions. The $F_1$ generations of all these crossings showed intermediate floral phenotypes, i.e. 2nd whorl organs with features of petaloidy and stamenoidy, while the

organs in all other floral whorls remained wild-typic (Figure 1U-W). The organs of the 2nd whorl differed in their degree of stamenoidy, ranging from weakly stamenoid organs mainly composed of petal blades (Figure 1V, right) to more strongly stamenoid organs composed of chimaeric antheroid/petaloid blades (Figure 1V, left). These more or less severely affected organs occurred apparently in a random way within single flowers. The surface of the chimeric organs shows isodiametric and dome-shaped cells characteristic for wild-type petals in the blade area as well as longitudinal ridged cells in the stalk epidermis which identify them as filament-like organs (data not shown). Even in case of the more severely affected stamenoid organs, however, pollen was not produced. These observations indicate that in 2nd whorl organs petaloidy and stamenoidy, respectively, are determined by wild-type and mutant alleles of *Spe* in a co-dominant way.

To determine the number of genetic loci controlling stamenoidy of organs in the 2nd floral whorl analyses of $F_2$ populations of wild-type x mutant plants and reciprocal testcrosses were performed (Table 1). Three phenotypic classes were observed: wild-type flowers, intermediate flowers as those observed in $F_1$ generations and full mutant flowers with 2nd whorl organs (almost) completely transformed into stamens, as known from 1947-*Spe* and 1948-*Spe* plants.

When the numbers of intermediate and full *Spe* phenotypes are taken together, the segregation patterns resulted in a ratio of one wild-type (genotype +/+) to about three mutant plants (*Spe*/+; *Spe*/*Spe*), thus fitting to a model of dominant inheritance of a single mutant locus (Table 1). This “1:3” model is well supported by p-values close to 1. If all three phenotypic classes are counted individually, the numbers result in a segregation ratio of one wild-type (+/+) to about two intermediate (*Spe*/+) and one *Spe* phenotype (*Spe*/*Spe*) with p-values ranging from 0.26 to 0.94. This “1:2:1” model reflects a co-dominant mode of inheritance (Table 1).

We took a closer look at the expressivity of the intermediate and the full *Spe* phenotypes in the cross where the lowest p-value was found (1a in Table 1). Among the $F_2$ generation, 31 individuals developed intermediate as well as *Spe* organs in one flower, which were initially counted as intermediate phenotypes and thus skewed segregation ratios. To test them for homozygosity at the *Spe* locus, they were selfed and the $F_3$ generations were analysed for segregation (Supplemental Table 3 online). Among the 31 $F_3$ generations seven were found, in which no segregation of the *Spe* phenotype was observed, indicating that their parental plants were homozygous. Taking these seven plants into account in an additional analysis, a new segregation ratio with a p-value of 0.95 was obtained (1b in Table 1). These data

demonstrate a reduced expressivity of 1947-*Spe*/1947-*Spe* homozygous mutant alleles when crossed into a 1947-wt background.

In contrast to these observations the analysis of crosses involving 1948-*Spe* showed a higher expressivity indicated by a better approximation to 1.0 in p-values. Additionally, differences in the severeness of intermediate organ transformation was usually visible in intermediate $F_2$ plants of the two different *Spe* line crossings, with 1948-*Spe* yielding stronger stamenoidy of 2nd whorl organs than 1947-*Spe* (Figures 1U, W).

**The same genetic locus is responsible for the *Spe* phenotype in two geographically distant populations**

1947-*Spe* and 1948-*Spe* represent plants from two populations in Germany which are about 200 km apart. To determine whether the same (or closely linked) loci are mutated in the two populations, we developed a specific crossing experiment that takes the co-dominant nature of the mutant alleles causing the *Spe* phenotype into account.

First, the two homozygous lines 1947-*Spe* and 1948-*Spe* were crossed in both directions (Table 2.1), resulting in $F_1$ generations that showed a perfect *Spe* phenotype. A plant of each $F_1$ generation was selfed and the offspring ($F_2$) was analysed. Concerning the genetic basis of the *Spe* phenotype in 1947-*Spe* and 1948-*Spe* we considered two extreme possibilities. If in both populations the same locus (or more than one but genetically closely linked loci) cause organ transformation in the 2nd floral whorl we expected only genotypes that generate a *Spe* phenotype (Table 2.2). If, however, the *Spe* phenotype is caused by different, genetically unlinked loci a segregation ratio of 1:15 typical for two-factor crosses, with 1 wild-type to 15 mutant (intermediate and *Spe*) phenotypes, was expected. In both offspring generations only *Spe* mutant phenotypes were found (Table 2.2), suggesting that the *Spe* loci in 1947-*Spe* and 1948-*Spe* are allelic (or closely linked).

To corroborate our conclusions the"mixed heterozygous" plants generated in the $F_1$ generations were backcrossed with homozygous 1947-wt plants in both directions, resulting in several offspring generations to be analysed for segregation (Table 2.2). Again two extreme scenarios were considered. In case of 1947-*Spe* and 1948-*Spe* being affected at the same locus an intermediate phenotype was expected for all offspring; in case of different, unlinked loci the typical two-factor backcross segregation pattern was expected, with genotypes 1 (+/+; +/+) to 1 (*Spe*/+; +/+) to 1(+/+; *Spe*/+) to 1(*Spe*/+; *Spe*+) that result in a relation of 1 wild-type to 3 mutant phenotypes (Table 2.2). Except in one case (see below) in the offspring

generations either only mutant phenotypes (selfed offspring) or only intermediate phenotypes (backcross with the wild-type) were found (Table 2.2), strongly supporting our hypothesis that in 1947-*Spe* and 1948-*Spe* the same loci are mutated. However, the intermediate plants showed a very broad spectrum of phenotypes, ranging from hardly distinguishable from wild-types to almost perfect *Spe*-like, with typical intermediate forms being more frequent than extreme ones. Generally, the offspring of reciprocal cross B had the distribution of phenotypes shifted towards less severe petal transformation compared to cross A. Many of the analysed offspring plants showed only few intermediate organs while the majority of flowers was wild-type. In one exceptional case (Table 2.2, cross no. 6) we even found a considerable number of plants without traces of mutant phenotype and hence classified them as "wild-type". We assume, however, that this represents just an extreme case of low penetrance of the mutant phenotype. Hence, our data suggests that expression of the *Spe* mutant phenotype depends on mutant background.

**Expression patterns of floral organ identity genes in *Spe* compared to wild-type flowers**

To determine whether the expression of floral homeotic genes is changed in *Spe* compared to wild-type flowers, detailed studies employing *in situ* hybridization were done. To generate specific hybridization probes we isolated cDNAs of the genes of interest with a focus on members of the *AGAMOUS* clade of genes. In *C. bursa-pastoris* we found two distinct sequences for every gene from *A. thaliana*, which very likely reflects the tetraploidy of the *C. bursa-pastoris* genome. Since *C. bursa-pastoris* has disomic rather than tetrasomic inheritance we treated the sequence variants as different loci rather than alleles here, distinguished by suffixes "a" and "b".

Complete coding sequences where isolated from *CbpAGa* and *CbpAGb* (putative co-orthologues of *AGAMOUS*), *CbpSHP1a* and *CbpSHP1b* (putative co-orthologues of *SHATTERPOOF1*), *CbpSHP2a* and *CbpSHP2b* (putative co-orthologues of *SHATTERPOOF2*), and *CbpSTKb* (putative co-orthologue of *SEEDSTICK*). Of *CbpSTKa* only a partial sequence containing 5´-UTR, MADS-box and part of the I-region was isolated from cDNA. To test wether additional *AGAMOUS* clade members are present in the *C. bursa pastoris* genome we performed genomic DNA gel blot ('Southern') hybridization experiments using genomic sequences (including introns) spanning most part of the I region to the start of the C-terminal region as probes. Except for *CbpSHP1* probes, where four bands were observed in case of one restriction enzyme being used (Supplemental Figure 1C online,

lanes 1, 7), just one or two bands were detected with both wild-type and *Spe* genomic DNA (Supplemental Figure 1). In some cases a two band pattern in a lane can be traced back to cutting sites in the digested genomic DNA (Supplemental Figure 1). These results corroborate that at least three of the four *AG*-clade members from *A. thaliana* have two co-orthologues in the *C. bursa-pastoris* genome, which could also be true for *SHP1*, in which case the four bands would represent allelic polymorphisms rather than additional genetic loci. However, the presence of additional copies of *CbpSHP1* genes beyond *CbpSHP1a* and *b* cannot be excluded at the moment.

Correct classification of the gene pairs as co-orthologues of their *A. thaliana* counterparts was verified through phylogenetic reconstructions with these sequences in comparison to other angiosperm *AG* clade members (sequence alignment and phylogenetic tree in the Supplemental Figures 2 and 3 online, respectively).

For *CbpAP3a/b, CbpPI*a/b and *CpbAP1a/b*, orthologues of the respective floral homeotic genes from *A. thaliana*, as well as for one *CbpH4* (*Histone 4*) gene only partial sequences were isolated. Due to high sequence similarity, co-orthologous sequences are expected to cross-hybridize. On the following, therefore, we do not distinguish between sequence variants "a" and "b".

Standard *in situ* hybridization protocols were adapted to the flowers of *C. bursa-pastoris* employing probes for a *CbpH4* gene, which is mainly transcribed in replicating cells (Brandstädter et al., 1994; Groot et al., 2005). Wild-type and *Spe* floral tissues showed the same characteristic punctuate expression pattern in which only replicating cells are strongly stained (Figure 3A, B), suggesting that cell division activity is not drastically altered in *Spe* compared to wild-type flowers.

*CbpAP3* and *CbpPI* showed very similar expression patterns in *C. bursa-pastoris* as the class B floral homeotic genes *AP3* and *PI*, their orthologues in *A. thaliana*. Differences between wild-type and *Spe* flowers were not observed (Figure 3C-J). Starting already at developmental stage 2 and stage 3, floral buds express *CbpAP3* in a circular zone between centre and margin of the bud, where organs of the 2nd and 3rd flower whorl will arise (Figure 3C, D). In the following stages, the signal was mainly restricted to developing organs of those whorls (Figure 3E-H). From about stage 8 onwards, when 2nd whorl organ primordia are still small but filaments and thecae of 3rd whorl stamens already developed, weak expression of *CbpAP3* was also detected in developing carpel tissue (Figure 3F, H). Expression of *CbpAP3* was detected in the upper parts of petals and in anthers of 2nd and 3rd whorl stamens until stage 12 (Figure 3G, H). At early developmental stages of wild-type and

mutant plants expression of *CbpPI* showed the same pattern as *CbpAP3*, in that the signal was detected in regions where 2nd and 3rd whorl organs will arise (Figure 3I, J).

In both wild-type and *Spe* inflorescences, strongest *CbpAP1* expression was found in the floral primordia of stage 1 and 2 (Figures 3K-M). At stage 3 expression of *CbpAP1* was still detectable in the sepal primordia and the adjacent cells leading to the central dome of the floral bud in wild-type plants (Figure 3N). But at stage 6, when first primordia of 2nd whorl became visible, *CbpAP1* expression was not detectable anymore (Figures 3O, P). Differences in *CbpAP1* expression between wild-type and *Spe* flowers were not apparent.

The expression of *CbpAG* in wild-type floral buds (Figure 4A) was not easy to detect and to distinguish from background staining probably because of a low expression level. Earliest *CbpAG* expression was detected in stage 3 and 4 buds in the centre of the floral meristem of both wild-type and *Spe* plants (Figure 4A-C). At stage 6, when stamen and carpel primordia in the 3rd and 4th floral whorl are well developed and the tiny primordia of 2nd whorl organs arise, *CbpAG* expression was detected in the organs of the two inner whorls of both wild-type and *Spe* flowers (Figure 4D-F). Only in *Spe* flowers, however, *CbpAG* shows also a staining signal in the small organ primorida of the 2nd whorl (Figure 4E). Even at later developmental stages *CbpAG e*xpression remains to be restricted to the organs of the 3rd and 4th whorl in wild-type flowers, whereas in *Spe* flowers also in developing 2nd whorl organs *CbpAG* expression was detected (Figure 4G-I). In flowers after stage 9 expression is limited to rapidly developing tissues like ovules and stigmatic papillae in carpel and anthers of the 3rd whorl stamens and, in *Spe*, also in the 2nd whorl stamens (Figure 4J-L). In flowers of this age we also detected *CbpAG* expression throughout the nectaries at the base of the 3rd whorl stamens in both the wild-type and in *Spe* (Figure 4J, and data not shown).

*CbpSTK* expression at stage 4 was detected in young stamen and carpel primordia (Figure 5A-C). At stage 9 a signal was not detectable any more in wild-type flowers (Figure 5D), but in *Spe* flowers mild expression in developing stamens and carpels was still visible (Figure 5E). In addition to the expression in 3rd and 4th whorl organs, ectopic expression of *CbpSTK* was also found in the stamens developing in the 2nd whorl, where expression appears stronger than in the 3rd whorl (Figure 5E). However, strongest expression of *CbpSTK* was detected at late stages in developing ovules of both wild-type and *Spe* plants as it was predictable from the knowledge about *Arabidopsis* flower development (Figures 5G-I).

Neither expression of *CbpSHP1*, nor of *CbpSHP2* was detected in any of the early developmental stages of floral buds in wild-type (data not shown) and *Spe* plants (Figures 5J-

L). Only at later stages, when ovules developed in the carpel, we could detect weak expression signals at stage 8 floral bud of the *Spe* variant in the marginal cell layers of the placental zones of the ovule (Figure 5M). This demonstrated that expression of *CbpSHP1* and *CbpSHP2* could be detected under our conditions, but since such a late expression is very likely irrelevant for the *Spe* phenomenon (organ identity in the 2nd whorl), we did not study it in a comparative way. Nevertheless, we examined fruits after fertilisation and found *CbpSHP1* and *CbpSHP2* expression in the valve margins as well as in the endothelium of ovules, exemplarily shown in Figure 5N-Q. In summary, *CbpSHP1* and *CbpSHP2* expression patterns are similar to those in developing *Arabidopsis* flowers (Flanagan et al., 1996).

**_CbpAGa_, but none of the seven other _AG_-like genes, co-segregates with the _Spe_ mutant phenotype**

Our previous results suggested that a mutation in either an orthologue of *AGAMOUS* or *SEEDSTICK*, or a negative regulator of the transcription of these genes in the 2nd floral whorl is responsible for the *Spe* phenotype. We thus checked the co-segregation of mutant- and wild-type-specific alleles of all *AG*-like genes in an $F_2$ population obtained by a cross between 1947-wt and 1947-*Spe*. To identify sequence polymorphisms distinguishing alleles from the wild-type and *Spe* parent we amplified and sequenced about 5 – 7.5 kbp of genomic sequence from each of the parents for loci *CbpAGa*, *CbpAGb*, *CbpSHP1a*, *CbpSHP1b*, *CbpSHP2a*, *CbpSHP2b*, *CbpSTKa* and *CbpSTKb*. For each locus sequence comparisons identified 4-16 candidates for sequence polymorphisms distinguishing parental alleles. All except one were located in non-coding regions such as introns and regions upstream of the coding region. As marker for the co-segregation analysis at least one SNP (Single Nucleotide Polymorphism) per gene was verified by pyrosequencing genomic DNA of the parents and the $F_1$ generation.

Genotyping of 9-11 wild-type plants of the $F_2$ population by pyrosequencing revealed recombination between the candidate loci under investigation and the floral phenotype for all genes except one, *CbpAGa* (Table 3). This implies that *CbpAGb*, *CbpSHP1a*, *CbpSHP1b*, *CbpSHP2a*, *CbpSHP2b*, *CbpSTKa* and *CbpSTKb* cannot represent the *Spe* locus, leaving *CbpAGa* as the only remaining candidate. We thus checked for co-segregation between the informative SNP and the *Spe* phenotype in the whole $F_2$ population, comprising 196 plants (Supplemental Table 4 online). Segregation of both phenotype and SNP followed again a 1:3 ratio model supported by a p-value of 0.9. For 191 plants both genotype and phenotype data could be determined, revealing a perfect co-segregation between SNP character state and

phenotype (Supplemental Table 4). This reveals that *Spe* and *CbpAGa* are genetically closely linked, or even identical, loci.

A detailed sequence comparison between the *CbpAGa* alleles from the parental plants revealed a total of 16 sequence polymorphisms, all upstream of the coding region and in the 1st and 2nd intron. 15 of the corresponding mutations are very unlikely the cause of the *Spe* phenotype. Most of these represent short microsatellites or polybase pair-stretches in little conserved regions, the others are SNPs representing autapomorphies in little conserved regions of the *CbpAGa* sequence of wild-type plants, as revealed by multiple sequence alignments (data not shown). The only remaining sequence difference is a combined putative deletion of 22 bp and a substitution of three bp in the 2nd intron (Figure 6). Considerable conservation of the corresponding region in *AG* orthologues throughout the Brassicaceae reveals that this indel almost certainly represents a deletion in the *CbpAGa* allele of the mutant (*Spe*) parent rather than an insertion in the allele from the wild-type parent (Figure 6). This characteristic deletion is absent in the *CbpAGa* allele of 1948-*Spe*, the other *Spe* line investigated here, as revealed by PCR analysis (see Supplemental Figure 4 online).

## DISCUSSION

We are establishing the *Spe* variant of *C. bursa-pastoris*, a plant that is known from wild habitats for at least about 200 years, as a model system to investigate the evolutionary potential of floral homeotic mutants (see also Theißen, 2006; Nutt et al., 2006; Hintz et al., 2006). Within this framework, the goal of the studies described here was to clarify the phenotype and molecular developmental genetics of the *Spe* variant.

### The *Spe* variant as a new model for a widespread phenomenon in flowering plants

A detailed morphological investigation involving plants from two populations revealed that the *Spe* lines represent a natural floral homeotic variant in which 2nd whorl organs develop into (almost) perfect stamens from both a morphological and functional point of view. Changes in other floral whorls, or any other pleiotropic effects on vegetative organs, were not observed. This phenotype is remarkable in that it displays a full homeotic conversion in organ identity in only one floral whorl, since in the typical floral homeotic mutants of the close relative *A. thaliana* always two adjacent whorls are affected (Coen and Meyerowitz, 1991).

The syndrome of petals transformed into stamens is known for many flowering plants, such as the monocots tulip (*Tulipa*), hyacinth (*Hyacinthus*), daffodil (*Narcissus*), meadow saffron (*Colchicum*), *Iris, Crocus* and different orchids, as well as for many eudicots, including *Ranunculus*, *Papaver* and a number of higher eudicots (Murbeck, 1918; Ronse De Craene, 2003). While our analysis strongly suggests that the *Spe* phenotype of *C. bursa-pastoris* is based on mutation of a single locus, it has not been observed so far in a single gene mutant of *A. thaliana*, despite the numerous mutagenesis experiments that have been carried out with this species. Except for transgenic plants a *Spe* phenotype in *A. thaliana* is only known from the *rbe roxy1* double recessive mutant affected in two loci, *RABBIT EARS* (*RBE*) and *ROXY1* (Xing et al., 2005). Therefore, with the molecular cloning of the *Spe* gene potential regulatory differences between *C. bursa-pastoris* and *A. thaliana* concerning the specification of organ identity in the 2nd floral whorl, may soon become apparent. Such differences may exist, for example, due to the tetraploidy of the *C. bursa-pastoris* genome. However, conservation of the site mutated in *CbpAGa*, the *Spe* candidate locus, suggests that similar mutations could be generated also in other Brassicaceae. It would be interesting to investigate, therefore, whether the *CbpAGa* mutation identified in the 2nd intron of *Spe* plants in a full-length genomic clone of the *AGAMOUS* locus transformed into wild-type *A. thaliana* generates a dominant *Spe* phenotype. If so, one could conclude that it's only by chance that such a mutation did not appear in *A. thaliana* yet.

In any case, the mutant variant of *C. bursa-pastoris* described here provides us with a new, experimentally tractable model system to investigate the *Spe* syndrome, a phenomenon that is widespread in flowering plants, but has been neglected by molecular plant research so far, possibly because of its absence in the model plant *A. thaliana*.

### *Spe* genetics - simple but revealing

Our genetic analyses demonstrated that co-dominant inheritance of the *Spe* phenotype in both populations, 1947-*Spe* and 1948-*Spe*, is stable and caused by a change in DNA sequence (rather than an epimutation) at a single genetic locus, even though the involvement of two or more closely linked loci cannot be completely ruled out. The segregation ratios observed corroborated the long held but little tested view that *C. bursa-pastoris*, despite its tetraploid genome, has disomic rather than tetrasomic inheritance (Hurka et al., 1989).

Our analyses revealed that 1947-*Spe* and 1948-*Spe* are mutated at loci that are at least closely linked, with the by far most simple and likely hypothesis being that the *Spe* loci in plants from both populations are allelic.

Segregation patterns of $F_2$ populations that fit into 1:2:1 models demonstrated that in a heterozygous condition the *Spe* allele controls stamenoidy of the organs in the 2nd floral whorl in a co-dominant fashion. This mode of inheritance indicates that the *Spe* effect is gene (better: allele) dose dependent. Moreover, we hypothesize that a co-dominant allele is more likely to represent a gain-of-function mutation than a recessive allele. Remarkably, gain-of-function often results from the ectopic expression of a gene. Prominent examples involving genes encoding transcription factors include *Knotted1*, *Gnarley1* and *Rough Sheath1* from maize, and *Hooded* from barley (Vollbrecht et al., 1991; Foster et al., 1995; Schneeberger et al., 1995; Müller et al., 1995). We consider it quite likely, therefore, that the *Spe* phenotype is brought about by the ectopic expression of a developmental gene controlling stamen identity. An intriguing precedent case is provided by the *Ovulata* (also known as *Macho*) mutant of snapdragon (*Antirrhinum majus*), which results from the insertion of a transposon into the 2nd regulatory intron of the *AGAMOUS* orthologue *PLENA* (Bradley et al., 1993). This created a dominant gain-of-function allele of *PLENA* that is ectopically expressed in the outer two whorls of the flower, resulting in mutant flowers in which sepals are replaced by carpels, and petals by stamens (Bradley et al., 1993). Despite the fact that stamenoidy of 2nd whorl organs is accompanied by carpelloidy of 1st whorl organs, the similarity to the *Spe* system at the genetic level is striking. We therefore tested how far this similarity goes down to the molecular level.

**Ectopic expression of two organ identity genes accompanies organ transformation in *Spe* flowers**

According to the ABC model we hypothesize that a class C gene, or a close relative, is ectopically expressed in the 2nd floral whorl of *Spe* flowers, while the expression of class A genes is reduced, and that of all other organ identity genes is unchanged in all whorls.

To test our hypothesis we studied the expression of orthologues of floral organ identity genes of *A. thaliana* in *Spe* and wild-type flowers of *C. bursa-pastoris*. In addition to orthologues of *AGAMOUS* (class C), *APETALA1* (class A), and *APETALA3* and *PISTILLATA* (class B) we considered also *SEEDSTICK* and *SHATTERPOOF1* and *2*, since they show high sequence similarity to *AG*. Expression of class E (*SEPALLATA*) genes was not determined,

since their function is essential for the development of all floral organ identities. Also the A-class gene *AP2* was not tested, because in *A. thaliana* it is expressed in all floral organs and regulated post-transcriptionally (Jofuku et al., 1994).

Expression of the putative class A floral homeotic gene *CbpAP1* was analysed in order to test whether the antagonistic regulation between class C and class A floral homeotic genes known from *A. thaliana* (Mandel et al., 1992, Gustafson-Brown et al., 1994) is also operating in *C. bursa-pastoris*. We detected *CbpAP1* only at early developmental stages, which is in contrast to the *AP1* expression in *A. thaliana*. Our observations did not provide any evidence for regulatory interactions between *CbpAP1* and *CbpAG*, including repression of *CbpAP1* by *CbpAG* activity. We hypothesize that in *C. bursa-pastoris* early expression of *CbpAP1* is predominantly responsible for conferring floral meristem identity, whereas a function as organ identity gene is less well developed or even absent, due to the early termination of expression.

Since the development of petals and stamens requires class B floral homeotic gene expression, differences in the expression of these genes between wild-type and *Spe* flowers were not expected. And indeed, expression patterns of the putative class B floral homeotic genes *CbpAP3* and *CbpPI* were found to be indistinguishable in wild-type and *Spe* flowers of *C. bursa-pastoris*, and very similar to those of *AP3* and *PI*, respectively, in *A. thaliana* flowers (Jack et al., 1992; Goto and Meyerowitz, 1994).

The most obvious candidate genes for providing the ectopic class C gene activity are an orthologue of the canonical *Arabidopsis* class C gene *AGAMOUS*, or one of its closely related paralogues *SHATTERPROOF1* (*SHP1*), *SHP2*, and *SEEDSTICK* (*STK*), which are all members of the clade of *AG*-like genes (Becker and Theissen, 2003). Our expression analysis of the genes of the *AG*-clade demonstrated that *CbpAG* and *CbpSTK*, orthologues of *AG* and *STK*, are ectopically expressed in the organs developing in the 2nd floral whorl of *Spe* flowers. Besides this ectopic expression *CbpAG* transcription signals in the inner two whorls of the flower were very similar to that in wild-type *C. bursa-pastoris* and *A. thaliana* flowers (Bowman et al.,1991; Drews et al., 1991). In contrast to that, the expression of *CbpSTK* in young stages of 3rd and 4th whorl organs of both wild-type and *Spe* flowers shown here has not been described for *Arabidopsis* flowers (Rounsley et al, 1995; Colombo et al., 1995). For orthologues of the *SHP* genes, *CbpSHP1* and *CbpSHP2*, no signals of expression in flower buds before developmental stage 8 were detected, and never in developing stamens, strongly suggesting that the *SHP* orthologues are not involved in the homeotic transformation in *Spe* flowers.

It is known that ectopic expression of *AG*, *SHP1* or *SHP2*, but not *STK*, is sufficient to transform 2nd whorl floral organs into stamens in the flowers of *A. thaliana* (Pinyopich et al., 2003). Remarkably, transformation of petals into stamens without other changes, was observed in transgenic plants that express *AG* under control of the promoter of the class B gene *AP3*, driving gene expression in the 3rd floral whorl – where *AG* is expressed anyway -, but also ectopically in the 2nd whorl (Jack et al., 1997). This demonstrates that ectopic expression of *AG* restricted to the 2nd floral whorl is, at least in *Arabidopsis*, sufficient to bring about a phenotype perfectly resembling *Spe*. Our findings suggest that a similar mechanism brings about the *Spe* phenotype.

Assuming that also in *C. bursa-pastoris* the *AG* gene, but not the *STK* gene confers stamenoidy, the question remains why both genes are ectopically expressed in *Spe* flowers. We have shown that the *Spe* phenotype is very likely caused by mutation at a single locus, and it is *a priori* unlikely that both genes are mutated in *Spe* plants. Our mapping data demonstrate that the *Spe* phenotype, and hence ectopic expression of *CbpAG*, is linked to the *CbpAGa* locus, but not to *CbpSTK*. Therefore, it appears plausible that *CbpAG* expression activates *CbpSTK*. This is in line with the fact that in *A. thaliana*, *AG* has the broader function in specifying stamen as well as carpel development, while the *STK* function is restricted to aspects of carpel (i.e., ovule) development (Pinyopich et al., 2003, Favaro et al. 2003). This means that in *C. bursa-pastoris*, *CbpSTK* being a target of *CbpAG* is quite conceivable.

Ito et al. (2007) have shown that in *A. thaliana* stamen development requires prolongued *AG* activity, as *AG* works there over different transcriptional cascades in different floral stages. Our finding that *CbpAG* is continuosly expressed during 2nd whorl stamen development in *Spe* flowers strengthens our hypothesis that the development of ectopic stamens is controlled by *CbpAG*.

Taken together, our findings strongly suggest that the *Spe* phenotype in *C. bursa-pastoris* is brought about by the ectopic expression of a *CbpAG* gene in the organs of the 2nd floral whorl, as summarised in a model in Figure 7, even though an involvement of *CbpSTK* cannot be excluded.

**Molecular cloning of the *Spe* gene by a candidate gene approach**

Concerning the ectopic expression of a *CbpAG* gene in the 2nd floral whorl, the *Spe* variants may be either mutated in one of the two *CbpAG* loci, or in a direct or indirect negative regulator of *CbpAG*. Our linkage analysis (Table 3) eliminates with one strike 7 out of 8 *AG*-

like genes (one *CbpAG*, four *CbpSHP* and two *CbpSTK*) as candidate genes for the *Spe* locus. In contrast, the *CbpAGa* allele from mutant plants perfectly co-segregates with the mutant *Spe* allele in a mapping population involving 191 plants, indicating that the respective loci are genetically closely linked. We conclude that *Spe* and *CbpAGa* are located on the same chromosome in close vicinity with a distance probably below 1 cM. The simplest hypothesis would be that *Spe* is just a mutant allele of *CbpAGa* rather than a *trans*-acting regulator closely linked to *CbpAGa*. On the following, we provide cumulative evidence strongly supporting that view.

In *A. thaliana*, quite a number of negative *trans*-acting regulators of *AG* are known, such as *APETALA2* (*AP2*) (Jofuku et al., 1994), *LEUNIG* (*LUG*) (Liu und Meyerowitz, 1995), *SEUSS* (*SEU*) (Franks et al., 2002), *CURLY LEAF* (*CLF*) (Goodrich et al., 1997), *BELLRINGER* (BLR) (Bao et al., 2004), *EMBRYONIC FLOWER1* and *2* (*EMF1, 2*) (Calonje et al., 2008), *RABBIT EARS* (*RBE*) (Krizek et al., 2006), *STERILE APETALA* (*SAP*) ( Byzova et al., 1999) and *ROXY1* (Xing et al. 2005). In contrast to *Spe* from *C. bursa-pastoris*, however, mutant alleles are usually recessive, and mutant plants often show incomplete transformation of petals into stamens (if any), and/or display considerable pleiotropic effects beyond stamenoidy of petals, sometimes even outside of the flower. For example, Fray et al. (1997) described a double mutant of *Brassica napus* (rape seed) possibly affected in an orthologue of *CLF*. Mutant flowers very much resemble the *Spe* phenotype, but vegetative leaves show a strong curly leave phenotype. In the *roxy1* and *rbe* single mutants of *A. thaliana* mainly organs of the 2nd floral whorl are affected, but complete changes in organ identity (homeotic transitions) are not observed (Xing et al. 2005; Krizek et al., 2006). In *BELLRINGER* mutants, carpelloid sepals rather than stamenoid petals are observed (Bao et al., 2004). We hypothesize, therefore, that the complete floral homeotic transitions seen in *Spe* are more likely due to a mutation in a floral homeotic gene rather than a *trans*-acting regulator. Moreover, we observed that none of the negative regulators of *AG* known so far is genetically closely linked with the *AG* locus. Since the order, orientation and sequence of genes are very similar in the genomes of *Arabidopsis* and *Capsella* (Boivin et al., 2004), it is likely that the same is true for orthologous genes in the genome of *C. bursa-pastoris* as well. In that case, *Spe* cannot be such a regulator of *CbpAG*, because we have shown that both loci are closely linked. It thus appears more likely that the *Spe* phenotype is caused by a mutation in a *cis*-regulatory element in the sequence of *CbpAGa* itself. Such a regulatory element would be required to keep expression of the gene out of the 2nd floral whorl, e.g. by binding a negative *trans*-acting factor.

It thus appears striking that the only major sequence difference distinguishing the *CbpAGa* allele of wild-type and *Spe* plants that we could identify affects a highly conserved region in the 2nd intron. The very long intron of *AG* genes (about 3.5 kb in *A. thaliana*) is well known for its regulatory function not only in *Antirrhinum* (*PLENA* gene, see above), but also in *A. thaliana* (see e.g. Sieburth and Meyerowitz, 1997; Busch et al., 1999; Deyholos and Sieburth, 2000, Hong et al., 2003). This intron contains numerous binding sites for *trans*-acting factors, comprising both activators and repressors of *AG* activity. The sequence polymorphism distinguishing *CbpAGa* from *Spe* and wild-type plants is located in the 5' region of the second intron, at about 30 % of relative intron length. In line with our findings, this part of the intron has previously been demonstrated to be responsible for stamen development in *A. thaliana*, whereas the 3' part was shown to be involved in regulating carpel and ovule development (Deyholos and Sieburth, 2000). We hypothesize that in case of the polymorphism discussed here a sequence motif responsible for negative regulation of *CbpAGa* in the 2nd floral whorl of *C. bursa-pastoris* is partially or fully deleted. This sequence motif may well bind one (or more) *trans*-acting factor, most likely a protein working as a transcription factor or transcriptional co-regulator.

Quite a number of *trans*-acting factors directly or indirectly binding to the 2nd intron of *AG* are known (reviewed in Liu and Karmakar, 2008). Positively acting regulators, such as *LEAFY* (*LFY*) and *WUSCHEL* (*WUS*), are not considered here further, because the *Spe* phenomenon is thought to be based on abolishment of binding of a negative regulator. Also for some, but not all, negative regulators acting via the 2nd intron binding sites are known (Bao et al, 2004, Nole-Wilson and Krizek 2000). *APETALA2* (*AP2*) and *AINTEGUMENTA* (*ANT*) are both members of the AP2/EREBP-gene family encoding transcription factors and are known as negative regulators of *AG* in *A. thaliana*, but their consensus binding site differs strongly from the polymorphic site identified here (Jofuku et al 1994; Krizek et al. 2000; Nole-Wilson and Krizek 2000)**.** *LEUNIG* (*LUG*) and *SEUSS* (*SEU*), encoding interacting members of different protein families, are two other negative regulators of *AG* (Franks et al. 2002). They do not bind directly to DNA, however, but are probably recruited to the DNA of the 2nd intron by binding to dimeric complexes of MADS-domain proteins AP1, AGL24, SEP3 or SHORT VEGETATIVE PHASE (SVP) (Gregis et al., 2006; Sridhar et al., 2006; Liu and Karmakar, 2008). MADS-domain proteins bind to CArG-boxes (consensus 5'-$CC(A/T)_6GG$-3'), but such DNA-sequence elements are not obvious in the *CbpAGa* polymorphic site considered here (Figure 6). The BELLRINGER (BLR) protein is also a supposed binding partner of LUG and/or SEU, but its known binding site in the *AG* 2nd

intron of *A. thaliana* is located about 500 bp downstream of the polymorphic site and differs clearly in its sequence (Bao et al., 2004). *PETAL LOSS* (*PTL*) and *ROXY1* are also negative regulators of *AG* in *A. thaliana*, but are very likely not involved in recognising the polymorphic site as they work indirectly by posttranslational modification of other repressors (Brewer et al., 2004, Xing et al., 2005).

Currently the arguably most likely candidate for a factor binding to the sequence deleted in the putative *Spe* allele could be the orthologue of *RABBIT EARS* (*RBE*), because the function of *RBE* is restricted to the 2nd whorl of the flower and not much information is currently available that rules out its ability to bind to a putative binding motif located in the sequence polymorphism of *Spe*. *RBE* encodes a Zn-finger protein whose DNA-binding motif contains a AGT-core sequence (Krizek et al., 2006). Such a motif is apparent in the polymorphic site (Figure 6), and another one in close proximity, but the probability to find such a short motif by chance is already quite high in any random sequence of three nucleotides (1:64). Moreover, the *rbe* loss-of-function phenotype in *A. thaliana* is much weaker than the *Spe* phenotype, so in *CbpAGa* more than the binding site of just one factor has possibly been deleted.

Nevertheless, *Spe* could be a yet unknown gene that, just by chance, is closely linked to the *CbpAGa* locus, and not related to the ectopic *CbpAGa* expression. Taken together, however, all pieces of evidence fall so nicely into place that we are quite confident that with *CbpAGa* we have cloned the *Spe* locus already. We thus favour the by far most simple and arguably most likely hypothesis that *Spe* is a mutant allele of *CbpAGa* in which a previously unrecognized negative *cis*-regulatory element in the 2nd intron, which is involved in keeping class C homeotic gene expression out of the 2nd floral whorl, has been deleted.

If the sequence change in the 2nd intron of *CbpAGa* is the critical mutation of the *Spe* locus in 1947-*Spe*, we can already conclude that 1948-*Spe* contains a different allele that lacks the respective deletion (Supplemental Figure 4). This suggests that both alleles, and thus probably also both populations of *Spe* plants, originated independently from wild-type alleles/populations. Whether it is just by chance or due to some intrinsic instability that the same locus has been affected in both *Spe* populations remains to be seen until even more populations have been investigated.

Definitive evidence that *Spe* is an allele of *CbpAGa* requires further experimentation, e.g. transformation of the mutant genomic locus from a *Spe* plant, including all regulatory sequences upstream and downstream of the coding region, into a wild-type plant of *C. bursa-pastoris*. Transformation protocols for *C. bursa-pastoris* that may facilitate such work have

recently been developed (Bartholmes et al., 2008). Since *Spe* is co-dominant, transformants should show a *Spe* or intermediate phenotype, thus demonstrating that *CbpAGa* is *Spe*. If different populations with a *Spe* phenotype show different mutations, but in the same locus, this would also corroborate the hypothesis that *Spe* is an allele of *CbpAGa.*

In any case, cloning of the *Spe* locus opens the door for investigations on the molecular evolution of a gene that may facilitate saltational evolution by homeosis.

## METHODS

### Plant material

*Capsella bursa-pastoris* (L.) Medik. selfed offspring was used from individuals of the wild-type line 'wt 6', and individuals from *Spe*-variant lines '*Spe* 9 and *Spe* 8. All these lines originated from population '1947', located in Gau-Odernheim (Rheinhessen, Germany). Additional selfed offspring used was from line '*Spe* 2', of the population '1948', located near Warburg (Westfalen, Germany) (Nutt et al., 2006). The plant lines are here referred to as ´1947-*Spe*´, ´1947-wt´ and ´1948-*Spe*´, respectively (Bartholmes et al., 2008). *Capsella* population and plant line numbers refer to the Brassicaceae Germ Plasm Collection of the Department of Systematic Botany, University of Osnabrück, Germany.

To test for stable inheritance members of each plant line were arbitrarily chosen and continuously selfed for four or six generations, resulting in two complete inbred lines of 1947-*Spe* and one in each case of 1948-*Spe* and 1947-wt, respectively.

### Genetic crosses

Plants serving as female parents were emasculated by removal of all organs except for the carpel a day before the floral buds would have been opened and after a day off for maturation of the stigma they were pollinated. Members of all $F_2$ generations were selfed after analysis and seeds stored at 4°C. Pedigree diagrams in Supplemental Figure 5 online show how genotypes for the genetic analysis were generated.

**Microscopy**

Flowers, floral organs and pollen staining were observed under a binocular microscope LEICA MZ-FLIII. *In situ* hybridizations have been documented with a LEICA DM 5500B microscope. Samples for Scanning Electron Microscopy (SEM) were fixed in FAEG Fixative (formaldehyde 3%, acetic acid 5%, ethanol 65%, glutaraldehyde 0.2%) containing 0.1% Tween 20 for 16-20 h at 4°C. The tissue was dehydrated through an ethanol series up to 100% alcohol followed by an alcohol-acetone series up to 100% acetone. After critical point drying with $CO_2$, samples were spottered with gold. Pictures were collected on a Phillips XL 30 ESEM scanning electron microscope.

**Test for Pollen functionality**

Viability of the pollen grains was tested with Alexander´s Stain (Alexander, 1969), containing malachite green which stains cellulose in pollen walls turquoise, and acid fuchsin which stains the pollen protoplasm purple. The stock stain solution was diluted 1:50 with 10% acidic acid for differentiation. Already opened anthers with the pollen mass presented were soaked with the diluted stain solution and immediately photographed under a binocular microscope. 2nd and 3rd whorl stamens both from varieties 1947-*Spe* and 1948-*Spe* (five flowers per plant; three plants per line) were analysed, as well as stamens from one 1947-wt plant (five flowers).

In fertility tests flowers from pollen recipients ("mother" plants) were emasculated by removing sepals and all stamens in the latest bud stages shortly before opening to avoid self-fertilisation in the crosses. Thereafter carpels of these plants were pollinated with pollen from 2nd whorl stamens of pollen donor ("father") plants. The tested plant lines and control crossings are listed in Supplemental Table 1 online. This procedure was carried out daily over a time span of two to three weeks. As a measure for fertilisation success seed numbers per fruit were scored.

**Molecular cloning of cDNA fragments**

The cDNA from total RNA of *C. bursa-pastoris* inflorescences was generated with Oligo d(T)- containing primers according to standard protocols. The 3´ and 5`RACE (Rapid Amplification of c-DNA Ends) were performed using the 5´/3´RACE kit (Roche, Mannheim,

Germany) according to the manufacturer´s instructions. Primers used for amplification will be provided upon request.

For phylogenetic analysis of the genes *CbpAG, CbpSHP1, CbpSHP2* and *CbpSTK* full length sequences including the UTRs were generated with primers derived from UTR sequences obtained in the 3´ and 5´ RACE-cloning (for primer sequences see Supplemental Table 5 online).

In general all PCR-fragments (including genomic fragments) were gel-purified and cloned into pGEM-T (Promega), pJET1 or pBluescript II SK(+) (both Fermentas) and afterwards sequenced with the respective vector primers.

### *In situ* hybridization

Organ primordia of the 2nd and 3rd floral whorl and developmental stages have been checked for correct identification by inspection of cutting series through the respective flowers.

Influorescences were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, containing 0.1% Tween 20 (16h at 4°C). Post fixation steps, dehydration by Histo-clear, embedding, sectioning and prehybridization was carried out according to the protocol of Zachgo (2002) except triethanolamine treatment was skipped. Samples were digested with 1 µg/ml Proteinase K in 2 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.0, for 30 min. Templates for *in vitro* transcription were generated by PCR and contain a T7 RNA polymerase binding site either at the 3´end (antisense probes) or the 5´end (sense probes). Either at the 5´end (antisense probes) or the 3´end (sense probes) a recognition site for the restriction enzyme *XbaI* or *XhoI* was introduced and digested after template generation by PCR (for primers see Supplemental Table 5 online). All template sequences lack the MADS domain to avoid cross hybridization and have the 3´UTR included (for an alignment of probes see Supplemental Figure 6 online, and for primers Supplemental Table 5). *In vitro* transcription, hydrolysis of probes to an approximate length of 150 bp, hybridization, post hybridization washes and immunological detection were also performed according to the protocol of Zachgo (2002). Usually 0,5µg - 1µg DIG- labelled probe/ 100µl hybridization solution was used. Hybridization was carried out at 50-52°C for 12-16 h and the final washing steps were done for 2 x 30 min in 0,3 SSPE at 52-53°C. The detection buffer contained 10% PVA and slides were incubated for 14-20 h.

## Co-segregation analysis

To generate a segregating $F_2$ population *C. bursa-pastoris* 1947-wt and 1947-*Spe* (pollen donor) were crossed. Afterwards one plant of the resulting $F_1$ generation was selfed to obtain the $F_2$ population of which 191 (from a total of 196) plants analysed. For all $F_1$ and $F_2$ plants the phenotype was determined during flowering.

Isolation of genomic DNA from leaf material was done with the standard methods. Amplification of genomic DNA was done via three and two step standard PCR protocols. Putative promoter sequences were generated by genome walking following the protocol from CLONTECH Laboratories. Sequences of oligonucleotide primers used for amplification will be provided upon request.

Amplification of the short fragments from genomic DNA necessary for the subsequent genotyping was done via a standard PCR protocol. For pyrosequencing we followed the procedure published by Groth et al. (2006). Sequences of respective oligonucleotide primers used for fragment amplification and the following pyrosequencing reaction are listed in Supplemental Table 5 online. To genotype the complete $F_2$ population for the SNP in *CbpAGa* we ordered a biotinylated forward primer to pyrosequence directly without ligation step (Supplemental Table 5).

The alignment in Figure 6 for a part of the 2nd intron of *AG* orthologues was generated with CLUSTALX (Jeanmougin et al., 1998) and afterwards corrected by hand. Sequences used other than those of *C. bursa-pastoris* have been published by Hong et al. (2003).

## Accession numbers

Sequence Data from this article can be found in the GenBank/EMBL data libraries under accession numbers EU551759-EU551773 and EU662251-EU662266 (see Supplemental Table 6 online).

## Supplemental data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** DNA blot hybridization experiments with genomic DNA of 1947-wt and 1947-*Spe* plant leaf material**.**

**Supplemental Figure 2.** MADS domain protein alignment including the isolated members of the *C. bursa-pastoris AG* clade.

**Supplemental Figure 3.** Phylogeny reconstruction of *AG*-like proteins.

**Supplemental Figure 4.** Result of PCR experiment testing for a specific deletion in the 2nd intron of *CbpAGa* in the different *Spe* lines.

**Supplemental Figure 5.** Crossing schedule; overview of crossed performed.

**Supplemental Figure 6.** Nucleotide alignment of *in situ* hybridization probes of *AG*-like genes

**Supplemental Table 1.** Test for fertility of 2nd whorl stamens.

**Supplemental Table 2.** Summary of floral development in *Capsella bursa-pastoris*: Landmark events that occur at beginning of each developmental stage are summarised and assorted in chronological order.

**Supplemental Table 3.** Segregation patterns of $F_3$ generations obtained from $F_2$ plants with questionable intermediate phenotypes of cross 1a.

**Supplemental Table 4.** Results of phenotyping and genotyping of the parent plants 1947-wt and 1947-*Spe*, the $F_1$ generation and the $F_2$ mapping population.

**Supplemental Table 5.** Sequences of primers used in this work.

**Supplemental Table 6.** GenBank/EMBL accession numbers of isolated genes.

## ACKNOWLEDGEMENTS

We gratefully acknowledge Marco Groth and Matthias Platzer (Leibniz Institute for Age Research, Jena) for providing the pyrosequencing facilities and for technical assistance. We thank Lennart Olsson for help with SEM, Sabine Zachgo for technical advise on in-situ hybridization, Jürgen Kroymann for providing additional technical facilities, Maren Hintz for clones of Southern probes and Conny Bartholmes for technical assistance. Many thanks also to Barbara Neuffer and Steffen Hameister for valuable advise on *Capsella* biology, to Annette Becker and Kerstin Kaufmann for help during initial stages of this project and to all people in our lab for valuable discussions. This work was supported by a grand from the Deutsche Forschungsgemeinschaft (DFG) to G.T.

## FIGURE LEGENDS

**Figure 1.** Structure of *C. bursa-pastoris* wild-type and *Spe* mutant inflorescences, flowers and floral organs.

**(A)** Wild-type flower (line 1947-wt).
**(B)** Flower of a *Spe* variant (line 1947-*Spe*).
**(C)** Floral diagram of the wild-type. Green, sepals; pink, petals; yellow, stamens; brown, carpels with ovules.
**(D)** Floral diagram of the *Spe* variant. Organs defined as in **(C)**.
**(E)** Inflorescence of a 1947-wt plant with open flowers (top view).
**(F)** Inflorescence of a 1947-*Spe* plant (top view).
**(G)** From left to right, one lateral and two medial stamens of the 3rd whorl of 1947-wt.
**(H)** and **(I)** Left side: one lateral and two medial 3rd whorl stamens, right side: 2nd whorl stamens of flowers of **(H)** 1947-*Spe* and of **(I)** 1948-*Spe*.
**(J)-(L)** Adaxial anther surfaces; **(J)** mature 3rd whorl stamen of 1947-wt; **(K)** 3rd whorl stamen of 1948-*Spe*; **(L)** 2nd whorl stamen of 1948-*Spe*.
**(M)** Closed anther of 2nd whorl stamen of 1947-*Spe*, arrow: surface area enlarged in **(P)**.
**(N)** Anther of **(M)** after opening through drought, releasing pollen.
**(O)** Left: typical anther of 2nd floral whorl of 1947-*Spe*; right: weak 1947-*Spe* phenotype of 2nd whorl stamen, arrow: surface area enlarged in **(P)**.
**(P)** Detail of petal-like surface structure of anther, indicated by arrowheads in **(M)** and **(O)** (SEM picture, 1947-*Spe*).
**(Q)** Detail of wild-type adaxial petal surface with single epidermal cells enlarged in inset (SEM picture, 1947-wt).
**(R)** Malformed anther of a 1948-*Spe* flower.
**(S)** Pollen grains on 3rd whorl stamen of 1947-wt stained with Alexander´s Reagent; viable pollen stains pink to purple.
**(T)** Like in **(S)**, but using 2nd whorl stamen of 1947-*Spe*.
**(U)** Intermediate phenotypes of flowers from an $F_1$ plant of a cross 1947-wt x 1947-*Spe*.
**(V)** Range of intermediate organ phenotypes of flowers of a cross 1947-wt x 1947-*Spe*; left: staminoid, right: petaloid intermediate organs.
**(W)** Intermediate phenotypes of flowers from an $F_1$ plant of a cross 1947-wt x 1948-*Spe.*

**Figure 2.** Structure and development of *C. bursa-pastoris* wild-type and *Spe* mutant inflorescences, flowers and floral organs, as revealed by SEM. Scale bar: 20 µm.

**(A)** Wild-type main inflorescence apex.
**(B)** Mutant main inflorescence apex (1948-*Spe*).
**(C)** Wild-type flower bud at late stage 11, arrow points to young petal.
**(D)** Mutant flower bud at stage 9, arrow points to young 2nd whorl stamen (1947-*Spe*).

**Figure 3.** *In situ* expression analysis in longitudinal sections of developing flowers of *C. bursa-pastoris*. All sections hybridised with antisense probes if not indicated otherwise. Arrows indicate developing 2nd whorl organs. Scale bar: 100 µm, st: stamen.

**(A)** and **(B)** *CbpH4* : stage 8 flowers showing the typical punctuate expression pattern of *H4* homologs; **(A)** 1947-wt flower; **(B)** 1947-*Spe* flower.
**(C)** *CbpAP3*: 1947-wt flower at stage 3 showing expression signals in the area between the sepals and the central dome.
**(D)** Stage 2 1947-*Spe* flower showing the onset of *CbpAP3* expression in a ring shaped area between centre and margin of flower primordium.
**(E)** *CbpAP3*: stage 8 1947-wt flower showing later expression in young petal primordia of the 2nd whorl and in stamens of the 3rd whorl.
**(F)** *CbpAP3*: stage 8 1947-*Spe* flower with signal visible in the stamen primordia of the 2nd whorl, stamens of the 3rd whorl and weakly in the carpel.
**(G)** *CbpAP3: s*tage 10 1947-wt flower showing late expression in the petals in the 2nd whorl (arrows).
**(H)** Stage 10 1947-*Spe* flower showing late expression of *CbpAP3* in 3rd and 4th whorl organs and stronger expression in the developing 2nd whorl stamens.
**(I)** *CbpPI* expression in stage 6 1947-wt flower in stamen primordia.
**(J)** *CbpPI* expression in stage 3 (left) and stage 4 (right) 1947-*Spe* flowers, both showing signals in area where 2nd and 3rd whorl organs will develop.
**(K)** and **(L)** Inflorescence apex with *CbpAP1* expression signal in stage 1 and 2 flower primordia **(K)** in 1947-wt plant and **(L)** in 1947-*Spe* plant.
**(M)** *CbpAP1* sense control of inflorescence apex of 1947-wt plant.
**(N)** Stage 4 floral bud of 1947-wt flower with *CbpAP1* signal in sepal primordia and adjacent area.

**(O)** and **(P)** Stage 6 flower without *CbpAP1* expression signal in **(O)** 1947-wt plant and **(P)** 1947-*Spe* plant.

**Figure 4**. *In situ* analysis of *CbpAG* expression in longitudinal sections of developing flowers of wild-type and *Spe* variant. All sections hybridised with antisense probe if not indicated otherwise. Arrows pointing to 2nd whorl organs. Scale bar: 100 µm.

**(A)** Late stage 4 flower of 1947-wt plant, expression is visible in central dome of the floral meristem.
**(B)** Early stage 3 flower of 1947-*Spe* plant, onset of *CbpAG* expression is visible in central dome of the floral meristem.
**(C)** Early stage 3 flower of 1947-*Spe* plant, hybridization with *CbpAG* sense negative control.
**(D)** Stage 6 flower of 1947-wt plant, expression visible in whorls three and four.
**(E)** Stage 6 flower of 1947-*Spe* plant, expression visible in the 3rd and 4th whorl and in young 2nd whorl organ primordia (arrow).
**(F)** Stage 6 1947-*Spe* flower, sense control.
**(G)** Stage 8 flower of 1947-wt plant, weak expression in 3rd and 4th whorl organs visible.
**(H)** Stage 7 flower of 1947-*Spe* plant, expression in 2nd, 3rd and 4th whorl organs.
**(I)** Stage 8 1947-*Spe,* sense control.
**(J)** Stage 10 flower of 1947-wt plant, expression visible in the developing stigmatic papillae, style tip and nectary; weak expression in ovules and filaments.
**(K)** Stage 9 flower of 1947-*Spe* plant, expression visible in developing carpel tip and ovules, in stamens and anthers of 2nd and 3rd whorl stamens.
**(L)** Stage 9 1947-*Spe,* sense control.

**Figure 5.** *In situ* analysis of *CbpSTK, CbpSHP1 and CbpSHP2* expression in longitudinal sections of developing flowers and fruits of wildtype and *Spe* variant. All sections hybridised with antisense probe if not indicated otherwise. Scale bar: 100 µm; p: placental tissue; e: endothelium; arrows pointing to 2nd whorl organs.

**(A)** *CbpSTK*: stage 7 flower of 1947-wt plant, expression signal is visible in the developing carpel and stamens.
**(B)** *CbpSTK*: stage 6 flower of 1947-*Spe* plant, expression signal visible in 3rd and 4th whorl organs.

**(C)** *CbpSTK*: stage 6 1947-*Spe* sense control.

**(D)** *CbpSTK*: stage 9 flower of 1947-wt plant, no expression signal visible.

**(E)** *CbpSTK*: stage 9 flower of 1947-*Spe* plant, expression signal visible in developing carpel, 2nd and 3rd whorl stamens.

**(F)** *CbpSTK*: stage 10 1947-*Spe* sense control.

**(G)** *CbpSTK*: stage 11 flower of 1947-wt plant, expression visible exclusively in the ovules.

**(H)** *CbpSTK*: stage 11 flower of 1947-*Spe* plant, expression visible exclusively in the ovules.

**(I)** *CbpSTK*: stage 10 1947-*Spe* sense control.

**(J)-(L)** No expression signal of *CbpSHP1* and *CbpSHP2* in longitudinal sections of young floral buds; **(J)** *CbpSHP1*: stage 8, 1947-*Spe*; **(K)** *CbpSHP2*: stage 6, 1947-*Spe*; **(L)** *CbpSHP2*: stage 8, 1947-*Spe*.

**(M)** Cross section of stage 6 flower with *CbpSHP2* expression signal in the placental area of the carpel where ovules will develop, weak staining of stamens and carpel walls represents unspecific background signal, 1947-*Spe*.

**(N)** Cross section of fertilised young 1947-*Spe* ovule with *CbpSHP2* expression in the endothelium tissue.

**(O)** Cross section of a fertilised young fruit of a 1947-wt plant, expression of *CbpSHP1* visible at the valve margins.

**(P)** Enlarged detail of box in **(O)** with *CbpSHP1* expression in the valve margin visible.

**(Q)** Sense control with a 1947-*Spe* fruit.

**Figure 6.** Alignment of a 2nd intron section of *AG* orthologs in Brassicaceae, showing a 22 bp deletion combined with three substitutions in *CbpAGa* of the 1947-*Spe* variant in *C. bursa-pastoris*.

Upper part: schematic view of the genomic organisation of the *CbpAG* genomic locus with sequence motifs known from *A. thaliana* (Bao et al. 2004; Hong et al. 2003) indicated; boxes: exons; filled boxes: coding sequence; empty boxes: UTR; connecting lines: non coding sequence; a, j, k: LFY/WUS-binding site; b, d, e: BLR-binding site; c, l: CCAAT-box; f, h: LFY-binding site; g: AGAAT-box; i, m: CArG-box. Lower part alignment section with *CbpAGa* highlighted.

**Figure 7.** Modified ABC models for the specification of organ identity in the flowers of *C. bursa-pastoris* wild-type and *Spe* plants.

**Figure 1.**

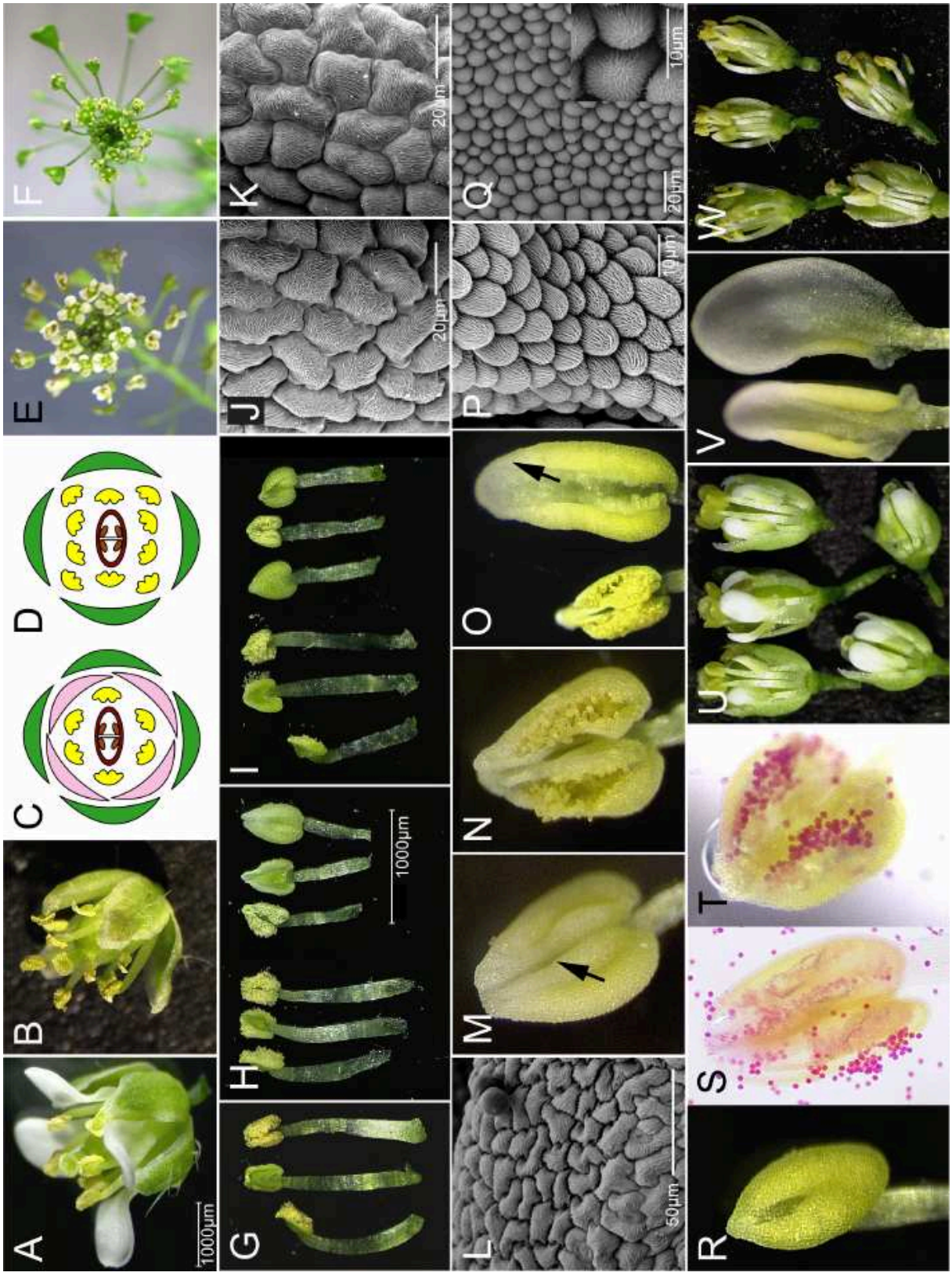

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
1000µm
1000µm
20µm
20µm
10µm
10µm
50µm

**Figure 2.**

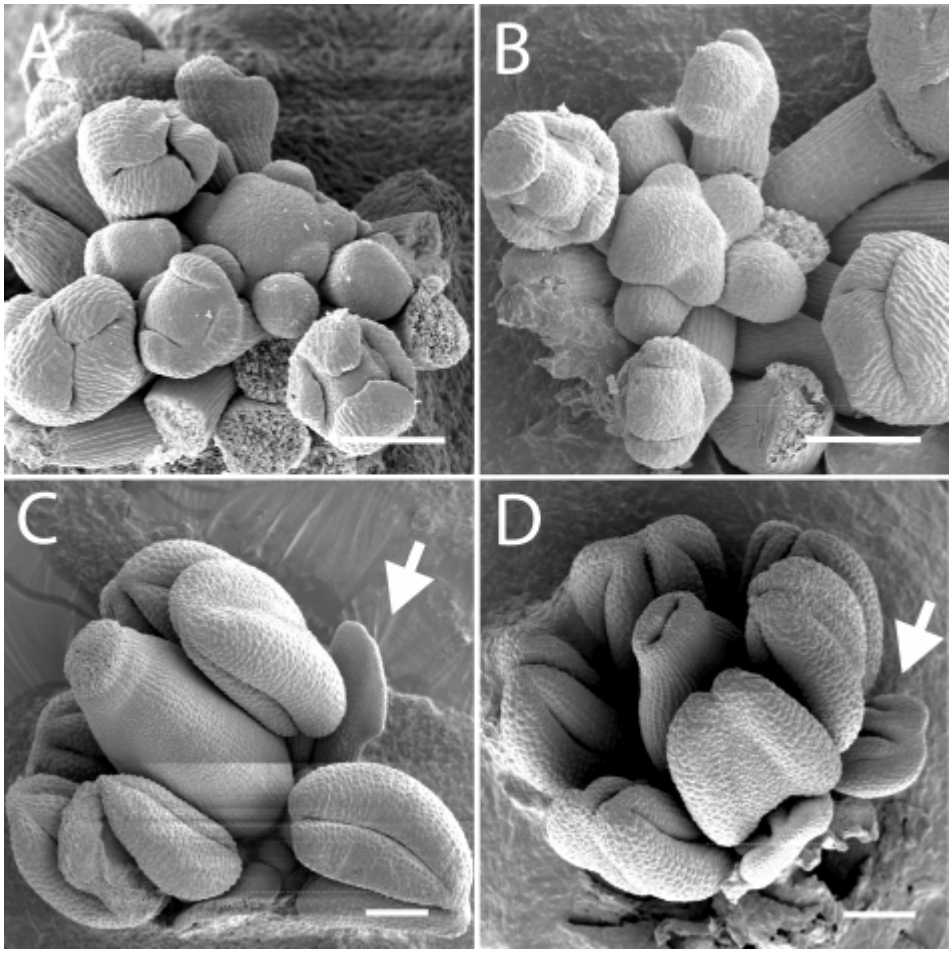


**Figure 3.**

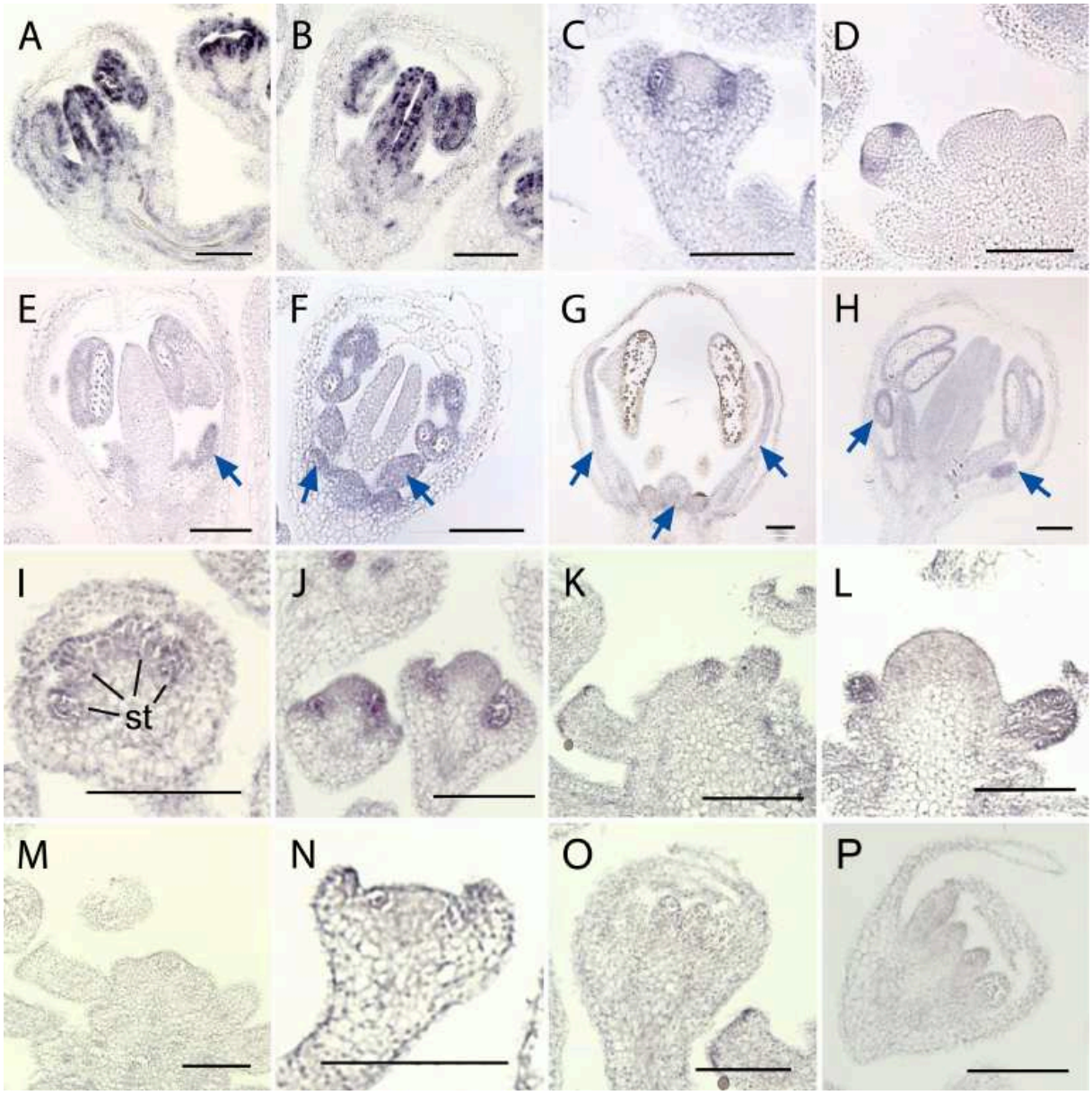


**Figure 4.**

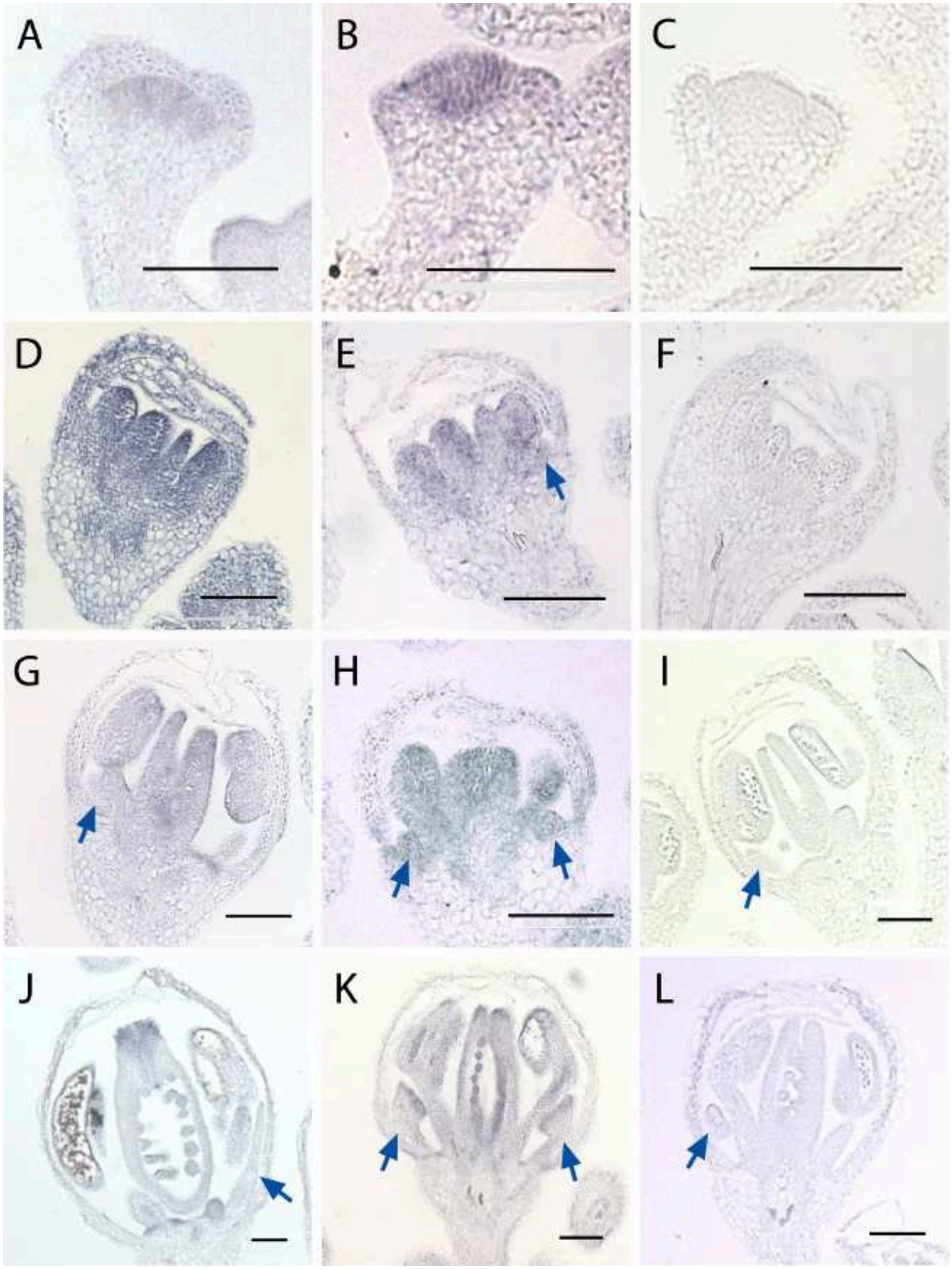


**Figure 5.**

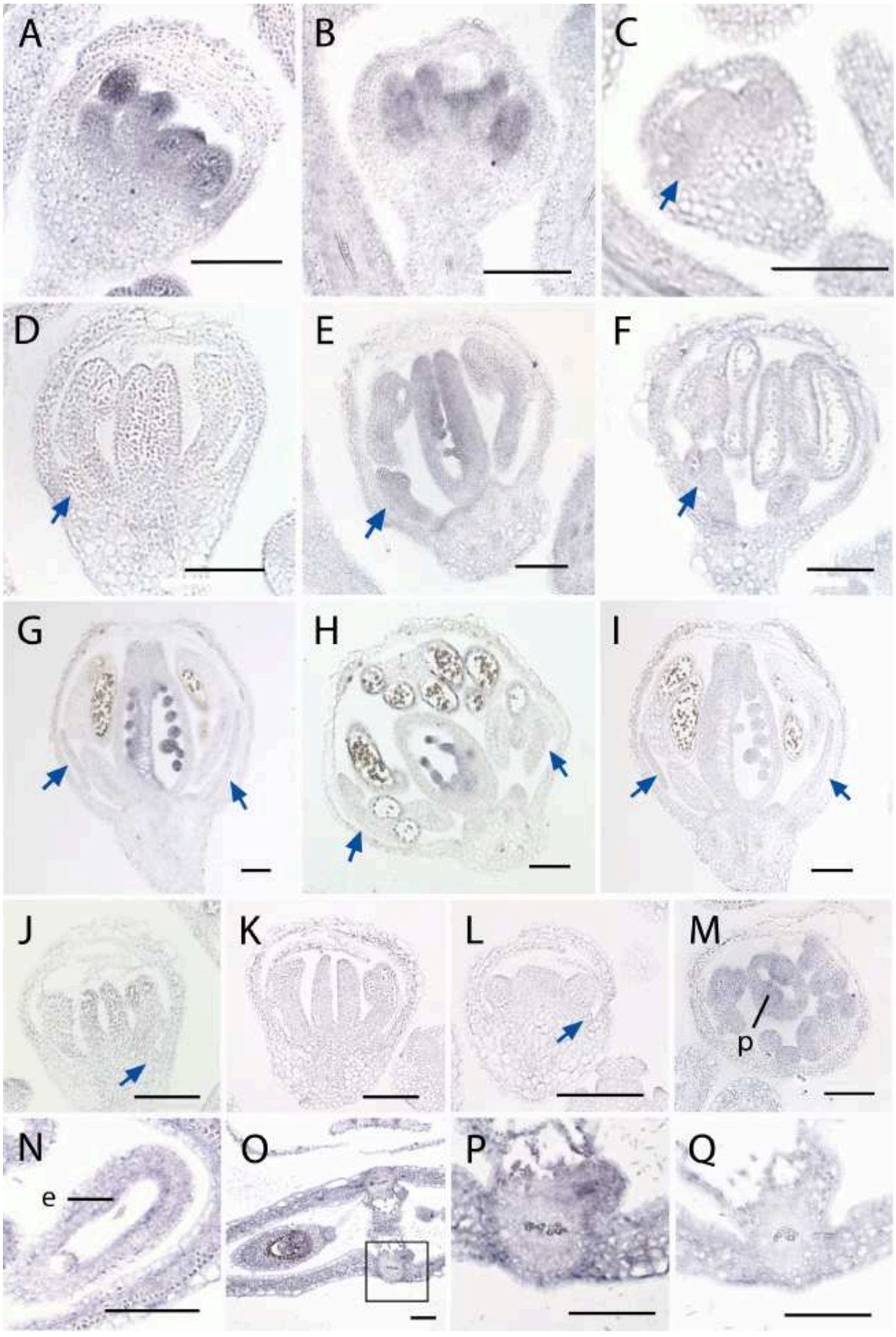


**Figure 6.**

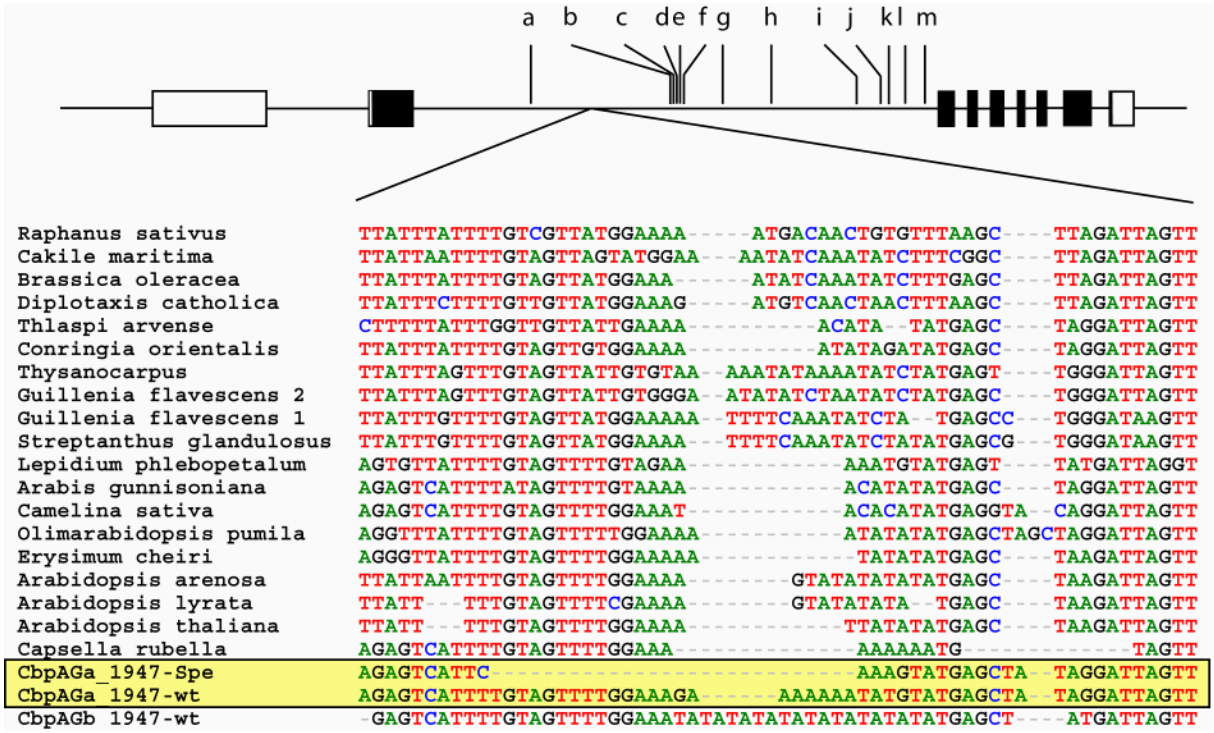
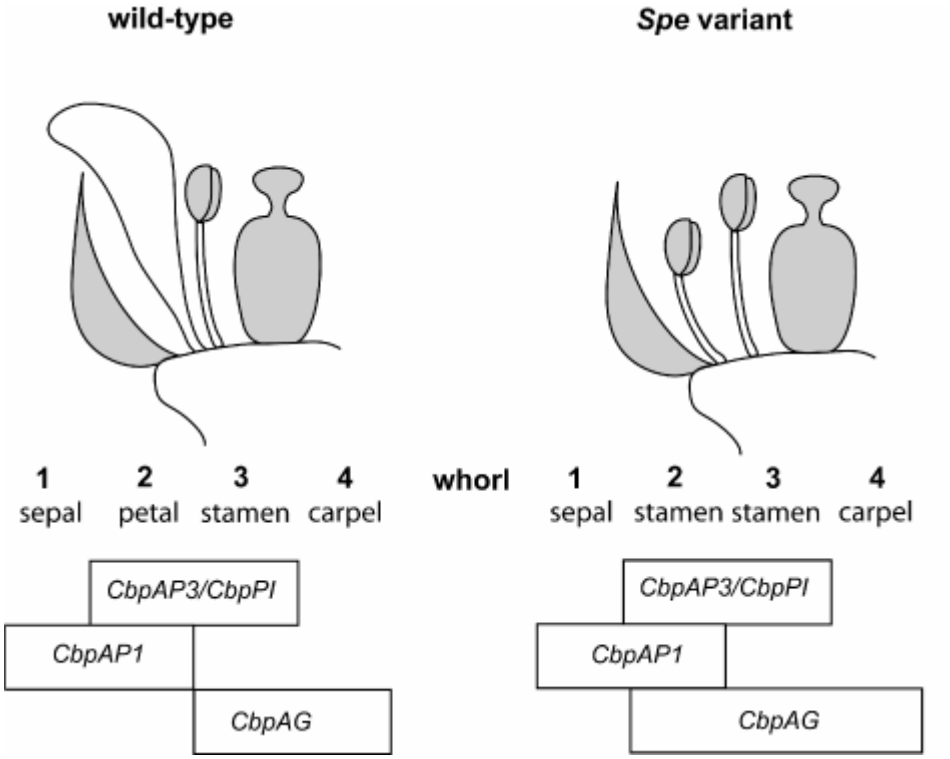



**Figure 7.**



## TABLES

**Table 1.** Genetic analysis of the number and nature of the *Spe*-inducing loci. Segregation patterns of visually analysed phenotypes of $F_2$ generations originating from the listed $F_0$ parental lines crossed.

| Cross No. | $F_0$ Parents | $F_2$ Phenotypes | | | | Model | $\chi^2$ | P-value |
|---|---|---|---|---|---|---|---|---|
| | | Wild-type | Intermediate and *Spe* | Intermediate | *Spe* | | | |
| 1 | 1947-*Spe* x 1947-wt | 42 | 125 | | | 1:3 | 0,002 | 0,96 |
| 1a | | 42 | | 92 | 33 | 1:2:1 | 2,701 | 0,26 |
| 1b* | 1947-*Spe* x 1947-wt * | 42 | | 85[#] | 40[#] | 1:2:1 | 0,102 | 0,95 |
| 2 | 1947-wt x 1947-*Spe* | 42 | 124 | | | 1:3 | 0,008 | 0,93 |
| 2 | | 42 | | 87 | 37 | 1:2:1 | 0,687 | 0,71 |
| 3 | 1948-*Spe* x 1947-wt | 37 | 118 | | | 1:3 | 0,105 | 0,75 |
| 3 | | 37 | | 85 | 33 | 1:2:1 | 1,658 | 0,44 |
| 4 | 1947-wt x 1948-*Spe* | 39 | 115 | | | 1:3 | 0,007 | 0,93 |
| 4 | | 39 | | 75 | 40 | 1:2:1 | 0,117 | 0,94 |

* Segregation pattern after test for homozygosity of candidate *Spe* plants in questionable intermediate phenotypes (see Supplemental Table 3 online).
[#] corrected number after test for homozygosity.

**Table 2.** Test for allelism of 1947-*Spe* and 1948-*Spe*.

**2.1.** Initial crosses of the two different *Spe* lines and observed phenotypes.

| Initial crosses | | Possible $F_1$ genotype models: Different loci | Same locus | Observed phenotype |
|---|---|---|---|---|
| *Spe*/Spe**; 1947 x *Spe/Spe*, 1948 | Cross **A** | *Spe*/+*, *Spe/+* | *Spe*/Spe* | 100% *Spe* |
| *Spe/Spe*, 1948 x *Spe*/Spe**; 1947 | Cross **B** | *Spe/+*, *Spe*/+* | *Spe/Spe** | 100% *Spe* |

**2.2.** Analysis of the $F_2$ generations resulting from selfing of initial cross offspring $F_1$ (see above) and resulting from the backcrosses of $F_1$ members with 1947-wt plants.

| Selfing or backcross with offspring of initial cross (Table 3) | Possible segregation models (genotypes): Different loci | Same locus | Observed phenotypes: Wild-type | Intermediate | *Spe* |
|---|---|---|---|---|---|
| 1: F1 Cross **A** selfed | 1 : 15[#] | *Spe*/Spe*<br>*Spe/Spe*<br>*Spe*/Spe** | - | - | 105 |
| 2: $F_1$ Cross **A** x 1947-wt | 1 : 1 : 1 : 1[x] | *Spe*/+*, *Spe/+* | - | 175 | - |
| 3: 1947-wt x F1 Cross **A** | 1 : 1 : 1 : 1[x] | *Spe*/+*, *Spe/+* | - | 180 | - |
| 4: F1 Cross **B** selfed | 1 : 15[#] | *Spe*/Spe*<br>*Spe/Spe*<br>*Spe*/Spe** | - | - | 90 |
| 5: 1947-wt x F1 Cross **B** | 1 : 1 : 1 : 1[x] | Spe/+, Spe*/+ | - | 176 | - |
| 6: F1 Cross **B** x 1947-wt | 1 : 1 : 1 : 1[x] | Spe/+, Spe*/+ | 62 | 83 | - |
| 7: F1 Cross **B** x 1947-wt | 1 : 1 : 1 : 1[x] | Spe/+, Spe*/+ | - | 43 | - |
| 8: F1 Cross **B** x 1947-wt | 1 : 1 : 1 : 1[x] | Spe/+, Spe*/+ | - | 188 | - |

* *Spe* locus originating from line 1947-*Spe* background

[#] **1** (+/+, +/+) **: 15** (*Spe*/Spe**, *Spe/Spe*; *Spe*/Spe**, *Spe* /+; *Spe*/Spe**, +/+; S*pe*/+*, S*pe/Spe*; *Spe*/+*, *Spe/+*; Spe*/+, +/+; +/+, Spe/+ )

[x] **1** (+/+, +/+) **: 1** (Spe*/+, +/+) **: 1** (+/+ , Spe/+) **: 1** (Spe*/+, Spe/+)

**Table 3.** Results of pyrosequencing analysis for co-segregation of wild-type phenotypes of the segregating $F_2$ population with the *AG*-like gene SNPs (genotypes) of the wild-type $F_0$ parent plants.

| Gene | SNP (genotype): Wild-type homo-zygous | *Spe* homo-zygous | hetero-zygous | Number of investigated wild-type plants | Plants homozygous for the wild-type SNP | Percentage of co-segregation |
|---|---|---|---|---|---|---|
| *CbpAGa* | C/C | -/- | C/- | 9 | 9 | 100 % |
| *CbpAGb* | A/A | C/C | A/C | 9 | 3 | 33,3 % |
| *CbpSHP1a* | T/T | G/G | T/G | 9 | 4 | 44,4 % |
| *CbpSHP1b* | A/A | C/C | A/C | 9 | 2 | 22,2 % |
| *CbpSHP2a* | G/G | T/T | G/T | 9 | 1 | 11,1 % |
| *CbpSHP2b* | T/T | A/A | T/A | 9 | 2 | 22,2 % |
| *CbpSTKa* | T/T | G/G | T/G | 11 | 5 | 45,5 % |
| *CbpSTKb* | T/T | C/C | T/C | 9 | 2 | 22,2 % |

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## 5. Manuskript III:

J. Ziermann, M. Ritz, S. Hameister, C. Abel, M. H. Hoffmann, B. Neuffer and G. Theißen (2009): Floral visitation and reproductive traits of *Stamenoid petals*, a naturally occurring floral homeotic variant of *Capsella bursa-pastoris* (Brassicaceae). *Planta* 230: 1239-1249.

Planta (2009) 230:1239–1249
DOI 10.1007/s00425-009-1018-z

ORIGINAL ARTICLE

# Floral visitation and reproductive traits of *Stamenoid petals*, a naturally occurring floral homeotic variant of *Capsella bursa-pastoris* (Brassicaceae)

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Received: 23 June 2009 / Accepted: 4 September 2009 / Published online: 23 September 2009


**Abstract** Homeotic changes played a considerable role during the evolution of flowers, but how floral homeotic mutants initially survive in nature has remained enigmatic. To better understand the evolutionary potential of floral homeotic mutants, we established as a model system *Stamenoid petals* (*Spe*), a natural variant of *Capsella bursa-pastoris* (Brassicaceae). In the flowers of *Spe* plants, petals are transformed into stamens, whereas all other floral organs are unaffected. In contrast with most other homeotic mutants, the *Spe* variant occurs in relatively stable populations in the wild. In order to determine how the profound change in floral architecture influences plant performance in the wild, we performed common garden experiments running over 3 years. Here, we show that *Spe* and wild-type plants attract the same assemblage of floral visitors: mainly hoverflies, wild bees and thrips. However, floral visitation is about twice as frequent in wild-type plants as in *Spe* plants. Nevertheless, the numbers of seeds per fruit were about the same in both variants. Wild-type plants produced more flowers, fruits and seeds per plant than *Spe* plants, whereas the germination capacity of *Spe* seeds was higher than that of the wild-type. Determination of volatile composition revealed monoterpenes and 3,4-dimethylbenzaldehyde, which were detected only in wild-type flowers, presumably because they are produced only by petals. Our data indicate that the similar fitness of *Spe* and wild-type *C. bursa-pastoris* in the field results from complex compensation between plant architecture and germination capacity. In contrast, flower structure and floral visitation are only of minor importance, possibly because *C. bursa-pastoris* is mainly self-pollinating.

**Keywords** *Capsella* · Floral homeotic mutant · Floral visitation · Germination · Plant fitness · Volatiles

**Electronic supplementary material** The online version of this article (doi:10.1007/s00425-009-1018-z) contains supplementary material, which is available to authorized users.

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## Introduction

Homeotic mutants are a frequent phenomenon in plants, where both vegetative and reproductive organs can be affected (Sattler 1988; Meyerowitz et al. 1989). Well-known are floral homeotic mutants: mutant plants with flowers that have more or less normal floral organs in places where organs of another type are typically found. The molecular and genetic analysis of such mutants has been of great help in understanding how different floral organs acquire their specific identity during flower development (Ferrario et al. 2004; Krizek and Fletcher 2005).

Many floral homeotic variants, either based on non-heritable developmental modifications or heritable mutations, have been described in the literature. Often, however,

heritability of the deviant phenotype—a requirement for evolutionary relevance—remained unknown (e.g. Darwin 1876; Murbeck 1918; Meyerowitz et al. 1989). Most of the 'classical' floral homeotic mutants, notably those of *Arabidopsis thaliana* or *Antirrhinum majus*, were not isolated from the wild. Moreover, compared with the corresponding wild-type, their strongly reduced fitness is often obvious even under laboratory conditions, especially in case of male and/or female sterile plants (Meyerowitz et al. 1989), and would almost certainly hamper their long-term survival in nature (Nutt et al. 2006).

Naturally occurring floral homeotic variants have been described for *Clarkia* and *Linaria*, but they have a very limited distribution and their fitness in the wild is probably significantly lower than that of the wild-type (Ford and Gottlieb 1992; Cubas et al. 1999). In contrast, *Capsella bursa-pastoris* (L.) Medik. (shepherd's purse) is known for the occurrence of the homeotic variant *Stamenoid petals* (*Spe*), which has been described for almost 200 years from different locations throughout Europe, many of which maintain stable populations of remarkable size (Murbeck 1918; Reichert 1998; Hintz et al. 2006; Nutt et al. 2006). Flowers of *C. bursa-pastoris* follow the architecture of other, better understood members of the Brassicaceae family, notably *A. thaliana*. Using the ABC model, which explains how homeotic genes control floral organ identity (Coen and Meyerowitz 1991), it has been argued that in the *Spe* variant ectopic expression of a class C organ identity gene causes the homeotic transformation of the second whorl petals into functional stamens (Hintz et al. 2006; Nutt et al. 2006).

There is circumstantial evidence that homeotic changes played a considerable role during the evolution of flowers (Sattler 1988; Theißen 2006). For example, petaloidy of outer whorl tepals has been associated with the heterotopic expression of class B floral homeotic genes in tulips (Kanno et al. 2003). Nevertheless, the relevance of homeotic transformations during the origin of morphological novelties has remained controversial. An especially intriguing example is provided by the origin of the flower, which is assumed to have involved, e.g., homeotic changes of male into female reproductive organs, or vice versa (Theißen et al. 2002; Frohlich 2003; Bateman et al. 2006; Baum and Hileman 2006). Studying the *Spe* variant should tell us more about whether, and if so how, such drastic morphological changes become established in natural populations and thus lead to evolutionary novelties (Theißen 2000).

Due to the presence or absence of additional stamens or petals, respectively, the *Spe* variant could in theory attract less, more or the same number of floral visitors as the wild-type. Bees or bumblebees are often attracted by the form, color or scent of petals, suggesting that the number of these floral visitors is decreased in mutant plants. In contrast, many beetles prefer eating pollen rather than drinking nectar, so that *Spe* flowers, which provide more pollen than wild-type flowers, could be more attractive to them. Both scenarios would result in a change of pollination strategy that could separate the evolutionary trajectory of the two variants.

Here, we investigated the performance of *Spe* plants in arranged field plots in the Botanical Gardens of Jena and Halle (Saale). We determined the floral visitor assemblage and frequency, fruit and seed set, and germination capacity, in each case comparing wild-type and *Spe* plants. In addition, the flower visitor assemblage was examined in one of the natural populations of *Spe* plants occurring in Germany. Floral volatile compounds are also ecologically important features of plant species, playing a decisive role in attracting pollinators or repelling a diverse array of pathogens (Dudareva et al. 2006; Knudsen et al. 2006). We, therefore, determined whether the spectrum of volatile compounds differs between wild-type and *Spe* plants.

## Materials and methods

### Plant material, experimental design and analysis of floral visitation

All wild-type (1947-wt) and floral homeotic (1947-*Spe*) plants used in this study trace back to one wild-type and one *Spe* plant, respectively; these were originally isolated from natural populations of *C. bursa-pastoris* growing in and near vineyards close to Gau-Odernheim, 30 km southwest of the city of Mainz (Rheinhessen, Germany) (Hintz et al. 2006; Nutt et al. 2006; Bartholmes et al. 2008). In this study, the term 'floral phenotype' is defined either as 'wild-type' if second whorl floral organs are petals or as '*Spe*' or 'mutant' if these organs are stamens. To achieve synchronous flowering, the seeds of *Spe* plants were sown 3 weeks before that of the wild-type (Hameister et al. 2009). Plants were cultivated in the greenhouse until the initiation of inflorescence development during May or June. Individuals were then planted in two different habitats, each encompassing two plots that contained either 25 wild-type or 25 *Spe* plants. The two habitats were a meadow and a normally cultivated bare soil (Fig. 1a, b). The distance between plots within a habitat was approximately 1 m, and plants were organized in a 5 × 5 arrangement within plots (Fig. 1a, b). This experimental setup was repeated from 2005 to 2007 in Jena and in 2005 and 2006 in Halle, in the respective Botanical Gardens. However, in Halle, only the meadow habitat was investigated and in 2006 both pairs of plots in Jena were surrounded by bare soil.


**Fig. 1** Plot design and floral visitors on inflorescences of *Capsella bursa-pastoris* in the year 2005. Plot design in the Botanical Garden Jena, with 25 plants of 1947-wt (wild-type) in the front and 25 plants of 1947-*Spe* (mutant) in the background in two different habitats: **a** bare soil, **b** meadow. **c** Mutant inflorescence with hoverfly. **d** Wild-type inflorescence with wild bee

Once flowering commenced both plots were observed simultaneously four times at each day in Jena (usually 0830–0900, 1100–1200, 1330–1430 and 1530–1600 hours) to identify and count visiting insects. Observations in Halle were made around noon for 1 h/day.

We used magnifying glasses to register small insects such as thrips inside flowers. Larger insects (e.g. Fig. 1c, d) and their visitation frequency per inflorescence were counted by eye. Because it is impossible to determine conclusively whether a particular visiting insect indeed did pollinate *Capsella* we use the term 'floral visitor' rather than 'pollinator' to describe an individual insect observed at a flower. We counted the number of these different individuals per plot and observation time, thus yielding the number of 'floral visitors' per time. However, one floral visitor often visited several flowers per plot. Therefore, we determined also the absolute number of 'floral visitations' during a survey (with number of floral visitations $\geq$ number of floral visitors). A visitation was only counted if the insect stayed at a flower for at least 2 s. Selected individuals, representing the diversity of floral visitors, were caught by net and stored in 70% ethanol for later species identification. Taxonomic determination of the most frequent species groups was done by E. Stolle (*Syrphidae*) and F. Burger (bees and wasps) (Institute of Zoology, Halle). In Halle, only the number of visitations and open flowers and the resulting visitation per flower were determined.

The number of open flowers was counted everyday and plots were surveyed until flowering had finished, resulting in different observation periods for the different habitats and years. Meteorological conditions during surveys were taken from the automated weather station of the University of Applied Sciences Jena, 1.8 km southwest of the study site, which records data every 10 min.

Supplementary qualitative data on floral visitors were obtained during fieldwork in the natural habitat of the *Spe* variant on field paths in vineyards close to Gau-Odernheim. Within this large population, *Spe* variants are admixed with wild-type plants. In May 2006, floral visitors of *C. bursa-pastoris* inflorescences were captured by net on two successive days at five sites of the Gau-Odernheim population, which differed in the abundance of wild-type and *Spe* flowers. Observed floral phenotype was denoted for each insect voucher. Sampling was supplemented in 2007 and 2008. In each case, two plots were examined for 30 min at two successive days in May. For qualitative evaluation of floral visitors of *C. bursa-pastoris*, specimens were identified to genus level. Determination of insects from the natural population in Gau-Odernheim was done by one of the authors (S.H.).

### Analysis of reproductive traits

Investigations of reproductive traits were performed only with plants from habitats located in Jena. We collected the withered plants at the end of the reproductive period, once all fruits had ripened, and counted the number of fruits per plant. On plants where fruits had already released seeds, the bare septa were counted as fruit. Due to unusually wet weather conditions, only few plants of the required stage were available in 2006. To estimate plant fitness, we determined the number of inflorescences per plant and the number of fruits per plant. Moreover, we determined the average number of seeds per fruit by cutting ten fruits from the main branch roughly 10 cm below the top of the inflorescence after those inflorescences had stopped flowering, counting their seeds and dividing the total number by ten. Based on our fruit production data, we then calculated the number of seeds per plant and used these data as a proxy for individual fitness. Furthermore, we checked the germination capacity of 100 seeds per plant, using plants that grew in the year 2005. Seeds were available for 37 plants of each floral phenotype. After 2 years of storage at room temperature, seeds were sown in pots containing moist soil and put in a dark room at 4°C for 4 days. Seed pots were then transferred to the greenhouse (22°C, 12 h light/day) with a plastic cover over the tray. Another charge of 100 seeds from the same plants was treated the same way, but without cooling, in order to determine whether usually applied stratification is really required. The germination test without cooling in a dark room was done only for 32 *Spe* plants and 35 wild-type plants, because 200 seeds were not available for all plants. We checked for germination daily during 1 month to ensure that we also registered late germination events. Five of the seedlings were checked for flower development after growing, because the number of seedlings able to reproduce represents a better proxy for fitness than just seedling number (Wolf and Wade 2001). We calculated the number of potential seedlings (another fitness proxy) of *Spe* and wild-type plants by multiplying marginal means (least square means) of seeds per plant with germination rate.

### Qualitative analysis of volatiles

For a qualitative volatile analysis, 16 flowers from wild-type and 30 from *Spe* plants were transferred to separate 1 ml glass vials that were closed with a septum cap. The septum was punctured, a PDMS-100 SPME (solid-phase microextraction) needle (Supelco, Munich, Germany) was inserted and the fiber was exposed for 30 min to the headspace of the sample. The procedure and the following gas chromatography–mass spectrometry (GC–MS) analysis were performed as described by Abel et al. (2009). 3,4-Dimethylbenzaldehyde was identified by comparison with an authentic standard (Fluka, Munich, Germany).

### Statistical analysis

#### *Data of floral visitation*

Observation time per day and plot ranged from 1.5 to 3 h (mean = 2.8 h). The summarized number of floral visitors and the number of floral visitations per plot per day were normalized to 3 h prior to analysis. To account for different numbers of flowers between plots during surveys, the two dependent variables were divided by the number of open flowers per plot. We did not use open flowers as a covariate because the influence of this factor is very strong, so much so that other effects could lose their significance in the model. The influence of the factors floral phenotype ('wild-type' or 'mutant' = '*Spe*'), year and habitat including their interacting effects, and four weather parameters (temperature, radiation, humidity, wind) was investigated using a generalized linear model with a gamma probability distribution and a logit link function in the program SPSS 15. Non-significant terms were successively removed as long as the model fit was significantly improved. Due to a reduced experimental design in Halle, the model for this location included only the factors floral phenotype and year.

A generalized linear model with binomial errors was used to test for influences of floral phenotype, year and habitat on the relative frequency of each species group of floral visitors. Starting with the three-way interaction, non-significant interaction terms were removed from the model as long as Akaike's information criterion (AIC) indicated an improvement of the model. Calculations were done using the R 2.5 program package (R Development Core Team 2006).

#### *Data of reproductive traits*

The factors floral phenotype, year and habitat influencing reproductive traits and inflorescence architecture (number of branches) were also investigated using generalized linear models in the same way as for the insect visitations. Dependent variables were transformed to match normal distribution if necessary. Again, non-significant interaction terms were excluded as long as this increased the $R^2$ of the model. The influence of floral phenotype, treatment of seeds and habitat on germination rate was investigated via a generalized linear model with binomial errors, using the R 2.5 program.

## Results

### Floral visitation of *Spe* and wild-type plants

In the year 2007, we counted up to 200 visitations for the wild-type and up to 100 visitations for the *Spe* plot in the meadow habitat during an observation interval of 3 h/day (Fig. 2a), which were realized on average by about 35 and 15 floral visitors, respectively (Figs. 1c, d, 2b). These observations were surprising because *C. bursa-pastoris* is a predominantly self-pollinating plant. As expected, the greatest number of insects was detected in the middle of the day (data not shown). The number of floral visitors and the number of floral visitations per plot were summarized per day. Generally, we observed more individuals on wild-type than on mutant plants, but the wild-type plants also provided more open flowers (Fig. 2c; Suppl. Fig. 1). Wild-type flowers were visited by nearly three times more individuals than were mutant flowers in 2006 (Suppl. Fig. 1), but by an equal number of insects per flower in 2005 and 2007 (Fig. 2d; Suppl. Fig. 1). These observations are confirmed with the only significant interaction term in the analysis, the two-way interaction phenotype × year ($\chi^2_{2,168} = 21.99$, $P < 0.001$) (Table 1). When the number of floral visitors and their visitation frequency were set in relation to the number of open flowers, both variables varied significantly between the factors year ($\chi^2_{2,168} = 52.8$, $P < 0.001$; $\chi^2_{2,167} = 70.8, P < 0.001$, respectively) and habitat ($\chi^2_{1,168} = 13.0$, $P < 0.001$; $\chi^2_{1,167} = 7.1$, $P = 0.007$, respectively) (Table 1). Temperature during observation was the only highly significant weather parameter in the final model (Table 1) with more floral visitors and visitations at higher temperature ($\chi^2_{1,168} = 33.9$, $P < 0.001$; $\chi^2_{1,167} = 50.0$, $P < 0.001$, respectively). This was not surprising, because in general insect flight is obviously positively correlated with increased environmental temperature. However, while the important factor floral phenotype had only a marginally non-significant influence on floral visitors ($\chi^2_{1,168} = 3.6$, $P = 0.057$), its impact on visitation frequency was significant ($\chi^2_{1,167} = 10.5, P = 0.001$), meaning that the number of visitations per visitor was higher for wild-type plants than for *Spe* plants (Table 1; Fig. 2d, e; Suppl. Fig. 1).

Observations in Halle revealed visitation patterns qualitatively similar to those observed in Jena (Fig. 2f; Suppl. Fig. 1). Thereby, only the factor phenotype influenced the visitation frequency significantly (wild-type > *Spe* mutant), whereas the interaction term phenotype × year was of minor importance (phenotype: $\chi^2_{1,44} = 4.31$, $P = 0.038$; phenotype × year: $\chi^2_{1,44} = 1.96, P = 0.161$). In contrast to Jena, the factor year had no significant effect on floral visitations (year: $\chi^2_{1,44} = 1.85, P = 0.173$).

The most common species groups visiting *C. bursa-pastoris* flowers were hoverflies (*Syrphidae*) and wild bees (*Apidae*), though *Thysanoptera* species were also observed regularly (Supplementary Tables 1, 2; Fig. 1c, d). Furthermore, we found some beetles (mainly one species of the family *Curculionidae*), a few *Heteroptera* species and several *Diptera* species other than hoverflies, which were mostly found resting on the inflorescences during morning surveys (Suppl. Table 1). The number of *Aranea* species (spiders) was also very low and they were usually found on nets hanging between inflorescences rather than on individual flowers (Suppl. Table 1).

For further analysis, we concentrated on the species-rich groups wild bees and hoverflies, which visited the plots in considerable numbers (Suppl. Tables 1, 2). Bees represented a larger proportion of flower visitors to wild-type plants compared with *Spe* plants ($Z_{1,11} = 2.53$; $P = 0.011$), whereas the converse was true for hoverflies ($Z_{1,11} = 5.61$; $P < 0.001$) (Suppl. Table 1). The difference in bees was not constant across years (phenotype × year: $Z_{1,11} = 2.53$; $P = 0.011$) (Table 2). The proportion of bees and hoverflies differed between years (bees: $Z_{2,11} = 2.67$; $P = 0.007$; hoverflies: $Z_{2,11} = 5.02; P < 0.001$) (Table 2). Bees represented a larger proportion in the bare soil habitat compared with meadow ($Z_{1,11} = 7.85$; $P < 0.001$) at varying degrees between years (habitat × year: $Z_{1,11} = 7.85$; $P < 0.001$) (Table 2). In general, beetles could be detected on both *Spe* and wild-type plants (Suppl. Table 1), but the influence of the phenotype itself was not strongly supported ($Z_{1,11} = 2.02$; $P = 0.044$). Habitat, year and the interaction term phenotype × habitat had a much stronger influence on beetle occurrence than the phenotype alone (habitat: $Z_{1,11} = 6.99$; $P < 0.001$; year: $Z_{1,11} = 9.05; P < 0.001$; phenotype × habitat: $Z_{1,11} = 4.45$; $P < 0.001$) (Table 2). With a closer look on absolute numbers of beetles, we observed opposite scenarios when comparing bare soil and meadow habitat (Suppl. Table 1). In particular, we found decreased beetle presence on wild-type plants in bare soil, but increased presence on wild-type plants in meadow habitats.

A preliminary survey of the taxa assemblage in the natural habitat at Gau-Odernheim revealed a similar pattern. More than half of the identified individuals belonged to *Syrphidae* and *Apidae* (wild bees) (Suppl. Table 3). However, smaller insects such as thrips were not surveyed. In addition to the above-mentioned groups, further individuals from *Diptera* and *Coleoptera* were determined either on wild-type or *Spe* inflorescences (Suppl. Table 3). A small floral phenotype-specific difference of flower visitors was observed for wild bees, because on wild-type flowers more individuals were captured.

### Fruit and seed production

The number of inflorescences per plant was significantly higher in wild-type plants ($\chi^2_{1,181} = 12.0$, $P = 0.001$),

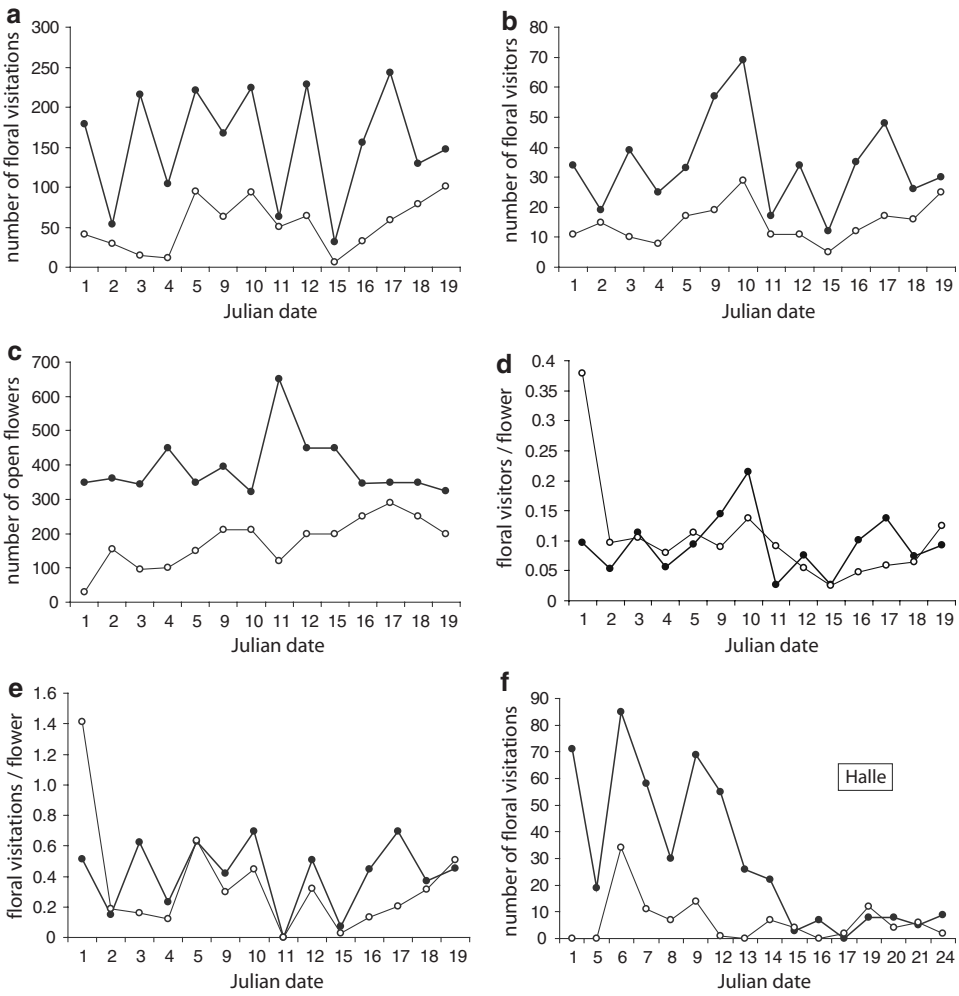


**Fig. 2** Observations at the meadow habitat 2007 in Jena (**a**–**e**) and 2005 in Halle (**f**) of 1947-wt (*black circles*) and 1947-*Spe* (*open circles*) plant plots per daily observational interval. Number of floral visitations (**a**), floral visitors (**b**) and open flowers (**c**), as well as the frequencies of floral visitors (**d**) and visitations (**e**) per flower (Julian date 1 = 25 June 2007). **f** Number of floral visitations (Julian date 1 = 9 June 2005)

higher in 2005 than in 2007 ($\chi^2_{1,181} = 51.2$, $P < 0.001$), and higher in bare soil plots compared with meadow plots ($\chi^2_{1,181} = 53.9$, $P < 0.001$) (Table 1; Fig. 3a). However, the interaction term phenotype × habitat was also significant ($\chi^2_{1,181} = 5.4$, $P = 0.019$), being the only interaction factor that influenced some of the reproductive traits investigated here (Table 1). Separate models for the two habitat types confirmed that a larger number of wild-type inflorescences was found only in the bare soil habitat. The number of inflorescences correlates positively with the number of flowers and hence with the number of fruits ($r_{189} = 0.76$, $P < 0.001$). Consequently, the number of fruits per plant showed a similar pattern (phenotype: $\chi^2_{1,181} = 29.7$, $P < 0.001$; year: $\chi^2_{1,181} = 38.1$, $P < 0.001$; habitat: $\chi^2_{1,181} = 58.0$, $P < 0.001$; phenotype × habitat: $\chi^2_{1,181} = 7.9$, $P = 0.005$) (Table 1). The difference in fruit number between wild-type and mutant plants was much higher in the bare soil habitat (701 vs. 408 in wild-type vs. *Spe* mutant; $\chi^2_{1,181} = 30.8$, $P < 0.001$), even though it was also detectable in the meadow habitat (354 vs. 298; $\chi^2_{1,181} = 3.8$, $P = 0.048$) (Table 1; Fig. 3b). Therefore, the interaction term phenotype × habitat is significant even though both habitats showed increased fruit production by wild-type. The average number of seeds per fruit and plant showed temporal variation in relation to floral phenotype. Whereas mutant plants had more seeds per fruit in 2005 ($F_{1,72} = 4.2$, $P = 0.042$), they had marginally non-significant fewer seeds per fruit in 2006 ($F_{1,83} = 3.1$, $P = 0.083$). Consequently, the factor floral phenotype was not significant in the full model ($F_{1,155} = 0.004$, $P = 0.951$) (Table 1; Fig. 3c).

Total seed production per plant yielded similar patterns to those for the trait fruits per plant. Seed production was also influenced by year and habitat, and wild-type plants produced more seeds than *Spe* plants (Table 1). We found more seeds per plant in the bare soil habitat than in the meadow habitat, and in the year 2005 than in 2007. Beyond that, the increased seed production by wild-type plants was much stronger in the bare soil habitat (Fig. 3d).

## Germination capacity

Seeds from *Spe* mutant plants had higher germination rates (phenotype: $Z_{1,134} = 7.51$, $P < 0.001$; Fig. 4), and this

**Table 1** ANOVA summaries of influences of floral phenotype, year and habitat on the number of floral visitors and visitations and the investigated fitness parameters of *Capsella bursa-pastoris* (not significant *P* values given in bold, $\chi^2$ values are generated from gamma distributed data sets and *F* values from normal distributed data sets)

| | Floral visitors | | Visitations | | Fruits | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | *P* | $\chi^2$ | *P* | $\chi^2$ | *P* |
| Phenotype | 3.616 | **0.057** | 10.509 | 0.001 | 29.792 | <0.001 |
| Year | 52.882 | <0.001 | 70.864 | <0.001 | 38.172 | <0.001 |
| Habitat | 13.02 | <0.001 | 7.157 | 0.007 | 58.055 | <0.001 |
| Phenotype × year | 21.99 | <0.001 | 3.091 | **0.213** | 2.056 | **0.152** |
| Phenotype × habitat | 0.006 | **0.938** | 1.095 | **0.295** | 7.928 | 0.005 |
| Temperature | 33.905 | <0.001 | 50.077 | <0.001 | | |

| | Inflorescences | | Seeds per fruit | | Seeds per plant | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | *P* | *F* | *P* | *F* | *P* |
| Phenotype | 12.055 | 0.001 | 0.004 | **0.951** | 14.022 | <0.001 |
| Year | 51.224 | <0.001 | 26.89 | <0.001 | 17.693 | <0.001 |
| Habitat | 53.942 | <0.001 | 9.607 | 0.002 | 49.111 | <0.001 |
| Phenotype × year | 3.176 | **0.075** | 5.682 | 0.018 | 1.471 | **0.232** |
| Phenotype × habitat | 5.475 | 0.019 | 0.047 | **0.829** | 8.447 | 0.004 |
| Temperature | | | | | | |

**Table 2** Influences of floral phenotype, year and habitat on the proportion of bees, hoverflies and beetles among floral visitors of *Capsella bursa-pastoris* (not significant *P* values given in bold)

| | Bees | | Hoverflies | | Beetles | |
|---|---|---|---|---|---|---|
| | *Z* | *P* | *Z* | *P* | *Z* | *P* |
| Phenotype | 2.535 | 0.011 | 5.606 | <0.001 | 2.018 | 0.044 |
| Year | 2.671 | 0.008 | 5.022 | <0.001 | 9.054 | <0.001 |
| Habitat | 7.854 | <0.001 | 1.793 | **0.073** | 6.988 | <0.001 |
| Phenotype × habitat | 0.075 | **0.94** | 0.567 | **0.571** | 4.446 | <0.001 |
| Phenotype × year | 2.531 | 0.011 | 0.094 | **0.925** | 0.458 | **0.647** |
| Habitat × year | 7.847 | <0.001 | 1.791 | **0.073** | 0.047 | **0.962** |

difference was more pronounced when the seeds had undergone a cold treatment (phenotype × cold treatment: $Z_{1,134} = 2.44$, $P = 0.015$). The cold treatment decreased the germination rate ($Z_{1,134} = 3.99$, $P < 0.001$), whereas the habitat (bare soil vs. meadow) had no influence on the germination ($Z_{1,134} = 0.89$, $P = 0.372$). On average, 65% of seeds of wild-type plants and about 90% of seeds of mutant plants germinated under our conditions with cold treatment. Without this pre-treatment, the germination capacity was even higher in case of both wild-type (80%) and *Spe* plants (about 95%) (Fig. 4). All selected plants of both types produced inflorescences and flowers in the usual way.

The expected number of *Spe* seedlings per plant based on germination rate and marginal means (least square means) of seeds per plant was slightly higher than that of the wild-type for the meadow habitat (7,893 mutant vs. 7,588 wild-type seedlings without cold treatment and 7,563 mutant vs. 6,161 wild-type with cold treatment). However, for the bare soil habitat, we detected a reduced number of *Spe* seedlings (10,709 mutant vs. 15,864 wild-type seedlings without treatment and 10,261 mutant vs. 12,882 wild-type with treatment).

### Volatiles

Determination of volatile composition revealed that monoterpenes (*Z*-ocimene, (*E*)-$\beta$-ocimene) and the benzenoid 3,4-dimethylbenzaldehyde were emitted by wild-type flowers (Fig. 5a). In contrast, flowers of the *Spe* variant showed none of these compounds, even in extracts based on twice the number of flowers than in case of the wild-type (Fig. 5b). Tiny peaks in Fig. 5b are unspecific background signals other than monoterpenes or dimethylbenzaldehyde. We concluded that flowers of the *Spe* mutant do not emit appreciable amounts of floral volatiles under the imposed conditions. Therefore, volatile emission correlates positively with the presence of petals in the flowers of wild-type *C. bursa-pastoris*.

## Discussion

Homeotic changes of flowers could play a substantial role in flower evolution since they may bring about major morphological innovations while requiring only minor genetic changes (Theißen 2006, 2009). However, investigations of mutants carrying such a transformation in the wild are quite under-represented. Many of these floral homeotic mutants are seriously affected in their reproductive fitness, or are even completely sterile, so that their evolutionary importance is probably negligible (Sattler 1988; Meyerowitz et al. 1989; Nutt et al. 2006; Hintz et al. 2006; Theißen 2006). The situation is obviously different for *Spe* plants, since these can co-exist with wild-type plants of *C. bursa-pastoris* for periods of at least decades (Hintz et al. 2006; Nutt et al. 2006). We assumed that the special type of floral homeosis represented by *Spe* (complete transformation of petals into stamens without changes of any other floral organs) contributes to the co-existence of mutant and wild-type plants. Since outcrossing rates of up to 12% have been observed for *C. bursa-pastoris* (Hurka et al. 1989), we initially hypothesized that compensatory changes in the floral visitor assemblage, such as bee versus beetle preference in case of wild-type and mutant plants, respectively, contribute to the co-existence

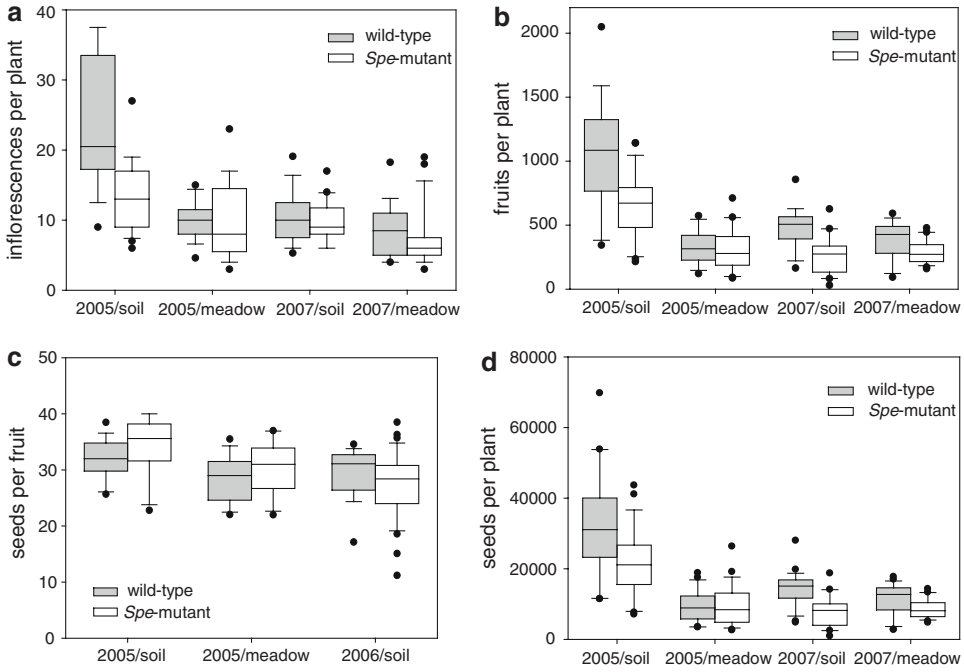


**Fig. 3** *Box plots* of morphological and reproductive traits of 1947-wt and 1947-*Spe* plants from the two different habitats, 'bare soil' and 'meadow', in Jena from 2005 and 2007 (**a**, **b**, **d**) and from 2005 (both habitats) and 2006 (only bare soil) (**c**). **a** Number of inflorescences per plant bearing at least one developed fruit. **b** Number of fruits per plant. **c** Number of seeds per fruit. **d** Number of seeds per plant. (*Middle line* of each *box* represents the median value. *Boxes* cover the middle 50% of observations. The *whiskers* outside the box extend between the highest and lowest values in the sample that are within 1.5 box lengths from the edge of the box. Data outside this limit are shown by *circles*)

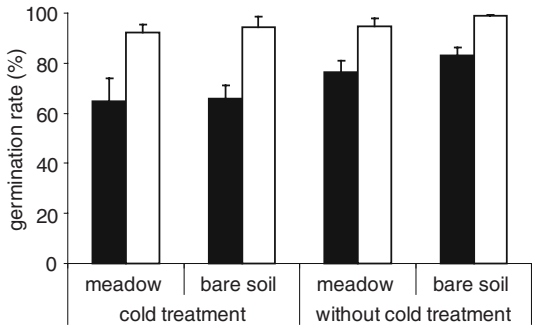


**Fig. 4** Germination rate (in percent) of seeds of 1947-wt (*black columns*) and 1947-*Spe* (*white columns*) gathered from the plants that grew in Jena Botanical Garden in the year 2005; 'meadow' and 'bare soil' refer to the two different habitats, '(without) cold treatment' refers to seeds that have been (or have not been) treated at 4°C for 4 days, as described in "Materials and methods"; *error bars* represent standard error

of both phenotypes over many years. However, as will be outlined below, this assumption could not be confirmed.

In principle, the absence of petals and their coincident replacement by stamens in the *Spe* mutant could have considerable consequences for the establishment of the mutant population in the wild. A general survey of the potential pollinator community of *C. bursa-pastoris* conducted in block experiments in two different locations (Jena, Halle) and by fieldwork in a natural population (Gau-Odernheim) revealed an unexpectedly high diversity of large insects visiting flowers of both phenotypes of shepherd's purse. Our studies show that wild bees (*Apidae*) and hoverflies (*Syrphidae*) are the most common insect visitors to the flowers. This assemblage was similar for the different observation conditions and even in the natural habitat. Our findings are also in line with previous observations made near Gau-Odernheim by Reichert (1998). We suggest, therefore, that outcrossing revealed in previous investigations (Hurka et al. 1989) is probably due to pollination by insects, mostly by wild bees, *Syrphidae* and other *Diptera*, as well as the comparatively tiny thrips. Furthermore, we could show that the composition of the visitor assemblage of mutant and wild-type plants is not changed. In the case of *A. thaliana*, which has flowers similar to those of *C. bursa-pastoris*, floral visitation experiments revealed similar assemblages of visiting insect species such as wild bees, dipterans and thrips (Hoffmann et al. 2003).

Only the proportion between the visitor types differed with respect to year or plot habitat. The only significant interaction term concerning floral visitors (phenotype × year) shows that the effect of the phenotype is enhanced across the different years. Therefore, the habitat in which the plots were located was only of minor importance, whereas year affected floral visitors considerably, probably due to variable environmental conditions for the survival of insect progeny. Bees visited more frequently at the bare soil habitat. Some wild bees live in small groups of up to 12 members and often place their nests in the soil or between stones, often in walls. The area in which they collect nectar is quite small. Hoverflies were always present, but their proportion differed depending on phenotype and year; our data suggest that the wild-type plants are more attractive to them (Table 2; Suppl. Table 1).

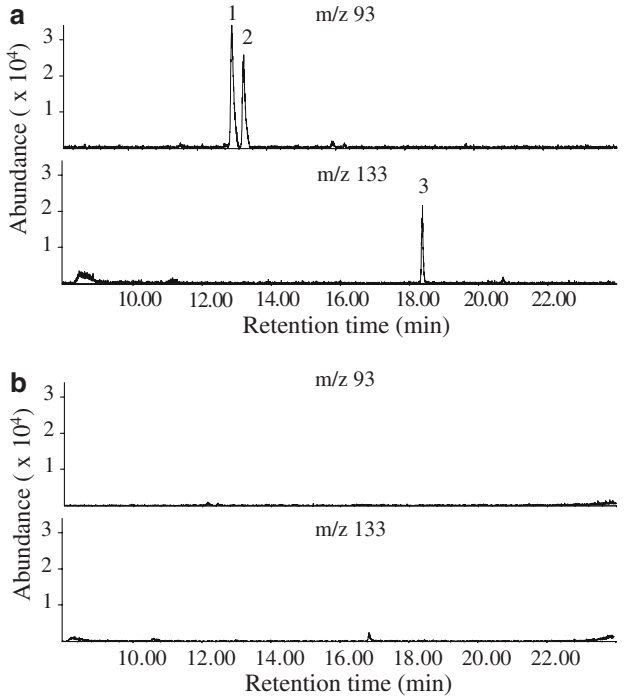


**Fig. 5** GC–MS (gas chromatography–mass spectrometry) chromatograms after headspace solid-phase microextraction (SPME) of floral volatiles of 24 1947-wt flowers (**a**) and 40 1947-*Spe* flowers (**b**). *Signal traces* are for the ion fragments *m/z* 93 and 133 extracted from a total ion chromatogram for ease of signal clarity. The fragment *m/z* 93 is characteristic for aliphatic monoterpenes, whereas *m/z* 133 was used to identify dimethylbenzaldehyde. 1, *Z*-ocimene; 2, (*E*)-*β*-ocimene; 3, 3,4-dimethylbenzaldehyde. Compounds were identified by comparison with authentic standards

Because the aim of this project was to test the attractiveness and fitness of *Spe* versus wild-type plants rather than to determine the amount of outcrossing (which will be considered in future investigations), we did not gather data from trapped insects to determine whether they were carrying *Capsella* pollen. As pollen of *Brassicaceae* is quite uniform (http://www.paldat.org), pollen of *C. bursa-pastoris* would be hard to distinguish from that of some other Brassicaceae such as *Brassica oleracea* and *B. rapa*.

We detected approximately twice as many floral visitations to wild-type plants than to mutant plants, suggesting that plants furnished with petals are more attractive. This could be due not only to optical cues, but also to other features such as scent. Production of floral volatiles plays an important role in the attraction of pollinators of many angiosperm species. Studies in *Petunia hybrida* and *Arabidopsis lyrata* demonstrated that benzenoid derivatives such as benzaldehyde are mostly emitted by petals rather than the other floral organs (Verdonk et al. 2003; Abel et al. 2009). Our data indicate that this is also the case in *C. bursa-pastoris*, because wild-type flowers produce *Z*-ocimene, (*E*)-*β*-ocimene and 3,4-dimethylbenzaldehyde, whereas *Spe* flowers do not, strongly suggesting that these substances are synthesized exclusively by petals. In *C. bursa-pastoris*, these substances are unlikely to be the only cue for pollinator attraction, however, given that petal-free *Spe* flowers devoid of volatiles are nonetheless visited by a substantial spectrum of insect species, even though that spectrum encompasses fewer individuals than the wild-type. Bees are often attracted by floral scent but they visited wild-type and mutant plants in a similar way, probably due to the fact that other flower traits attracting pollinators, such as pollen or nectar, are still present in both phenotypes.

Besides developing petals, the fact that wild-type plants generate larger numbers of open flowers per inflorescence and also more inflorescences per plant than *Spe* plants (Figs. 2c, 3a) may contribute to their superior attractiveness for visitors. However, the difference in visitation frequency per flower between both phenotypes was not large. One could argue that not only the inflorescence as a whole serves as the attraction unit but also individual flowers are of considerable importance.

Our initial assumption that pollen eating beetles visited *Spe* plants more frequently than wild-type plants due to the additional stamens which provide a greater amount of pollen in mutant flowers was not supported by our data. We detected an influence of the phenotype on the numbers of beetles, but in respect to the different habitats we observed opposite pictures (Table 2; Suppl. Table 1). However, *Spe* and wild-type plants of *C. bursa-pastoris* have occurred in mixed populations for decades, suggesting that they have a similar fitness, at least in the studied habitats. We thus wondered what factors other than floral visitor attraction critically contribute to their co-existence.

Fitness is often best defined as the expected lifetime reproductive output (Darwin 1859). Plant fitness is thus usually defined as lifetime production of viable seeds, but for operational reasons it is often estimated by measuring various growth parameters that are considered to be correlated with fitness (Silvertown 1987). In our case, however, we measured the production of germination-competent seeds of a monocarpic (hapaxanth) plant, and we even checked that the seedlings could develop into flowering adults, an important step often ignored in fitness calculations. Thus, our fitness proxy is unusually close to a most complete definition of plant fitness.

Even though the number of seeds per fruit was similar in case of wild-type and *Spe* plants, wild-type plants produced up to twice as many seeds as *Spe* plants because they generated more inflorescences and therefore more fruits per plant (Fig. 3). Thus, despite the fact that a strong variation between years and habitats was observed, these data seem to indicate that wild-type plants have a higher fitness than *Spe* plants, at least under the growth conditions tested by

us. Surprisingly, however, germination rate—another important character related to fitness—was found to be higher in mutant plants under the experimental conditions (Fig. 4).

The resulting theoretical number of potential seedlings for each floral phenotype does not provide a uniform picture. Although the *Spe* mutant produced the same number of potential seedlings or more than the wild-type in the meadow habitat, fewer seedlings were produced by the mutant in the bare soil habitat. In the investigated 'natural' habitat at Gau-Odernheim, we found a ruderal landscape supporting mixed plant populations where both phenotypes of *C. bursa-pastoris* compete with each others and other plants. The fact that wild-type and *Spe* co-exist for many years suggests that Gau-Odernheim represents a relatively complex habitat in which both aspects (bare soil vs. meadow) considered here may compensate for each other.

Only the interaction term phenotype × habitat was significant for investigated reproductive traits except seeds per fruit. In an ecological context, that means that not only does the floral phenotype itself play an important role in fruit or seed production but also the habitat in which the plants were growing—this has an influence on the reproductive success of both phenotypes. We conclude that for fruit or seed production the location of the developing plants is an important factor, in that the difference in fruit and seed production between wild-type and mutant plants was higher in the bare soil habitat than the meadow habitat (Fig. 3), possibly due to the absence of competition from other plant species. It was reported that the genetic diversity of *C. bursa-pastoris* is much higher in disturbed habitats, such as the natural location of the *Spe* mutant in Gau-Odernheim, than in undisturbed areas (Bosbach et al. 1982; Reichert 1998). Interspecific competition is relatively low on bare soil and hence selection pressure is reduced, so that sub-optimally adapted genotypes could also develop in such a disturbed landscape.

We hypothesize that the reduced number of inflorescences, fruits and seeds of *Spe* plants compared with wild-type plants (Fig. 3) is compensated for by the observed increase in germination rate (Fig. 4) and thus may not necessarily lead to a reduced overall fitness of *Spe* plants. One should note, however, that the germination capacity of *C. bursa-pastoris* seeds is regulated by dormancy and is generally characterized by a broad temperature tolerance, permitting intraspecific differences in germination strategy (Neuffer 1990; Hurka and Neuffer 1997). Furthermore, seeds of *C. bursa-pastoris* are components of a soil seed bank and so could germinate in different time intervals in order to benefit from optimal conditions (Salisbury 1963; Bosbach et al. 1982). Therefore, the lower germination capacity of wild-type seeds could be the result of a sophisticated seed dormancy strategy, whereas the *Spe* variant may follow the cruder principle of 'all or nothing', meaning immediate germination of all seeds.

We observed that the *Spe* variant has a later onset of flowering than the wild-type (Hameister et al. 2009). Therefore, we synchronized flowering prior to the observation studies in botanic gardens by sowing mutant seeds earlier than wild-type seeds. Under natural growth conditions, however, different flowering times may contribute to the relative fitness of *Spe* and wild-type plants. This aspect deserves future investigation.

Despite the fact that wild-type and *Spe* plants have a striking difference in floral architecture, both variants have a similar fitness, at least in those habitats where they co-exist for decades or more (Hintz et al. 2006; Nutt et al. 2006). In attempting to understand why this is so, we did not find an expected compensatory change in the floral visitor assemblage (e.g. bee vs. beetle preference in case of wild-type and mutant plants, respectively). Rather, a more complex scenario became obvious. We hypothesize that, at least in some habitats, wild-type and *Spe* plants have reached a kind of evolutionary 'stalemate' (David Roberts, Royal Botanic Gardens Kew, UK, pers. comm. in August 2008), in which a higher seed production of wild-type plants is compensated for by a higher germination rate of seeds of the *Spe* mutant. Future experiments are required to determine whether the observed linkages between floral phenotype, plant architecture and seed germination rate also hold at the population genetic level in natural habitats, and to identify its underlying genetic components.

For the co-existence of *Spe* and wild-type plants, floral visitors are apparently of minor importance. This is remarkable given that attracting pollinators is usually an essential function of petals. The fact that much lower numbers of floral visitors do not affect the fitness of *Spe* plants, at least in some habitats, could be easily explained by the fact that *C. bursa-pastoris*, such as *A. thaliana*, is a predominantly selfing rather than outcrossing species (Hurka et al. 1989), so that transformation of petals into stamens is not selected against because of neglect of the flowers by pollinators. Reduction or even loss of perianth organs and increase in pollen production is a typical syndrome during evolution of wind-pollinated groups such as grasses (Endress 1994), but whether this could apply also to the evolutionary future of the *Spe* variant remains highly speculative (Nutt et al. 2006).

The high rate of self-pollination in *C. bursa-pastoris* may act as a fairly effective gene flow barrier in wild populations of this plant. Together with the obvious dependence of the relative fitness of both variants on the habitat occupied, it is not unconceivable (though highly speculative) that with *Spe* we are watching a remarkable case of sympatric speciation in statu nascendi. It might therefore be of more than rare historical interest that *Spe*-like plants were already

described as a new species (*Capsella apetala*) almost 200 years ago by Opiz (1821) (see Nutt et al. 2006).

**Acknowledgments** We gratefully acknowledge E. Stolle and F. Burger (Institute of Zoology, Halle) for taxonomic determination of *Syrphidae* and bees plus wasps, respectively. We also thank the undergraduate students Stefan Lukas, Christine Steinbach, Katja Kühdorf, Ulrike Schure and Sophie Bartsch for help in observations of the plots, and Jeanette Keim and Ulrike Schure for counting fruits and seeds. Many thanks also to Richard Bateman, David Roberts and an anonymous reviewer for valuable comments and suggestions that helped considerably to improve a previous version of this manuscript. We are especially grateful to Richard Bateman for his continuous encouragement to work on this topic. This project was supported by grants from the Deutsche Forschungsgemeinschaft (TH 417/4-1 and -2 to G.T., NE 314/7-1 and -2 to B.N.).

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# 6. Gesamtdiskussion

Im Mittelpunkt dieser Arbeit stand die zentrale Frage nach der Bedeutung floraler homöotischer Mutationen für die Evolution neuer Strukturen in morphologischen Bauplänen, insbesondere neuer Blütenstrukturen. Untersuchungen an natürlich vorkommenden floral homöotischen Mutanten gab es bislang nur zu *Clarkia* und *Linaria* (Cubas *et al*. 1999, Ford & Gottlieb 1992). Das Untersuchungsobjekt, die floral homöotische Variante „*Stamenoid petals*“ (*Spe*) von *Capsella bursa-pastoris* mit dem Phänomen der staminalen Pseudapetalie, d. h. die Umwandlung der Petalen in Stamina, wurde bislang nur ungenügend berücksichtigt (Manuskript I, Hintz *et al*. 2006). Am Anfang wurde die *Spe*-Variante als eigene Art *C. apetala* (Opiz 1821) geführt und erst später als homöotische Mutante bezeichnet (Gottschalk 1971, Meyerowitz *et al*. 1989). Nur einige wenige Vererbungsanalysen mit kronblattlosen *Capsella* sind aus der Literatur bekannt (Manuskript I).

*Capsella* eignet sich dabei sehr gut als experimentelles Modellsystem, insbesondere aufgrund seiner nahen Verwandtschaft zum Modellorganismus *Arabidopsis thaliana* (Manuskript I). In dieser Arbeit wurde eine detaillierte molekulargenetische Analyse des *Spe*-Phänomens in *C. bursa-pastoris* durchgeführt, wobei höchstwahrscheinlich der für den homöotischen Phänotyp verantwortliche Locus identifiziert werden konnte (Manuskript II).

Abschließend wurde auf bestäubungsbiologische Aspekte und reproduktive Merkmale eingegangen, da drastische blütenmorphologische Veränderungen, hier der Verlust der Petalen und die erhöhte Staubblattanzahl, diese Faktoren erheblich beeinflussen können (Manuskript III).

## 6.1. Kandidatengenansatz

Zunächst war unklar, welche molekulargenetische Ursache dem *Spe*-Phänotyp zugrunde liegt. Für diese molekulare Charakterisierung eines Locus, d. h. die Identifizierung und Lokalisierung eines unbekannten Gens, das für die Erzeugung eines bestimmten Phänotyps verantwortlich ist, stehen zwei Möglichkeiten zur Verfügung (Haag & True 2001). Zum einen kann das im Fokus stehende Gen mittels aufwändiger klassischer Kartierungsanalyse auf einer Chromosomenkarte lokalisiert und anschließend der Locus über kartierungsgestützte Klonierung identifiziert werden. Im Gegensatz dazu steht der sogenannte Kandidatengenansatz. Dabei wird auf das enorme molekulargenetische Wissen über die Blütenentwicklung bei *A. thaliana* zurückgegriffen, indem man nach Genen sucht, deren Mutation in *A. thaliana* einen ähnlichen Phänotyp hervorrufen, wie der zu untersuchende.

Danach werden Orthologe dieser Gene in *C. bursa-pastoris* erkundet, wobei die große Ähnlichkeit zwischen den beiden Genomen von *Arabidopsis* und *Capsella* sehr hilfreich ist (Acarkan *et al*. 2000, Boivin *et al*. 2004, Koch & Kiefer 2005). Im Folgenden gilt es dann, mit experimentellen Nachweisen zu zeigen, dass eines dieser orthologen Gene für die Ausbildung der *Spe*-Variante verantwortlich ist.

Als Grundlage für die Arbeitshypothese diente das klassische ABC-Modell (Haughn & Sommerville 1988, Bowman *et al*. 1991, Coen & Meyerowitz 1991). Es wurde so modifiziert, dass es als molekularen Auslöser des *Spe*-Phänotyps eine Ausbreitung der Klasse C-Genfunktion auf den zweiten Blütenkreis postuliert, da offensichtlich nur dieser von der homöotischen Merkmalsausprägung betroffen ist (Manuskript I, Hintz *et al*. 2006). Weiterhin wurde davon ausgegangen, dass es sich um eine *cis*-regulatorische Mutation in einem oder mehreren Klasse C-Genen handeln könnte. Dem steht eine Veränderung in einem oder mehreren *trans*-wirksamen Faktoren, die Klasse C-Gene regulieren, gegenüber. Aufgrund der co-dominanten Vererbung, die für eine „*gain of function*“ spricht, wurde aber eine *cis*-regulatorische Veränderung in einem *AG* bzw. *AG*-ähnlichen Organidentitätsgen als wahrscheinlicher erachtet. Außerdem sind nur ein Locus oder eng gekoppelte Loci für den *Spe*-Phänotyp verantwortlich, da die $F_2$-Population, resultierend aus einer Kreuzung zwischen Wildtyp und *Spe*-Variante, 1:3 (bzw. 1:2:1) aufspaltet (Manuskript I und II). Aufgrund dieser Annahmen wurde der Fokus auf die Orthologen des Klasse C-Gens *AGAMOUS* (*AG*) und Orthologe der *AG*-ähnlichen Gene *SHATTERPROOF1* und *2* (*SHP1, 2*) sowie *SEEDSTICK* (*STK*) gelegt (Manuskript I und II, Hintz *et al*. 2006). Diese Gene sind alle Mitglieder der *AG*-Subklade.

Es wurden je zwei co-orthologe Gene der *AG*-Subklade isoliert (*CbpAGa* und *b*, *CbpSHP1a* und *b*, *CbpSHP2a* und *b*, *CbpSTKa* und *b*). Aufgrund des tetraploiden Genoms von *C. bursa-pastoris* musste überprüft werden, wie viele Genkopien jeweils zu detektieren sind. Mittels *Southern* Hybridisierung konnte keine höhere Kopieanzahl als zwei festgestellt werden (Pia Nutt, Manuskript II). Die Co-Orthologen konnten stets durch klare Unterschiede in der Basenabfolge nicht codierender Sequenzbereiche (Introns, Promotor) von einander getrennt werden. Dies traf auf codierende Bereiche (MADS-, I-, K-, C-Domäne) weit weniger zu.

## 6.2. Co-Segregationsanalyse

Die acht Kandidatengene wurden in ihrer Basenabfolge nach Sequenzunterschieden, beispielsweise *single nucleotide polymorphisms* (SNPs) zwischen Wildtyp und *Spe*-Variante untersucht. Diese waren so gering, dass beispielsweise für *CbpSHP2a* und *b* nur acht bzw. sieben SNPs über eine Sequenz von ca. 5200 Basenpaaren detektiert werden konnten (für *CbpAGa* siehe Manuskript II). Zudem war nur ein einziger SNP in der cDNA, speziell in der 5'-UTR von *CpbAGb* lokalisiert (Manuskript II). Dies kann darauf zurückzuführen sein, dass Wildtyp- und *Spe*-Pflanze von einem Standort (Gau-Odernheim) benutzt wurden, das wiederum für eine relativ junge Entstehung der *Spe*-Variante aus dem Wildtyp sprechen würde.

Unter den detektierten SNPs gab es eindeutige Einzelnukleotid-Substitutionen, aber auch Längenunterschiede in sich wiederholenden Basen oder Basenabfolgen, bei denen nie zweifelsfrei auszuschließen war, ob es sich dabei nicht auch um einen Fehler durch die Polymerase während der PCR oder bei der Sequenzierung handeln kann. Deshalb mussten die SNPs bewertet werden, da nur eindeutige SNPs zur anschließenden Genotypisierung verwendet werden konnten.

Durch Kreuzung von wildtypischen mit *Spe*-Pflanzen und darauf folgender Selbstung der $F_1$-Generation wurde eine segregierende $F_2$-Population erzeugt. Diese wurde hinsichtlich ihres Blütenphänotyps analysiert und anschließend auf Co-Segregation jeweils eines SNP (Genotyp) pro Locus mit dem *Spe*-Phänotyp untersucht.

Für diese Co-Segregationsanalyse oder SNP-Genotypisierung stehen viele verschiedene Methoden zur Verfügung (Gut 2001, Rafalski 2002). Im Wesentlichen sind das Hybridisierung und Ligation von Oligonukleotiden an der variablen Stelle, Nukleaseverdau an der Position des SNP, Primerextension und Sequenzierung. Diese Grundprinzipien können wiederum in verschiedenen Abwandlungen durchgeführt werden, z. B. CAPS und RFLP als Restriktionsmethoden. Eine schöne Übersicht und Erläuterungen bietet Gut (2001). Meist geht allen eine individuelle Amplifikation des SNP-tragenden Fragments mittels PCR voraus (Rafalski 2002). Die Methoden können auf verschiedenen Detektionsmöglichkeiten basieren, z. B. Fluoreszenz oder Massenspektrometrie. Welche Methode man anwendet, ist von der Fragestellung (Anzahl SNPs, Individuen usw.) und den zur Verfügung stehenden Mitteln (Finanzen, Geräte usw.) abhängig. In dieser Arbeit wurde die Pyrosequenzierung verwendet (Ronaghi *et al*. 1996). Die Methode wurde von Groth *et al*. (2006) so verändert, dass sie nicht nur schnell und im Maßstab von 96-Proben-Platten durchführbar ist, sondern die Möglichkeit

bietet, kostengünstig verschiedene SNPs zu analysieren. Außerdem können die Ergebnisse mit einer speziellen Software einfach und eindeutig ausgewertet werden.

In einer Co-Segregationsanalyse wird nach Übereinstimmung der Phänotypen aus der Kartierungs-Population mit den Genotypen der Elternpflanzen gesucht. Beim Auftreten einer Rekombinanten pro 100 Pflanzen, d. h. eine Pflanze bei der Phänotyp (*Spe*-Locus) und Genotyp (SNP) nicht übereinstimmen, liegt eine Rekombinationshäufigkeit von 1 % vor, was einem Abstand zwischen beiden Loci von 1 cM entspricht. Das *C. bursa-pastoris* Genom besitzt eine Größe von etwa 680-800 Millionen Basenpaaren (Bennett & Smith 1976, Johnston *et al*. 2005). Man geht davon aus, dass in *A thaliana* 1 cM etwa 200.000 Basenpaaren entspricht und eine Genomgröße von etwa 150-300 Millionen Basenpaaren vorliegt (Bennett & Smith 1976, Johnston *et al*. 2005). Folglich hätte man mindestens 10000 Pflanzen einer segregierenden $F_2$-Population von *C. bursa-pastoris* untersuchen müssen, um sich auf einen Bereich von circa 10000 Basenpaaren für die Lokalisation des betreffenden Locus konzentrieren zu können. Demzufolge war die von mir verwendete Kartierungs-Population von 191 Pflanzen zu klein, um sich auf einen segregierenden Locus eines der Kandidatengene als Ursache für das *Spe*-Phänomen festlegen zu können. Es muss demnach berücksichtigt werden, dass auch ein eng gekoppelter Locus verantwortlich sein kann.

In der Segregationsanalyse wurde die Staminoidie der Petalen als phänotypischer Marker genutzt. Unterschieden wurden dabei die Merkmalsklassen wildtypisch und mutant. Als molekularer Marker diente je ein detektierter SNP pro Gen, der jeweils homozygot wildtypisch, heterozygot oder homozygot mutant vorliegen konnte.

Als einziger co-segregierender Locus wurde *CbpAGa* identifiziert (Manuskript II). Dieser oder ein daran eng gekoppelter Locus sind somit für die Ausprägung des *Spe*-Phänotyps verantwortlich. Alle übrigen potentiellen Kandidatengene (*CbpAGb*, *CbpSHP1a* und *b*, *CbpSHP2a* und *b*, *CbpSTKa* und *b*) konnten durch die Analyse ausgeschlossen werden. Die zusätzlichen Daten von Pia Nutt aus den Expressionsanalysen mittels *in-situ* Hybridisierung (Manuskript II), in denen eine ektopische Expression von *CbpAG* im 2. Wirtel nachgewiesen wurde, unterstützen diese Aussage. Die ebenfalls im 2. Blütenkreis detektierte Überexpression von *CbpSTK* könnte durch eine Aktivierung von *CbpSTK* durch funktionelles *CbpAG* in diesem Wirtel verursacht und somit auch an den *Spe*-Phänotyp gekoppelt sein. Die beiden Co-Orthologen *CbpSTKa* und *b* selbst sind aber nicht für die Ausprägung von *Spe* verantwortlich, da sie in der Kopplungsanalyse nicht mit dem Phänotyp co-segregieren (Manuskript II).

### 6.3. Mutation im *cis*-regulatorischen Bereich von *CbpAGa*

In der Kontrolle eines Gens werden *trans*- und *cis*-regulatorische Mechanismen unterschieden. *Trans*-wirksame Regulatoren wirken meist auf mehrere Zielgene, so dass im Falle einer Mutation in solch einem übergeordneten Regulationsgen oftmals ausgeprägte pleiotrope Effekte zu beobachten sind (Wray *et al*. 2003). In Bezug auf die phänotypische Ausprägung von *Spe* konnten bisher aber keine pleiotropen Auswirkungen dokumentiert werden (Manuskript II). Zusätzlich konnte für die beiden Genome von *A. thaliana* und *Capsella rubella* nachweislich eine große Ähnlichkeit und Übereinstimmung bezüglich chromosomaler Anordnung und Orientierung gezeigt werden (Acarkan *et al*. 2000, Boivin *et al*. 2004). Sollte also ein *trans*-wirksamer Regulator für den *Spe*-Phänotyp verantwortlich sein, sollte auch im *Arabidopsis*-Genom einer der bekannten negativen Regulatoren enger mit dem *AG*-Locus gekoppelt sein. Gestützt auf diese Co-Linearität der oben genannten Genome kann man jedoch mit relativ hoher Wahrscheinlichkeit ausschließen, dass eine enge Kopplung eines negativen Regulators an den *CbpAGa* Locus in *Capsella* vorliegt, da sich im *Arabidopsis* Genom keiner der bekannten negativen Regulatoren stromauf- oder stromabwärts von *AG* befindet.

Mit den Mutanten *knotted1*, *gnarley1* und *rough sheath1* (Mais), *hooded* (Gerste) und *ovulata* (*Antirrhinum*) wurden schon mehrere Beispiele in Pflanzen untersucht, in denen Mutationen innerhalb *cis*-regulatorischer Regionen für die Ausbildung der jeweiligen Phänotypen verantwortlich sind (Vollbrecht *et al*. 1991, Bradley *et al*.1993, Foster *et al*. 1995, Schneeberger *et al*. 1995, Müller *et al*. 1995). Im Falle von *CbpAGa* lässt die intakte Funktion des Gens auf eine Regulationsstörung schließen, da Karpelle und Stamina der beiden inneren Wirtel offensichtlich nicht beeinträchtigt sind. Des Weiteren sind sowohl die Co-Segregation von *CbpAGa* mit dem *Spe*-Phänotyp aus einer segregierenden $F_2$-Population als auch die ektopische Expression von *CbpAG* im zweiten Organkreis starke Hinweise dafür, dass die Ursache für *Spe* höchstwahrscheinlich in einer Änderung der Regulation von *CbpAGa* zu suchen ist, und dass eine Veränderung in einem der *cis*-regulatorischen Sequenzelemente dieses Entwicklungskontrollgens vorliegt. Folglich müssen die negative Regulation des Klasse C-Gens und damit die räumliche Restriktion der Expression auf die inneren beiden Wirtel gestört sein.

Aufgrund dieser Annahme, dass eine Veränderung im *cis*-regulatorischen Bereich des *CbpAGa* Locus viel wahrscheinlicher der Auslöser für den *Spe*-Phänotyp ist als ein *trans*-wirksamer Regulator von *CbpAGa*, wurden nicht-codierende Bereiche mit regulatorischer Funktion (stromaufwärts der 5'-UTR, Introns) intensiv nach Sequenzveränderungen

untersucht. Im 2. Intron von *CbpAGa* konnte ein großer Sequenzpolymorphismus, bestehend aus einer Deletion von 22 Basenpaaren und 3 Substitutionen, lokalisiert werden (Manuskript II).

Umfassende Untersuchungen über den intragenischen Promotorbereich im 2. Intron des *AG* Gens von *Arabidopsis* beschäftigten sich mit positiver sowie negativer Regulation (z. B. Sieburth & Meyerowitz 1997, Busch *et al*. 1999, Deyholos & Sieburth 2000, Hong *et al*. 2003, Causier *et al*. 2009). Das 2. Intron kann danach in verschiedene Teilbereiche eingeteilt werden, wobei der 5'-Aktivierungsdomäne eher eine Bedeutung innerhalb der Staminaentwicklung und dem Bereich des 3'-Endes eine Rolle innerhalb der Ovulaentwicklung zugeschrieben wird (Deyholos & Sieburth 2000). Der Sequenzpolymorphismus befindet sich genau in dem Abschnitt, der eine entscheidende Rolle in der Staminaentwicklung spielt.

Die Regulation wird durch die Bindung von Transkriptionsfaktoren an *cis*-regulatorische Sequenzelemente, die in diesem 2. Intron lokalisiert sind, realisiert, wobei eine Vielzahl verschiedener Proteine als Faktoren in dieser Regulationsmaschinerie involviert sind (z. B. Sieburth & Meyerowitz 1997, Busch *et al*. 1999, Deyholos & Sieburth 2000, Hong *et al*. 2003, Causier *et al*. 2009). Diese Funktion ist dabei nicht nur auf *A. thaliana* begrenzt. Innerhalb der Angiospermen ist ein hoher Konservierungsgrad für bestimmte Bereiche in dieser intragenischen Region zu erkennen, denen eine regulierende Funktion zugesprochen wird (Hong *et al*. 2003, Causier *et al*. 2009). Das Sequenzalignment der polymorphen Region von *CbpAGa* mit dem homologen Sequenzbereich aus 18 verschiedenen Vertretern der *Brassicaceae* zeigt an dieser Stelle ebenso eine hohe Konservierung. Daher ist anzunehmen, dass es sich hierbei um einen *cis*-regulatorischen Bereich handeln kann. Bisherige Untersuchungen zu *cis*-Elementen zeigen allerdings, dass diese in ihrer Mehrheit von positiv wirkenden Regulatoren, beispielsweise *LEAFY* (*LFY*), *WUSCHEL* (*WUS*) oder *PERIANTHIA* (*PAN*) gebunden werden (Busch *et al*. 1999, Lenhard *et al*. 2001, Lohmann *et al*. 2001, Hong *et al*. 2003, Causier *et al*. 2009, Maier *et al*. 2009). Sie kommen für die Ursache des *Spe*-Phänotyps nicht in Betracht, da positive Regulation, gleichzusetzen mit Aktivierung, nicht gestört sein sollte, da Blütenorgane der inneren Wirtel korrekt entwickelt werden.

Mögliche Kandidaten für negative Regulatorgene sind zwar hinsichtlich ihrer Wirkungsweise und Struktur gut untersucht, über deren Bindestellen in *AG* ist aber meist nicht viel bekannt. Ihr Potential an das konservierte und in *Spe* deletierte Sequenzelement zu binden und somit als Vermittler der *AG*-Repression im 2. Wirtel zu dienen, ist sehr unterschiedlich. Beispielsweise benötigen Repressoren von *AG* oftmals einen

Interaktionspartner, um an DNA binden und infolge dessen regulieren zu können, wie z. B. *CURLY LEAF* (*CLF*), *LEUNIG* (*LUG*) und *SEUSS* (*SEU*) (Goodrich *et al*. 1997, Conner & Liu 2000, Franks *et al*. 2002, Sridhar *et al*. 2004). Die Heterodimerkomplexe aus *LEU* und *SEU* können mit den MADS-Domänen-Proteinen *AP1*, *SEP3*, *AGL24* und *SVP* zu höheren Komplexen aggregieren, welche an das Sequenzmotiv CArG-Box binden können (Gregis *et al*. 2006, Sridhar *et al*. 2006, Liu & Karmarkar 2008). Solche CArG-Boxen sind in *Arabidopsis* innerhalb der 3'-Aktivierungsdomäne des 2. Introns lokalisiert und zeichnen sich durch die Konsensussequenz ($CC(A/T)_6GG$) aus (Hong *et al*. 2003). Da sich diese Sequenz stark von der Sequenz in der polymorphen Region unterscheidet und an einer anderen Stelle im 2. Intron positioniert ist, sind *LUG* und *SEU* als Kandidatenproteine eher ungeeignet (Manuskript II).

*PETAL LOSS* (*PTL*) und *ROXY1* sind ebenfalls negative Regulatoren von *AG*. Sie regulieren Genaktivität auf indirektem Weg, in dem sie andere Repressoren post-translational modifizieren (Brewer *et al*. 2004, Xing *et al*. 2005). Daher ist es ziemlich unwahrscheinlich, dass sie mit dem *Spe*-Phänomen in Verbindung stehen, wenn keine direkte Bindung beider Faktoren an *AG* erfolgt. Für den Regulator *BELLRINGER* (*BLR*) ist sogar eine putative Bindesequenz bekannt. Sie konnte an homologer Stelle im *AG* Gen von *Arabidopsis* 500 Basenpaare stromabwärts vom Sequenzort des Polymorphismus in *CbpAGa* lokalisiert werden und besitzt auch keine Sequenzähnlichkeit, womit *BLR* ebenfalls ungeeignet ist (Bao *et al*. 2004, Manuskript II).

*RABBIT EARS* (*RBE*) und *STERILE APETALA* (*SAP*) sind zwei weitere Kandidatenproteine, die in *A. thaliana AG* negativ regulieren und somit eine Rolle bezüglich der Regulation von *CbpAGa* spielen könnten (Byzova *et al*. 1999, Krizek *et al*. 2006). Expressionsmuster beider Gene zeigen, dass ihre Funktion auf den 2. Wirtel beschränkt ist. Leider kann *SAP* bisher in keine der gut charakterisierten Proteinfamilien eingeordnet werden (Byzova *et al*. 1999). *RBE* wiederum ist ein Zink-Finger-Protein (Takeda *et al*. 2003, Krizek *et al*. 2006). Für einige dieser Proteine muss die Kernsequenz -AGT- im Bindemotiv enthalten sein, teilweise mehr als einmal, um eine Bindung zu gewährleisten (Takatsuji *et al*. 1994, Takatsuji & Matsumoto 1996). Innerhalb der polymorphen Stelle ist solch eine Basenfolge vorhanden. Krizek *et al*. 2006 fanden innerhalb des 2. Intron von *AG* 102 dieser -AGT- Kernsequenzmotive, da die Wahrscheinlichkeit, solch ein Motiv in einer zufällig verteilten Basenabfolge zu finden, sehr hoch ist (1:64). Aufgrund der bisherigen Evidenzen können *RBE* und *SAP* nicht ausgeschlossen werden.

Ein weiterer Repressor von *AG* ist *APETALA2* (*AP2*), ein Vertreter der *AP2/EREBP*-Genfamilie. Neben vielen anderen Funktionen verhindert *AP2*, dass *AG* über den 3. und 4. Wirtel hinaus exprimiert wird (Jofuku *et al*. 1994, Riechmann & Meyerowitz 1997). Die Konsensussequenz für die Bindestelle einiger *EREBP*-Faktoren ist bekannt, unterscheidet sich aber deutlich von der polymorphen Stelle in *CbpAGa* (Nole-Wilson & Krizek 2000). *AINTEGUMENTA* (*ANT*) gehört ebenfalls zur *AP2/EREBP*-Genfamilie und wirkt ähnlich in Bezug auf die Repression von *AG*, so dass *AP2* und *ANT* augenscheinlich keine besonders guten Kandidaten darstellen (Krizek *et al*. 2000).

Zusammengefaßt gibt es momentan keinen „heißen" Kandidat für die negative Regulation von *CbpAGa* durch Bindung an die mutmaßliche *cis*-regulatorische Region im 2. Intron, die in der *Spe*-Variante eine stark vom Wildtyp abweichende Sequenzveränderung aufweist. *RBE* und *SAP* kommen als negative Regulatorproteine in Frage, aber auch andere Faktoren können derzeit nicht ausgeschlossen werden. Man sollte außerdem stets bedenken, dass diese Informationen über negative Regulatoren von *AG* aus Analysen mit *A. thaliana* stammen und die Situation in *C. bursa-pastoris* ähnlich, aber auch abgewandelt sein kann.

### 6.4. *Spe*-Locus der Warburg-Population

Populationen der *Spe*-Variante von *C.*bursa-*pastoris* sind an verschiedenen europäischen Standorten beschrieben worden (Manuskript I und II). Kreuzungsexperimente mit den *Spe*-Linien aus Gau-Odernheim und Warburg in Form eines Allelietests zeigten, dass in beiden Linien der gleiche Locus oder eng gekoppelte Loci für die Ausprägung des *Spe*-Phänotyps verantwortlich sind (Pia Nutt, Manuskript II). Mittels spezifischem PCR-Test konnte die Sequenzdeletion in *CbpAGa* in der *Spe*-Variante aus Warburg ursprünglich nicht nachgewiesen werden (Manuskript II). Dieses Ergebnis konnte in nachfolgenden Tests nicht bestätigt werden (Daten nicht gezeigt). Möglicherweise wurde Pflanzenmaterial oder daraus isolierte DNA vertauscht. Anschließende Sequenzierung des 2. Introns zeigten nun exakt die gleiche Sequenzveränderung in *CbpAGa* aus der Warburger *Spe*-Linie im Vergleich zur Wildtyp-Sequenz, bestehend aus der Deletion und den drei Substitutionen, wie sie in der *Spe*-Variante aus Gau-Odernheim detektiert wurde (Manuskript II, Daten für *Spe*-Variante aus Warburg nicht gezeigt). Dieser Fund würde zumindest den Test auf Allelie untermauern, aber auch die Frage aufwerfen, in welchem genetischen Zusammenhang die beiden *Spe*-Varianten aus Gau-Odernheim und Warburg stehen. Dabei liegt die Vermutung nahe, dass beide Varianten auf einen gemeinsamen Vorfahren zurückgehen. Erste Analysen, basierend auf AFLP-Daten zeigen jedoch, dass Wildtyp und *Spe*-Variante von einem Standort näher

miteinander verwandt sind, als Individuen mit *Spe*-phänotypischer Ausprägung untereinander (Hameister 2009). Als weitere mögliche Ursache für den gleichen Sequenzunterschied in *CbpAGa* aus beiden *Spe*-Linien könnte auch das Einbringen des Allels der *Spe*-Variante aus Warburg in das Genom der Variante aus Gau-Odernheim oder *vice versa* infolge von Introgression sein.

### 6.5. Einblicke in die Bestäubungsbiologie und Fitness der *Spe*-Variante

Ob sich phänotypische Neuheiten, wie sie von homöotischen Mutationen hervorgerufen werden können, in einer Population manifestieren und in Sympatrie mit dem Wildtyp behaupten können, ist eine zentrale Frage in der Evolutionsbiologie. Berichte und nähere Untersuchungen über homöotische Mutanten, die über einen längeren Zeitraum natürliche Populationen bilden, sind aber mangelhaft (Manuskript I). Im Rahmen dieser Arbeit konnten an künstlich angelegten Plotexperimenten in den Botanischen Gärten Jena und Halle Beobachtungen der *Spe*-Variante im Freiland durchgeführt werden (Manuskript III).

Die *Spe*-Variante ist offensichtlich nicht in ihrer Reproduktionsfähigkeit beeinträchtigt im Gegensatz zu den meisten floralen homöotischen Mutanten, z. B. die typische Klasse C-Mutante, der alle zur Fortpflanzung nötigen Organe fehlen (Yanofsky *et al*. 1990). Die Reproduktionsorgane der beiden inneren Wirtel sind nicht betroffen und die zusätzlich ausgebildeten Stamina im 2. Wirtel sind zudem funktionsfähig (Pia Nutt, Manuskript II). Petalen dienen neben ihrer Schutzfunktion jedoch hauptsächlich der Anlockung potentieller Bestäuber, die aufgrund des Kronblattverlustes beeinflußt sein könnte. Daher galt mein Interesse vorrangig den Auswirkungen des *Spe*-Phänotyps auf die Attraktion potentieller Bestäuber sowie dem Vergleich der individuellen reproduktiven Fitness der *Spe*-Variante und des Wildtyps. Diese muss für Pflanzen beider Blütenphänotypen ähnlich sein, da sonst auf längere Sicht keine Co-Existenz möglich wäre.

In den durchgeführten Freilandversuchen konnte ein diverses Artenspektrum potentieller Bestäuber (v. a. Wildbienen, Schwebfliegen, Thripse) sowohl für wildtypische als auch *Spe*-Pflanzen dokumentiert werden. Im Vergleich der Spektren beider Phänotypen konnten keine nennenswerten Unterschiede festgestellt werden. Ebenso konnten keine signifikanten Verschiebungen bezüglich prozentualer Anteile der einzelnen Artengruppen für jeden Phänotyp gezeigt werden, beispielsweise mehr pollenfressende Käfer auf *Spe*-Pflanzen (Manuskript III).

Wildtypische *C. bursa-pastoris* wurden im Vergleich zur *Spe*-Variante doppelt so häufig besucht. Dabei ist nicht auszuschließen, dass aufgrund des Mehrangebots an

Infloreszenzen pro Pflanze, aber auch offener Blüten im einzelnen Blütenstand des Wiltypes eine höhere Anziehungskraft auf potentielle Bestäuber besteht. Die Unterschiede in der Besucherhäufigkeit pro offene Blüten sind nur noch geringfügig (Manuskript III).

Die Produktion von Blütenduftstoffen spielt neben dem optischen Reiz der Petalen ebenso eine wichtige Rolle beim Anlocken potentieller Bestäuber (Dobson 1994, Dobson & Bergström 2000). Typischerweise bestehen Düfte aus Terpenoiden, Fettsäurederivaten, Indolverbindungen und Benzenoiden (Knudsen & Tollsten 1993). Für die Gewinnung der Duftstoffe wurde eine Headspace-Festphasenmikroextraktion (headspace SPME) durchgeführt, die sich durch einfache Anwendung, Sensitivität und Robustheit auszeichnet (Manuskript III). Größter Vorteil dieser Methode ist aber die direkte Analyse der extrahierten Proben in der GC-MS (gaschromatographische Massenspektrometrie), die nach Abgleich mit erhältlichen Standards oder mit der NIST Bibliothek (National Institut of Standards and Technology) eine Zuordnung der detektierten Stoffe erlaubt (Zhang & Pawliszyn 1993, Chin *et al*. 1996, Verdonk *et al*. 2003). Die Tatsache, dass für wildtypische *C. bursa-pastoris* Terpene sowie Benzaldehyde, und für den *Spe*-Phänotyp gar keine Duftstoffe detektiert werden konnten, lässt vermuten, dass diese Stoffgruppen von den Petalen gebildet werden, wie es in Studien mit *Petunia hybrida*, *Mirabilis jalapa* und *Arabidopsis lyrata* bereits gezeigt wurde (Verdonk *et al*. 2003, Effmert *et al*. 2005, Abel *et al*. 2009, Manuskript III). Das bedeutet außerdem, dass Duftstoffe in Blüten von *C. bursa-pastoris* nicht entscheidend für die Anlockung potentielle Bestäuber sind, da *Spe*-Pflanzen immer noch besucht werden, wenn auch nur halb so oft wie der Wildtyp (Manuskript III).

Individuelle Fitness ist definiert als Anzahl reproduktionsfähiger Nachkommen einer Pflanze. Oft werden aber auch andere Parameter, z. B. pflanzliche Biomasse als Maß für Fitness genommen. In dieser Arbeit wurde zum einen die Anzahl gebildeter Samen pro Pflanze bestimmt, als auch potentiell zu erwartende Keimlinge, die die Folgegeneration darstellen. Repräsentativ wurden einige der Keimlinge zur Blüte gebracht, um ihre Reproduktionsfähigkeit zu testen. Wildtypische *C. bursa-pastoris* entwickeln eine höhere Anzahl Samen pro Pflanze, wobei Samenanzahl pro Frucht konstant ist (Manuskript III). Demnach besitzt der Wildtyp eine höhere Fitness als *Spe*-Pflanzen. Die *Spe*-Variante weist hingegen eine höhere Keimeffizienz auf (Manuskript III). Sie könnte daher durch Unterschiede in der Keimstrategie eine Möglichkeit gefunden haben, mit dem Wildtyp in Co-Existenz zu leben. Weitere Untersuchungen zeigen, dass auch der Blühzeitpunkt eine entscheidende Rolle in der gemeinschaftlichen Existenz von Wildtyp und *Spe*-Variante spielt, da *Spe*-Pflanzen signifikant später blühen als der Wildtyp (Hameister *et al*. 2009).

Sowohl Beobachtungen von S. Hameister (Hameister 2009) in den letzten Jahren am natürlichen Standort, als auch die Aufzeichnungen von Reichert (1998) belegen eine stabiles Vorkommen von *Spe* in Gemeinschaft mit wildtypischen *C. bursa-pastoris* (Manuskript I und III, Hintz *et al*. 2006). Zusätzlich wird diese Aussage von historischen Beschreibungen unterstützt (Murbeck 1918, Dahlgren 1919). Dieses beständige Auftreten wird durch anthropogene Einwirkungen, z. B. die wirtschaftliche Bearbeitung der Weinberge in Gau-Odernheim, stark begünstigt, welche die Verbreitung der Samen erheblich fördern (Hurka & Haase 1982).

### 6.6. Entwicklungs- und evolutionsbiologische Bedeutung

Die *Spe*-Variante von *C. bursa-pastoris* repräsentiert einen Fall von Homöosis ganz besonderer Art. Im Vergleich zu anderen floralen homöotischen Mutanten verfügt sie über ausreichend Fitness und somit Potential, um mit wildtypischen Pflanzen in verschiedenen natürlichen Populationen gemeinschaftlich existieren zu können. Das Einzigartige der homöotischen Veränderung, die vollständige Umwandlung der Petalen in Stamina, ist die Beschränkung auf nur einen Blütenkreis ohne offensichtlich zu erkennende pleiotrope Effekte, die das Überleben der *Spe*-Pflanzen beeinträchtigen würde. Die Veränderung in nur einem entwicklungsbiologisch relevanten Gen (hier: *CbpAGa*) reicht aus, um neue blütenmorphologische Strukturen zu erzeugen und somit die Entstehung einer neuen Abstammungslinie zu ermöglichen. Auf diese molekulargenetischen Ergebnisse gestützt, kann die *Spe*-Variante als Modell für einen nicht graduell verlaufenden, also sprunghaften (saltatorischen) Entwicklungsschritt innerhalb der Makroevolution interpretiert werden (Theißen 2006, 2009). Da für *Spe*-phänotypische Pflanzen keine offensichtlichen negativen Auswirkungen auf individuelle Fitness verzeichnet werden konnten, würde diese homöotische Mutante nach dem von R. Goldschmidt (1940) postulierten Konzept als ‚hopeful monster' einzuordnen sein, welches aber im Widerspruch zu Darwins Artbildung durch natürliche Selektion steht, in der sich Mutationen nur schrittweise in einer Population etablieren aufgrund von Vorteilen, die der veränderte Phänotyp für den Organismus mit sich bringt (Darwin 1859, Theißen 2006).

Außerdem könnte die *Spe*-Variante als Beispiel sympatrischer Artbildung diskutiert werden. Die hohe Selbstungsrate, unterschiedliche Keimstrategie und Verschiebung des Blühzeitpunktes tragen möglicherweise zu reproduktiver Isolation und somit sympatrischer Artbildung bei. Der Verlust der Petalen scheint offensichtlich bei der sich überwiegend selbst bestäubenden *C. bursa-pastoris* nur von geringer Bedeutung für die reproduktive Fitness der

*Spe*-Variante zu sein und folglich nicht unmittelbar unter evolutionären Selektionsdruck aufgrund des Ausbleibens diverser Bestäuber zu stehen. Die hohe Selbstbefruchtungsrate, die für *C. bursa-pastoris* bekannt ist (Hurka & Neuffer 1997), kann als erste Barriere für intraspezifischen Genfluß angesehen werden (Levin 1971). Diese Barriere kann durch Verschiebung des Blühzeitpunktes noch unterstützt werden (Levin 2006). Populationsgenetische Analysen zeigen, dass die genetische Differenzierung wildtypischer und *Spe*-Pflanzen aus Gau-Odernheim eine Behandlung beider Varianten als eigenständige Subpopulationen zulässt (Hameister *et al*. 2009).

Die *Spe*-Variante ist somit ein exzellentes Modell für die Entwicklungsbiologie, um einerseits makroevolutionäre Neuentstehungen und ihre zugrunde liegenden Mechanismen auf molekularer Ebene zu erforschen. Dabei bietet sie Möglichkeiten, Regulationsprozesse im Allgemeinen zu untersuchen, aber auch Prinzipien speziell im Netzwerk der Blütenentwicklungsgene zu verstehen. Andererseits könnte die *Spe*-Variante als neue Abstammungslinie in Artbildungsprozessen eine Rolle spielen.

### 6.7. Ausblick

Um die Aussage, dass der polymorphe Sequenzabschnitt in einer *cis*-regulatorischen Region des Blütenorganidentitätsgen *CbpAGa* für den *Spe*-Phänotyp verantwortlich ist, eindeutig zu beweisen, können Transformationsexperimente durchgeführt werden. Dafür muss der mutmaßliche mutante Locus (*CbpAGa*) vollständig kloniert und in wildtypische *C. bursa-pastoris* Pflanzen transformiert werden. Das bedeutet die Isolierung der kompletten genomischen DNA des *CbpAGa*-Locus mit putativer Promotorsequenz stromaufwärts der 5'-UTR, aber auch stromabwärts der 3'-UTR, um zumindest alle in unmittelbarer Nähe liegenden möglichen regulatorischen Bereiche zu berücksichtigen. Das Konstrukt tragende Nachkommen sollten dann einen *Spe*-Phänotyp ausbilden, da es sich um einen co-dominanten Erbgang handelt. Man kann dafür bereits auf für *C. bursa-pastoris* etablierte und publizierte Methoden zur Transformation zurückgreifen (Bartholmes *et al*. 2008).

Des Weiteren wäre es höchst interessant die orthologen Proteine der diskutierten Kandidatenproteine, z. B. *AP2*, *ANT*, *RBE* und *SAP* aus *A. thaliana* hinsichtlich ihres Bindeverhaltens an den Sequenzbereich, der für Wildtyp und *Spe*-Variante verschieden ist, zu untersuchen (z. B. mittels Gelretardierung). Zum einen könnte auf diese Art ein negativer Regulator von *CbpAGa* bestimmt werden, der die Expression dieses Gens im 2. Wirtel reprimiert. Zum anderen könnte ein neuartiges *cis*-Element beschrieben werden, wie es bisher

noch nicht für das 2. Intron oder spezifischer Bindestellen negativer Regulatoren von *A. thaliana* dokumentiert wurde.

*Spe*-phänotypische Pflanzen sind auch von anderen europäischen Standorten bekannt (z. B. Schweden, Prag, Wien) (Manuskript I). Es würde das Ergebnis dieser Arbeit unterstützen, wenn die Sequenz innerhalb des 2. Intron von *CbpAGa* aus diesen *Spe*-Populationen eine Veränderung in gleicher Weise gegenüber der Wildtypsequenz zeigt.

Idealerweise sollten Untersuchungen über Bestäuber und Fitness an den natürlichen Populationen und in Form von Langzeitstudien durchgeführt werden. Somit stellt diese Arbeit nur den Beginn einer solchen Dokumentation dar und kann in verschiedenen Punkten noch optimiert werden. Beispielsweise sind die durchgeführten Plotexperimente nur als semi-natürlich einzustufen, da beide Phänotypen am natürlichen Standort in Mischpopulationen auftreten. Für eine präzise Durchführung der Beobachtungen wurden *Spe*- und wildtypische Pflanzen jedoch mit einem Meter Abstand räumlich voneinander getrennt. Untersuchungen am natürlichen Standort haben zudem den Vorteil, dass hinsichtlich der untersuchten Parameter (z. B. Blüten- Infloreszenz- oder Fruchtanzahl) die intraspezifische Variationsbreite der wildtypischen und *Spe*-Population mit in die Analyse einfließen kann.

## 7. Zusammenfassung

In dieser Arbeit wurde die Möglichkeit genutzt, echte Homöosis im Hinblick auf ihre molekularen Auslöser sowie auf ihre Bedeutung in der Natur experimentell zu untersuchen. Die natürlich vorkommende, floral homöotische Variante *Spe* von *Capsella bursa-pastoris*, deren Phänotyp eine vollständige Umwandlung von Petalen in Stamina im 2. Blütenwirtel aufweist, stellt dabei ein geeignetes Untersuchungsobjekt für die Erforschung der Blütenentwicklung dar.

Mittels Co-Segregationsanalyse von Kandidatengenen der Klasse C-Gene der MADS-box-Genfamilie konnten Hinweise für den molekularen Mechanismus erbracht werden, der dem *Spe*-Phänomen zugrunde liegt. Es wurde gezeigt, dass der Locus *CbpAGa* in einer 3:1 aufspaltenden $F_2$-Population mit dem mutanten Phänotyp co-segregiert und somit für die Ausprägung des *Spe*-Phänotyps verantwortlich sein könnte. Alle weiteren Kandidaten der Klasse C-Gene wurden über die Co-Segregationsanalyse ausgeschlossen. Innerhalb eines hoch konservierten Bereichs des für seine *cis*-regulatorische Funktion bekannten 2. Introns von *CbpAGa* wurde ein Sequenzpolymorphismus lokalisiert, der möglicherweise Teil einer Bindestelle für einen der negativen Regulatoren von *CbpAGa* ist.

Beobachtungen im Freiland zeigten, dass sowohl *C. bursa-pastoris* Wildtyp als auch *Spe* vom gleichen Artenspektrum potentieller Bestäuber aufgesucht werden, allerdings mit einer doppelt so hohen Häufigkeit für den Wildtyp. Blütenduftstoffe konnten nur für Blüten des Wildtyps detektiert werden. Dies lässt auf eine Produktion der Duftstoffe in den Petalen schließen. Vergleichende Untersuchungen reproduktiver Merkmale, z. B. Anzahl der Samen pro Pflanze, weisen auf eine reduzierte Fitness der *Spe*-Variante hin. Das gemeinschaftliche Vorkommen am natürlichen Standort wird wahrscheinlich durch die erhöhte Keimrate der *Spe*-Variante realisiert. Somit scheinen die Blütenstruktur sowie Blütenbesuche potentieller Bestäuber der wildtypischen und *Spe*-phänotypischen *C. bursa-pastoris* eine untergeordnete Rolle zu spielen, möglicherweise aufgrund des Verlustes des Selbstinkompatibilitätssystems.

Am Beispiel der *Spe*-Variante von *C. bursa-pastoris* konnte gezeigt werden, dass homöotische Mutanten das Potential besitzen, sich in der Natur zu etablieren. Somit wurde eine Basis geschaffen, um Makro- und Mikroevolution im Bereich der angiospermen Blütenentwicklung molekulargenetisch und ökologisch erfolgreich zu untersuchen.

## Summary

In this study, the opportunity to examine real homeosis experimentally was taken, regarding its molecular trigger as well as its importance in nature. The naturally occurring floral homeotic variant *Spe* of *Capsella bursa-pastoris*, showing a complete transformation of petals into stamens in the second floral whorl, represents a suitable model organism for the investigation of flower development.

The molecular mechanism behind the *Spe* phenomenon was elucidated with a co-segregation analysis of candidate genes from orthologs of the C-class genes from the MADS-box gene family. Namely, it was shown that the *CbpAGa* locus co-segregates with the mutant phenotype of *C. bursa-pastoris* in a 3:1 segregating $F_2$ population. Therefore, the *CbpAGa* locus seems to be responsible for the development of the *Spe* phenotype. All other candidate C-class genes were ruled out in the co-segregation analysis. In a highly conserved region of the second intron of *CbpAGa*, well known for its *cis*-regulatory function in the *AG* gene of *Arabidopsis*, a sequence polymorphism is localized that could be a part of a binding element for a negative regulator of *CbpAGa*.

Observations in field studies revealed a similar spectrum of potential pollinators visiting wildtypic as well as *Spe* plants, but with a doubled number of wild-type visits. Floral volatiles were only detected in wild-type flowers. Therefore, volatiles in *C. bursa-pastoris* seem to be produced by petals. Investigations of reproductive traits, i.e. number of seeds per plant, suggest a slightly reduced fitness of *Spe* plants. However, the co-existence in the natural habitat could be due to a higher germination rate of the *Spe* variant, which compensates for produced seed number. Hence, flower structure and floral visitations of potential pollinators of wild-type and *Spe* plants seems to be only of minor importance, possibly because *C. bursa-pastoris* is mainly self-pollinating.

In summary, the potential of a homeotic mutant to establish themselves in nature was demonstrated using the *Spe* variant of shepherd's purse as an example. Therefore, we provide a basal platform to successfully investigate macro- and microevolution in the scope of flower development in angiosperms in a context of molecular genetics as well as in an ecological way.

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SUPPLEMENTAL ONLINE MATERIAL TO

# Ectopic expression and co-segregation of an *AGAMOUS* orthologue in *Stamenoid petals*, a natural floral homeotic variant of *Capsella bursa-pastoris*

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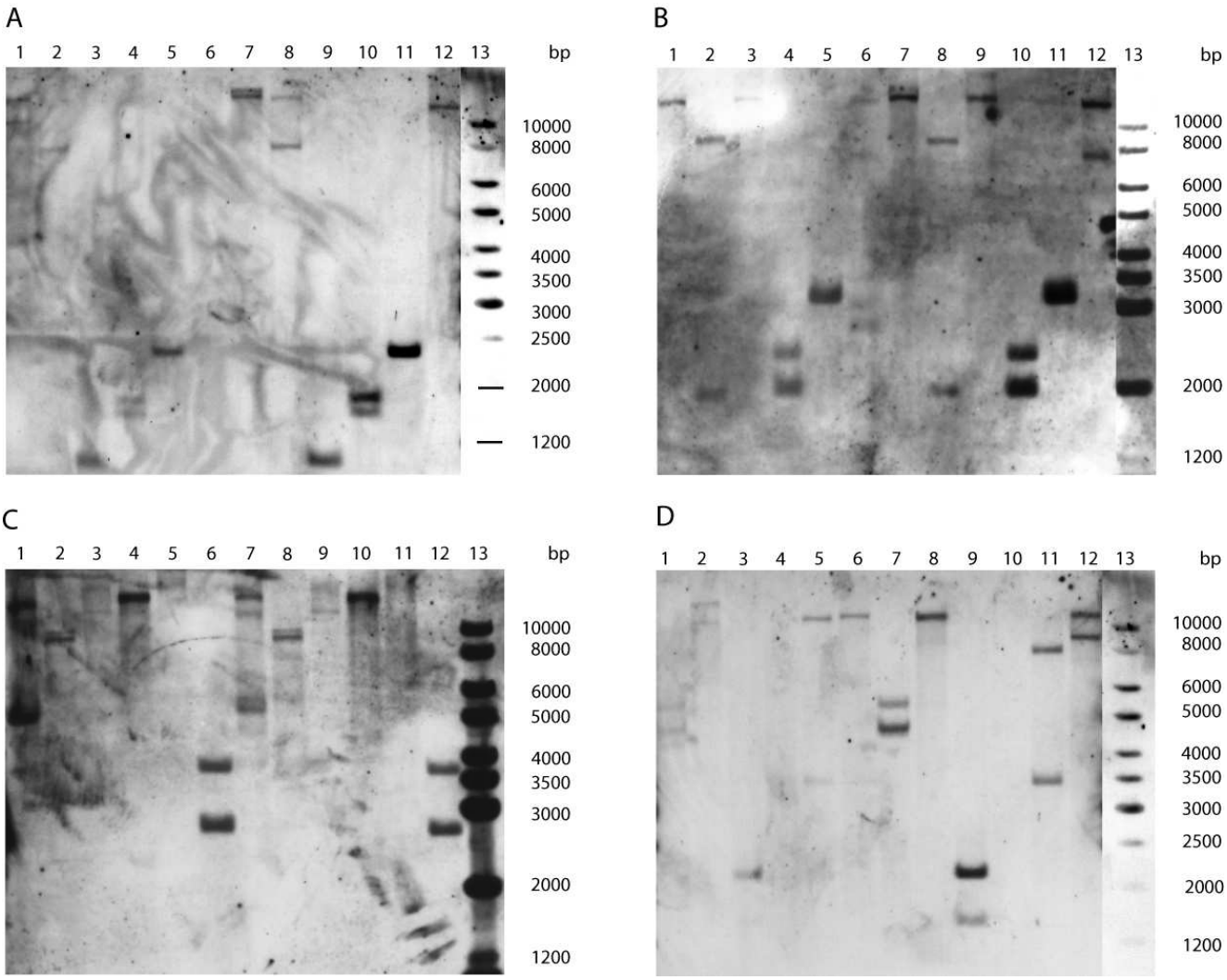



**Supplemental Figure 1.**

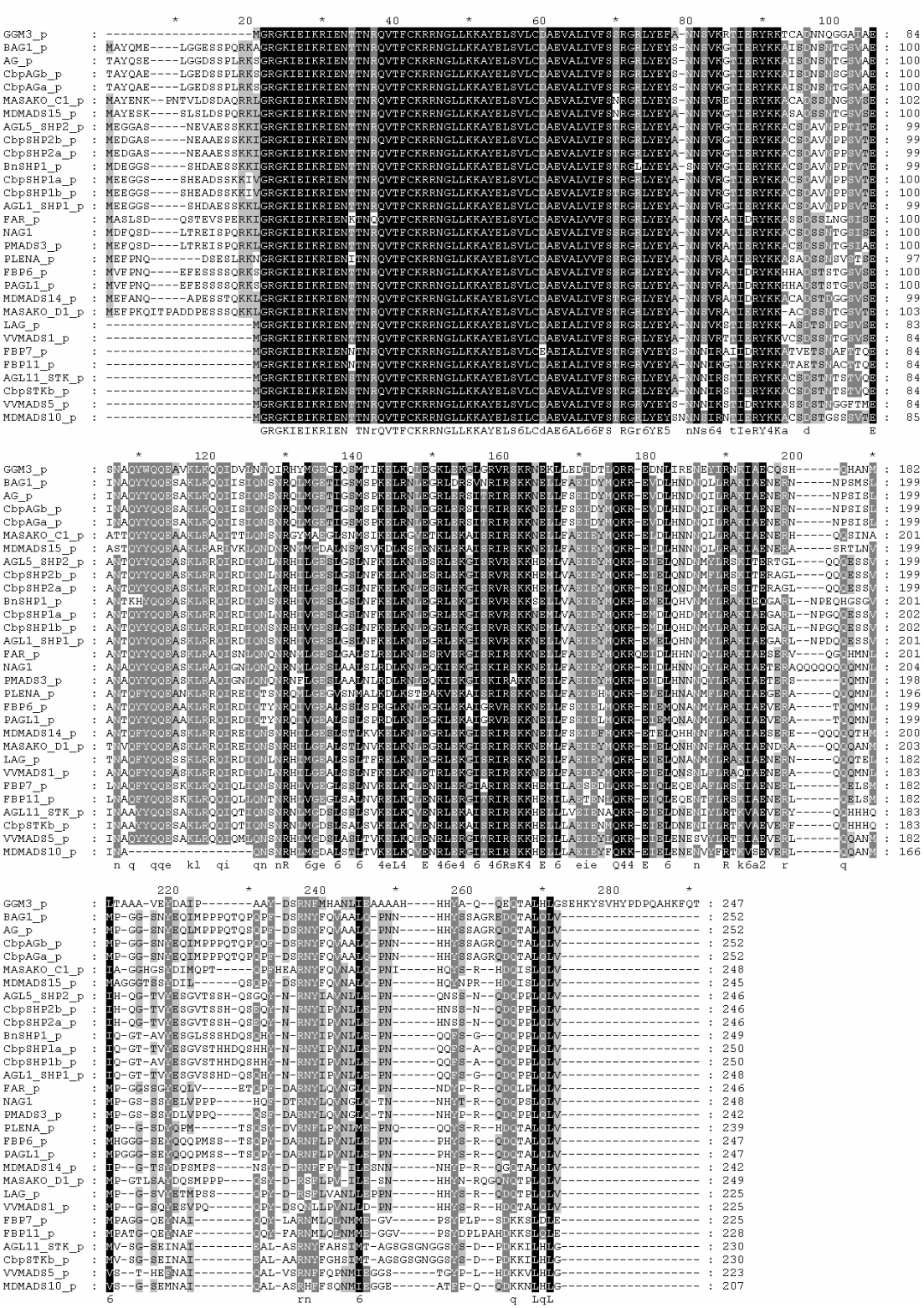
DNA blot hybridization experiments with genomic DNA (30µg) of 1947-wt and 1947-*Spe* plant leaf material digested with six different restriction enzymes, separated on 1% (w/v) agarose gel and blotted onto positively charged nylon membranes. Membranes were hybridised with DIG- labelled gene fragments according to manufacturer´s instructions. Hybridised fragments comprise genomic sequence from beginning of I-domain to the C-domain spanning four to five introns of the respective genes.
Lanes (1)-(6) genomic DNA of 1947-wt plants; lanes (7)-(12) genomic DNA of 1947-*Spe* plants.

**(A)** Hybridization with *CbpAG* fragment. DNA digested with restriction enzymes (1), (7) EcoRI; (2), (8) EcoRV; (3), (9) DraI. (4); (10) HincII *(cuts the probe once in K-domain)*; (5), (11) XbaI; (6), (12) XhoI.

**(B)** Hybridization with *CbpSTK* fragment. DNA digested with restriction enzymes (1), (7) EcoRI; (2). (8) EcoRV; (3), (9) DraII; (4), (10) HincII *(cuts the probe in intron between K1 and K2 domain)*; (5), (11)XbaI; (6), (12) XhoI.

**(C)** Hybridization with *CbpSHP1* fragment. DNA digested with restriction enzymes (1), (7) EcoRI; (2), (8) EcoRV (3), (9) SalI; (4), (10) XbaI; (5), (11) XhoI; (6), (12) HincII *(cuts the probe right at the 5´end of fragment).*

**(D)** Hybridization with *CbpSHP2* fragment. DNA digested with restriction enzymes (1), (7) EcoRI *(cuts the probe once in intron between K1 and K2 domain)*; (2), (8) EcoRV; (3), (9) DraI; (4), (10) HincII *(cuts the probe three times in intron sequences, explaining missing band)*; (5), (11) XbaI; (6), (12) XhoI.
**(13), all panels:** Length marker (DNA Ladder Mix, Fermentas, Germany). (bp), base pairs.



**Supplemental Figure 2.**

MADS domain protein alignment of isolated AG-like genes of *C. bursa-pastoris* and of homologous members of the *AG* clade from different angiosperm species. One *AG* homolog originated from the gymnosperm *Gnetum gnemon* and served later in the phylogeny reconstruction as rooting outgroup. Complete protein sequences were aligned including N-terminal extensions.

The alignment was constructed using the MUSCLE programme (Edgar, 2004) with default parameters. The protein alignment was checked manually for correctness.

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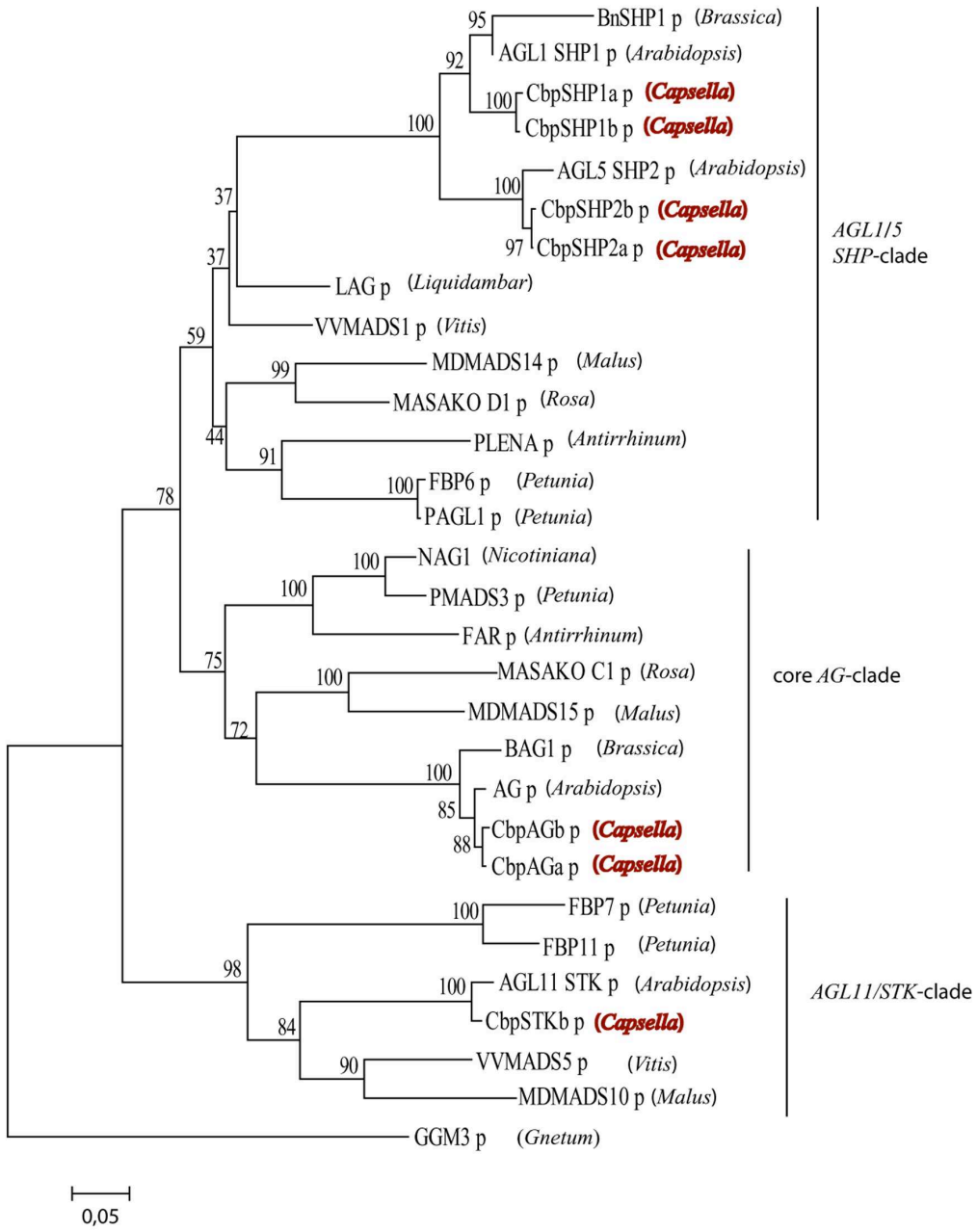


**Supplemental Figure 3.**

Phylogeny reconstruction of AG-like proteins of *C. bursa-pastoris* and of other angiosperm species as well as one gymnosperm species (*Gnetum gnemon*) as rooting outgroup. Phylogeny reconstruction was performed using Neighbour Joining method (Saitou and Nei, 1987) with the help of the MEGA3 software (Kumar et al., 2004). As model, poisson correction was chosen with gaps incorporated and gamma distribution of 1.0. Support values were estimated with 10000 bootstrap replicates (Felsenstein, 1985) and bootstrap-values are indicated at tree nodes. Branch lengths are equivalent to the substitution rates indicated in the scale bar; p: protein.

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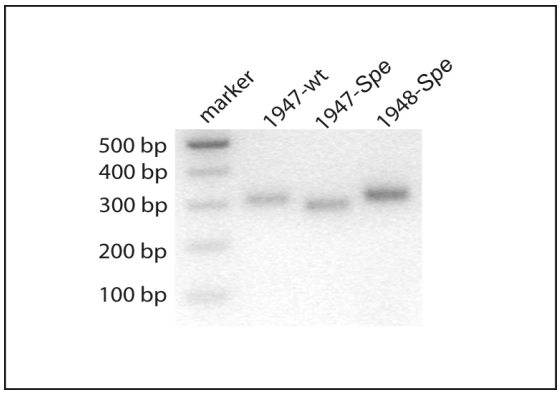
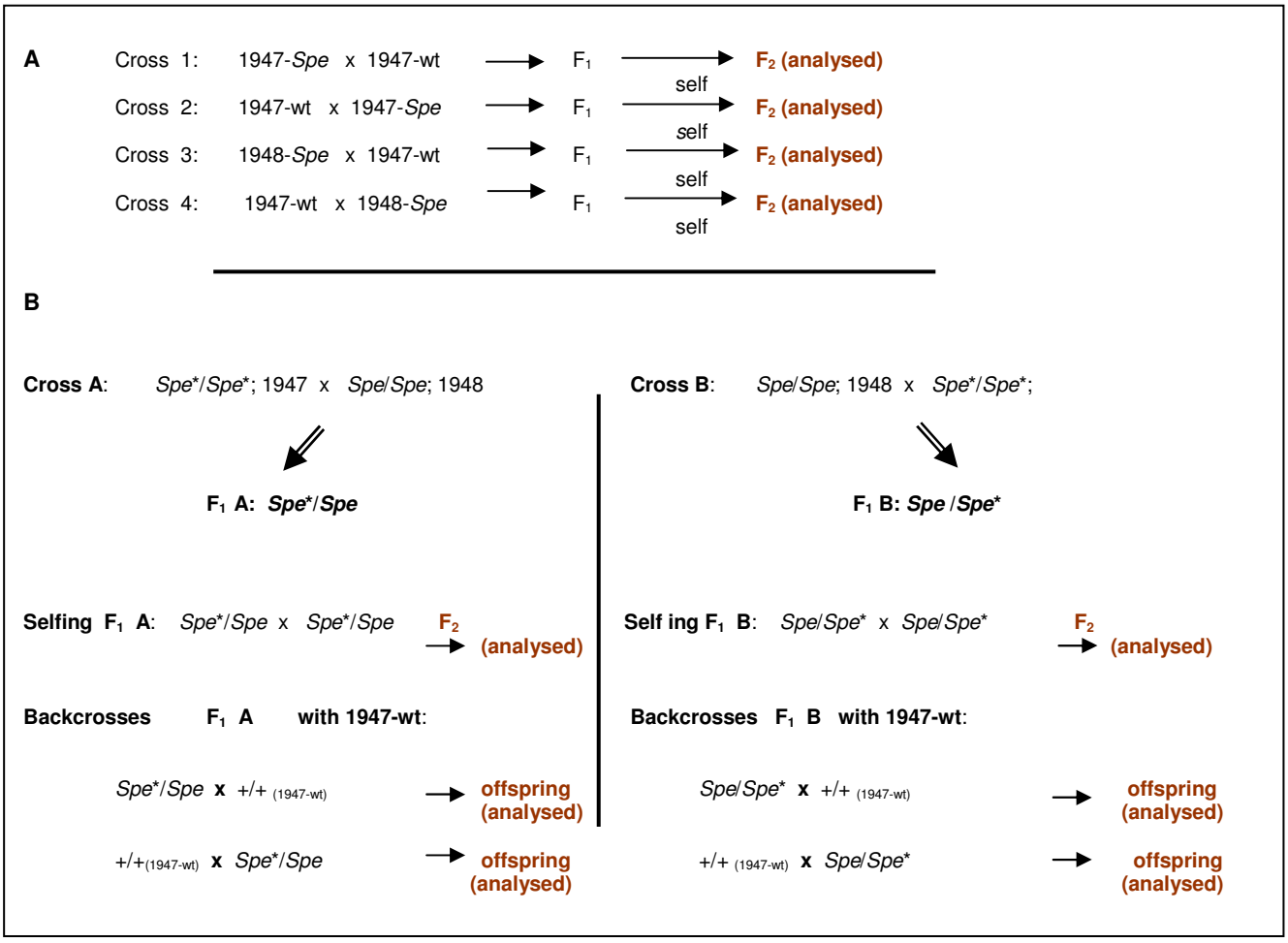
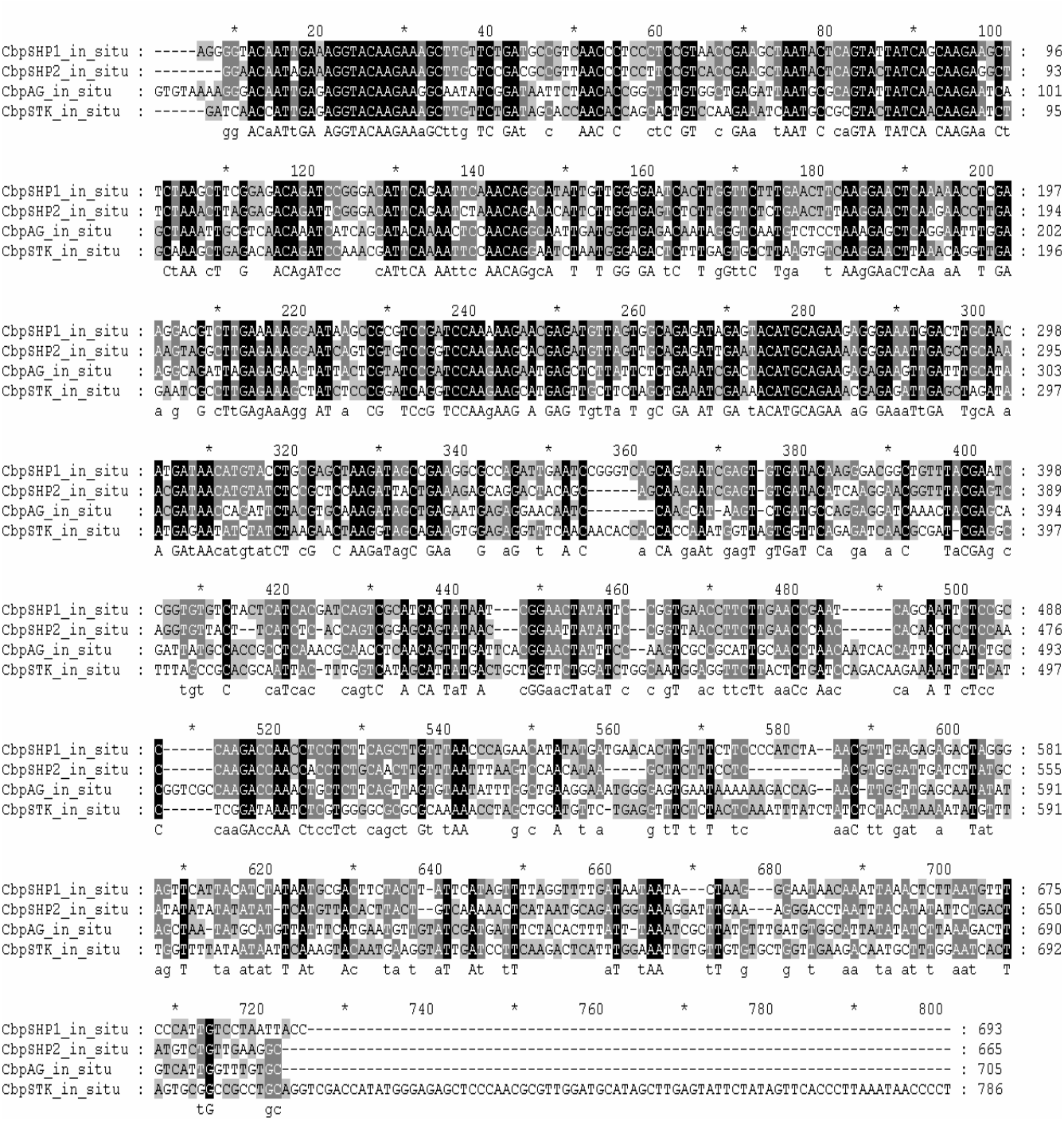



**Supplemental Figure 4.**

Result of a PCR experiment testing for a specific deletion in the 2nd intron of *CbpAGa* of the 1948-*Spe* line in comparison to the 1947-*Spe* line. Fragments were amplified with specific primers (Supplemental Table 4) for *CbpAGa* with a standard PCR program. Fragment lengths of 1947-wt: 299 bp, 1947-*Spe*: 277 bp, 1948-*Spe*: about 299 bp.




**Supplemental Figure 5.**
Crossing schedule; overview of crosses performed.
**(A)** Formal genetic analysis to test for the number of genetic loci responsible for the *Spe* phenotype.
**(B)** Analysis to test for identity (allelism) of the genetic *Spe* loci in the lines 1947-*Spe* and 1948-*Spe*, with *Spe* locus originating from line 1947 *Spe* marked with an asterisk for ancestry differentiation; +, symbol for wild-type allele.




**Supplemental Figure 6.**

Nucleotide alignment of *in situ* hybridization probes of *AG*-like genes. The alignment was generated with CLUSTALX (Jeanmougin et al., 1998).

**Jeanmougin, F.**, **Thompson, J.D.**, **Gouy, M.**, **Higgins, D.G. and Gibson, T.J**.(1998). Multiple sequence alignment with Clustal X. Trends Biochem Sci. **23**: 403-405.

**Supplemental Table 1.** Test for fertility of 2nd whorl stamens.

| Pollen recipient (emasculated) | Pollen donor | Plants selfed | Number of seeds/fruit |
|---|---|---|---|
| 1947-wt | 1947-*Spe* 2nd whorl stamens | | 13 |
| 1947-wt | 1947-*Spe* 2nd whorl stamens | | 20,9 |
| 1947-wt | 1948-*Spe* 2nd whorl stamens | | 13,2 |
| 1947-wt | 1948-*Spe* 2nd whorl stamens | | 0,3 |
| 1947-*Spe* | 1947-*Spe* 2nd whorl stamens | | 24,5 |
| 1947-*Spe* | 1947-*Spe* 2nd whorl stamens | | 22,0 |
| 1948-*Spe* | 1948-*Spe* 2nd whorl stamens | | 12,8 |
| 1948-*Spe* | 1948-*Spe* 2nd whorl stamens | | 0,9 |
| 1947-wt | 1947-*Spe* 3rd whorl stamens | | 17,5 |
| 1947-wt | 1948-*Spe* 3rd whorl stamens | | 13,4 |
| 1947-*Spe* | 1947-*Spe* 3rd whorl stamens | | 8,4 |
| 1947-wt | 1947-*Spe* all stamens | | 19,4 |
| 1947-wt | 1948-*Spe* all stamens | | 21,6 |
| 1947-*Spe* | 1947-*Spe* all stamens | | 23,0 |
| 1948-*Spe* | 1948-*Spe* all stamens | | 19,3 |
| | | 1947-wt | 26,6 |
| | | 1947-wt | 25,1 |
| | | 1947-wt | 22,7 |
| | | 1947-*Spe* | 24,8 |
| | | 1948-*Spe* | 22,6 |
| | | 1948-*Spe* | 24,0 |
| 1947-wt | Not pollinated (neg. control) | | 0 |
| 1947-wt | Not pollinated (neg. control) | | 0 |
| 1947-*Spe* | Not pollinated (neg. control) | | 0,2 |
| 1947-*Spe* | Not pollinated (neg. control) | | 0 |
| 1947-*Spe* | Not pollinated (neg. control) | | 2,3 |
| 1948-*Spe* | Not pollinated (neg. control) | | 0,4 |
| 1948-*Spe* | Not pollinated (neg. control) | | 0,4 |

**Supplemental Table 2.** Summary of floral development in *Capsella bursa-pastoris*: Landmark events that occur at beginning of each developmental stage are summarised and assorted in chronological order.

| Developmental stage | Landmark event at begin of developmental stage. |
|---|---|
| 1 | Flower primordium arises at edge of inflorescence meristem as a small dome of cells. |
| 2 | Flower primordium develops by differentiation from the inflorescence meristem through a groove and through length growth in an right angle to the main apex. |
| 3 | Sepals begin to bulge out as ridges, first the axial ones followed by the lateral ones, pedicel begins to form (and will elongate concurrently to the increasing bud size in all following stages) → formation of the 1st floral organ whorl. |
| 4 | Abaxial sepal ridge lengthens and curves inward to overlie the flower primordium closely followed by the other sepals in aforementioned order. |
| 5 | Stamen primordia begin to bulge out, first the four medial stamens parallel to the axial sepals followed by the two lateral stamen primordia; the central carpel dome starts to bulge out → formation of the 3rd and 4th organ whorl. |
| 6 | Floral bud is fully enclosed by sepals, primordia of long medial stamens bulge out further and become distinct from central dome, lateral stamen primordia follow through stage duration, central dome is developing a rim growing upward to form an oval tube; petals begin to arise with small primordia in the sinuses of the sepals, → formation of 2nd floral whorl becomes visible. |
| 7 | Growing primordia of medial stamens begin to become stalked giving rise to the filament. |
| 8 | Thecae start to bulge out from adaxial anther surfaces as longitudinal ridges in medial stamens followed by the lateral ones, petal primordia still small and hemispherical. |
| 9 | Petal primordia begin to be stalked, rapid growth of stamens. |
| 10* | The oval carpel tube is closed at the tip with a smaller circular constriction and starts to develop stigmatic papillae on top. |
| 11* | Petal tips reach level with the tips of lateral stamens. |
| 12 | Petals reach level with the longer medial stamens, rapid length growth of all floral organs. |
| 13 | Begin of anthesis: Sepals begin to open to a small circular hole through which the already receptive stigma is growing to overtop the sepal tips. This behaviour shows clear proterogyny, like it is known from *Arabidopsis* flowers. During stage 13 sepals open completely by extrusion trough length growth of the inner organs. |
| 14 | Long anthers and petals extend the carpel tip in length; sepals petals and stamens are bracing from the carpel. |
| 15 | Sepals, petals and stamens cling to the carpel surface and by doing so long medial stamens release pollen to the carpel stigma and finally ensure pollination. |
| 16* | Stigma extends above the long anthers, silicules bulge out at the valve tips on each side of the carpel leading to the typical triangular and later heard shaped fruit form. |
| 17 | Sepals, petals and stamens wither and start to fall off the flower. |
| 18 | Silicules turn yellow. |
| 19 | Valves separate from the dried silicules when agitated causing seed fall. |

* Stages in which developmental events in flowers of *C. bursa-pastoris* deviate from the developmental stages of *Arabidopsis thaliana* flowers as described in Smyth et al. (1990).

**Supplemental Table 3.** Segregation patterns of $F_3$ generations obtained from $F_2$ plants with questionable intermediate phenotypes of cross 1a.

| $F_3$ generation | wt | int/*Spe* | $F_3$ generation | wt | int/*Spe* | $F_3$ generation | wt | int/*Spe* | $F_3$ generation | wt | int/*Spe* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| F3-4 * | 0 | 18 | F3-64 | 3 | 13 | F3-112 * | 0 | 30 | F3-129 * | 0 | 14 |
| F3-7 | 3 | 13 | F3-70 | 3 | 14 | F3-115 * | 0 | 17 | F3-142 * | 0 | 19 |
| F3-8 | 6 | 10 | F3-71 | 3 | 15 | F3-116 | 2 | 15 | F3-156 | 8 | 11 |
| F3-11 | 6 | 9 | F3-75 | 9 | 9 | F3-121 | 4 | 5 | F3-165 | 3 | 15 |
| F3-21 | 5 | 12 | F3-77 | 4 | 9 | F3-122 | 6 | 9 | F3-166 | 6 | 12 |
| F3-26 | 1 | 15 | F3-98 | 2 | 3 | F3-126 | 5 | 12 | F3-168 * | 0 | 19 |
| F3-41 | 3 | 3 | F3-99 | 8 | 9 | F3-127 * | 0 | 19 | F3-171 | 4 | 14 |
| F3-56 | 4 | 13 | F3-111 | 4 | 10 | F3-128 | 3 | 14 | | | |

* $F_3$ generations where no segregation was observed in this analysis; they will most probably be homozygous for *Spe*, but with a lower expressivity in the *Spe* phenotype.

**Supplemental Table 4.** Results of phenotyping and genotyping of the parent plants 1947-wt and 1947-*Spe*, the $F_1$ generation and the $F_2$ mapping population; WT, wild-type; M, mutant (*Spe* or intermediate).

| Plant | Phenotype | Genotype | Plant | Phenotype | Genotype |
|---|---|---|---|---|---|
| 1947-wt | WT | C/C | F2-98 | M | C/- |
| 1947-*Spe* | M | -/- | F2-99 | M | -/- |
| F1 | M | C/- | F2-100 | M | C/- |
| F2-1 | M | C/- | F2-101 | M | -/- |
| F2-2 | M | -/- | F2-102 | M | -/- |
| F2-3 | no flower | | F2-103 | M | C/- |
| F2-4 | M | C/- | F2-104 | M | C/- |
| F2-5 | M | -/- | F2-105 | M | C/- |
| F2-6 | WT | no DNA | F2-106 | M | C/- |
| F2-7 | M | -/- | F2-107 | M | C/- |
| F2-8 | M | C/- | F2-108 | M | -/- |
| F2-9 | M | C/- | F2-109 | M | C/- |
| F2-10 | M | C/- | F2-110 | M | C/- |
| F2-11 | M | C/- | F2-111 | WT | C/C |
| F2-12 | WT | C/C | F2-112 | WT | C/C |
| F2-13 | M | C/- | F2-113 | WT | C/C |
| F2-14 | M | C/- | F2-114 | WT | C/C |
| F2-15 | M | C/- | F2-115 | M | -/- |
| F2-16 | M | -/- | F2-116 | M | C/- |
| F2-17 | M | C/- | F2-117 | M | C/- |
| F2-18 | no flower | | F2-118 | M | C/- |
| F2-19 | M | C/- | F2-119 | M | C/- |
| F2-20 | WT | C/C | F2-120 | WT | C/C |
| F2-21 | M | -/- | F2-121 | M | C/- |
| F2-22 | M | C/- | F2-122 | WT | C/C |
| F2-23 | M | C/- | F2-123 | M | C/- |
| F2-24 | M | C/- | F2-124 | M | C/- |

| | | | | | |
|---|---|---|---|---|---|
| F2-25 | M | C/- | F2-125 | M | C/- |
| F2-26 | M | C/- | F2-126 | M | -/- |
| F2-27 | WT | C/C | F2-127 | M | C/- |
| F2-28 | M | C/- | F2-128 | M | -/- |
| F2-29 | no flower | | F2-129 | M | -/- |
| F2-30 | no flower | | F2-130 | WT | C/C |
| F2-31 | M | C/- | F2-131 | M | -/- |
| F2-32 | M | -/- | F2-132 | M | C/- |
| F2-33 | M | C/- | F2-133 | M | C/- |
| F2-34 | WT | C/C | F2-134 | M | C/- |
| F2-35 | WT | C/C | F2-135 | WT | C/C |
| F2-36 | WT | C/C | F2-136 | M | -/- |
| F2-37 | WT | C/C | F2-137 | M | C/- |
| F2-38 | M | C/- | F2-138 | M | -/- |
| F2-39 | M | C/- | F2-139 | M | -/- |
| F2-40 | M | -/- | F2-140 | M | C/- |
| F2-41 | M | C/- | F2-141 | M | -/- |
| F2-42 | M | C/- | F2-142 | WT | C/C |
| F2-43 | M | C/- | F2-143 | WT | C/C |
| F2-44 | WT | C/C | F2-144 | M | C/- |
| F2-45 | M | C/- | F2-145 | M | C/- |
| F2-46 | M | C/- | F2-146 | M | C/- |
| F2-47 | M | C/- | F2-147 | M | C/- |
| F2-48 | M | -/- | F2-148 | M | C/- |
| F2-49 | M | C/- | F2-149 | M | C/- |
| F2-50 | WT | C/C | F2-150 | WT | C/C |
| F2-51 | M | C/- | F2-151 | WT | C/C |
| F2-52 | M | -/- | F2-152 | M | C/- |
| F2-53 | M | C/- | F2-153 | WT | C/C |
| F2-54 | M | C/- | F2-154 | WT | C/C |
| F2-55 | M | C/- | F2-155 | M | -/- |
| F2-56 | M | C/- | F2-156 | M | C/- |
| F2-57 | M | C/- | F2-157 | M | C/- |
| F2-58 | M | C/- | F2-158 | WT | C/C |
| F2-59 | M | -/- | F2-159 | M | C/- |
| F2-60 | M | C/- | F2-160 | WT | C/C |
| F2-61 | M | C/- | F2-161 | WT | C/C |
| F2-62 | M | C/- | F2-162 | M | C/- |
| F2-63 | WT | C/C | F2-163 | M | -/- |
| F2-64 | WT | C/C | F2-164 | M | -/- |
| F2-65 | M | C/- | F2-165 | M | C/- |
| F2-66 | WT | C/C | F2-166 | M | C/- |
| F2-67 | WT | C/C | F2-167 | WT | C/C |
| F2-68 | WT | C/C | F2-168 | M | C/- |
| F2-69 | M | C/- | F2-169 | M | -/- |
| F2-70 | WT | C/C | F2-170 | M | -/- |
| F2-71 | M | C/- | F2-171 | M | C/- |
| F2-72 | WT | C/C | F2-172 | M | C/- |
| F2-73 | M | C/- | F2-173 | WT | C/C |
| F2-74 | M | C/- | F2-174 | WT | C/C |
| F2-75 | M | -/- | F2-175 | M | C/- |
| F2-76 | M | C/- | F2-176 | M | -/- |
| F2-77 | WT | C/C | F2-177 | M | C/- |

| | | | | | |
|---|---|---|---|---|---|
| F2-78 | M | C/- | F2-178 | M | C/- |
| F2-79 | WT | C/C | F2-179 | WT | C/C |
| F2-80 | WT | C/C | F2-180 | M | -/- |
| F2-81 | M | C/- | F2-181 | WT | C/C |
| F2-82 | M | C/- | F2-182 | M | -/- |
| F2-83 | M | C/- | F2-183 | M | C/- |
| F2-84 | M | C/- | F2-184 | WT | C/C |
| F2-85 | M | C/- | F2-185 | M | C/- |
| F2-86 | WT | C/C | F2-186 | M | C/- |
| F2-87 | M | C/- | F2-187 | M | -/- |
| F2-88 | WT | C/C | F2-188 | M | C/- |
| F2-89 | M | C/- | F2-189 | M | C/- |
| F2-90 | M | -/- | F2-190 | M | C/- |
| F2-91 | M | C/- | F2-191 | M | C/- |
| F2-92 | M | C/- | F2-192 | M | C/- |
| F2-93 | M | C/- | F2-193 | WT | C/C |
| F2-94 | M | -/- | F2-194 | WT | C/C |
| F2-95 | M | C/- | F2-195 | M | -/- |
| F2-96 | WT | C/C | F2-196 | M | -/- |
| F2-97 | WT | C/C | | | |

**Supplemental Table 5.** Sequences of primers used in this work.

Primers for isolation of complete mRNAs of *AGAMOUS* (*AG)*-like genes.

| Primer names and fragment lengths (in parentheses) | Primer sequences ( Fwd: forward and Rev: reverse direction of primer) |
|---|---|
| *CbpAG*_ges (1051, 1055 bp) | Fwd: 5´-TTA GCA CAA CCT TAC CTT CC-3´ and<br>Rev: 5´-GCA CAA ACC AAT GAC AAG TC-3´ |
| *CbpSHP1*_ges1 ( 972 bp) | Fwd: 5´-GGA ATA TAG TTT TCT CAT CAC-3´and<br>Rev: 5´-AAG AGT TTA ATT TGT CAT TCC-3´ |
| *CbpSHP1*_ges2 (968 bp) | Fwd: 5´-CTT TCG GTG ATG TGA TAG G-3´ and<br>Rev: 5´-GGA ACT TTG GAT TTA AAT ATT G-3´ |
| *CbpSHP2*_ges (1001, 1005 bp) | Fwd: 5´-TCT CTC AGA TTT CAT CTT CC-3´and<br>Rev: 5´-GCC TTC AAC AGA CAT AGT C-3´ |
| *CbpSTK*_ges (945 bp) | Fwd: 5´-AGA TAG AGA TAG AGW GAG AG3´- and<br>Rev: 5´-CCA AAG CAT TGT CTT CAA CC-3´ |

Primers for generation of genomic template fragments for Southern hybridization, fragments spanning 4-5 introns from I-domain to K-domain of *AG*-clade genes.

| | |
|---|---|
| *CbpAG*_IKC (966-984 bp) | Fwd: 5´-GGA GAA TTC TAA CAC CGG CTC TGT G-3´ and<br>Rev: 5´AAC TCT AGA GCA GTT TGG TCT TGG CG-3´ |
| *CbpSHP1*_IKC (1188 bp) | Fwd: 5´-GTTCTC GAG CCG TCA ACC CTC CCT CC-3´ and<br>Rev: 5´-GAT TCT AGA GAT GCG ACT GAT CGT GAT G -3´ |
| *CbpSHP2*_IKC (877-879 bp) | Fwd: 5´-AAG AAT TCT TGC TCC GAC GCC GTT AAC-3´ and<br>Rev: 5´-GGT CTA GAT TGG AGG AGT TGT GGT TGG-3´ |
| *CbpSTK*_IKC (877-906 bp) | Fwd: 5´-CAA CTC GAG CAC TGT CCA AGA AAT CAA TGC-3´ and<br>Rev: 5´-TGT CTA GAT CAG AGT AAG AAC CTC CAT TGC-3´ |

Primers for isolation non-*AG*-clade genes for *in situ* hybridization.

| | |
|---|---|
| *CbpH4*_3´end (RT) (535~ bp) | 5´-CTC TTC TAG AGG ATT GGG AAA GGG AGG A-3´ |
| *CbpAP3*_3RACE (744 bp + poly-A) | 5´-CAC CAC AAC GAA GGA GAT C-3´ |
| *CbpPI*_3RACE (820, 830 bp + poly-A) | 5´-G GAA GGT GGG AGT AGT CAC-3´ |
| *CbpAP1*_3RACE (789, 792 bp + poly-A) | 5´-CTT ATT GCA CCT GAG TCC G-3´ |

Primers for construction of template fragments for in vitro transcription of *in situ* hybridization probes with T7-RNA-polymerase-promoter in the forward- and restriction site (XbaI, XhoI) in the reverse-primer.

| | |
|---|---|
| *CbpAG* antisense (705 bp) | Fwd: 5´-TAA TAC GAC TCA CTA TAG GGG CAC AAA CCA ATG ACA AGT C-3´ and Rev: 5´-AGTA TCT AGA GTG TAA AAG GGA CAA TTG AGAG-3´ |
| *CbpAG* sense (705 bp | Fwd: 5´-TAA TAC GAC TCA CTA TAG GGG TGT AAA AGG GAC AAT TGA GAG-3´ and Rev: 5´-ACTA TCT AGA GCA CAA ACC AAT GAC AAG TC-3´ |
| *CbpSHP1* antisense (713 bp) | Fwd: 5´-TAA TAC GAC TCA CTA TAG GGA ACT TTG GAT TTA AAT ATT G-3´ and Rev: 5´-AGTC TCT AGA AGG GGT ACA ATT GAA AGG TAC-3´ |
| *CbpSHP1* sense (713 bp) | Fwd: 5´-TAA TAC GAC TCA CTA TAG GGG TAC AAT TGA AAG GTA C-3´ and Rev: 5´-AGTC TCT AGA GGA ACT TTG GAT TTA AAT ATT G-3´ |
| *CbpSHP2* antisense (691 bp) | Fwd: 5´-TAA TAC GAC TCA CTA TAG GGG CCT TCA ACA GAC ATA GTC-3´ and Rev: 5´-AGTA TCT AGA GGA ACA ATA GAA AGG TAC AAG-3´ |
| *CbpSHP2* sense (691 bp) | Fwd: 5´-TAA TAC GAC TCA CTA TAG GGA ACA ATA GAA AGG TAC AAG-3´ and Rev: 5´-ATCT TCT AGA GCC TTC AAC AGA CAT AGT C-3´ |
| *CbpSTK* antisense (715 bp) | Fwd: 5´-TAA TAC GAC TCA CTA TAG GGC CAA AGC ATT GTC TTC AAC C-3´ and Rev: 5´-AGCT TCT AGA GAT CAA CCA TTG AGA GGT AC-3´ |
| *CbpSTK* sense (714bp) | Fwd: 5´-TAA TAC GAC TCA CTA TAG GGA TCA ACC ATT GAG AGG TAC-3´ and Rev: 5´-ACTA TCT AGA CCA AAG CAT TGT CTT CAA CC-3´ |
| *CbpAP1* antisense (820 bp) | Fwd: 5´-TAA TAC GAC TCA CTA TAG GGT TAT TGC ACC TGA GTC CGA C-3´ and Rev: 5´-TAGC CTC GAG TTC GTT CTC TCC AAC CTT C-3´ |
| *CbpAP1* sense (820 bp) | Fwd: 5´-TAA TAC GAC TCA CTA TAG GGT TCG TTC TCT CCA ACC TTC-3´ and Rev: 5´- TAGC CTC GAG TTA TTG CAC CTG AGT CCG AC-3´ |
| *CbpAP3* antisense (694 bp) | Fwd: 5´-TAA TAC GAC TCA CTA TAG GG GAA GTC TTG AAT ACA TTC CAC-3´ and Rev: 5´-TTCA TCT AGA CTG ATG TCG ATG TTT GGA G-3´ |
| *CbpAP3* sense (694 bp) | Fwd: 5´-TAA TAC GAC TCA CTA TAG GGC TGA TGT CGA TGT TTG GAG-3´ and Rev: 5´-TTCA TCT AGA GAA GTC TTG AAT ACA TTC CAC-3´ |
| *CbpPI* antisense (616 bp) | Fwd: 5´- TAA TAC GAC TCA CTA TAG GGA AGC AAA CAC ACC ACA TGC-3´ and Rev: 5´-TAGC CTC GAG ATG CTA AGC ATG AGA ACC-3 |
| *CbpPI* sense (616 bp) | Fwd: ´5´- TAA TAC GAC TCA CTA TAG GGA TGC TAA GCA TGA GAA CC-3´and Rev: 5´-TAGC CTC GAG AAG CAA ACA CAC CAC ATG C |

Primers for amplification of fragments from genomic DNA used for genotyping with

pyrosequencing. Marked in red: primer biotinylated at 5´end.

| | |
|---|---|
| *CbpAGa* (486 bp, 1st intron) | Fwd: 5' CTA TGT TCT TCT TTT TCG GTT TCC T 3' and Rev: 5' TA GGG CTA AAC TGA TTA AAC ATC 3' |
| *CbpAGb* (751 bp, 2nd intron) | Fwd: 5' TGA TCA TAC AAC ACT AGA CAT GTG 3' and Rev: 5' TAA ACT TTA CTT TTC TGT TTC GTT GC 3' |
| *CbpSHP1a* (544 bp, 4th intron) | Fwd: 5' TGT ATA CAA ATG GTG GCA TTC TGA AG 3' and Rev: 5' AG ATC GAG AGA GAG AGG TAC ACA C 3' |
| *CbpSHP1b* (297 bp, promoter) | Fwd: 5' CGA TTA GAC TCG GTT TTG GCA TGG 3' and Rev: 5' AGG ACA TTG AAG GAG GAC CAT CC 3' |
| *CbpSHP2a* (535 bp, 2nd intron) | Fwd: 5' GCT AGT GAT CAT TTT TTT CTT GTT GAA G 3' and Rev: 5' TGT CTC GGA CTA AAT ACC AAC G 3' |
| *CbpSHP2b* (482 bp, 2nd intron) | Fwd: 5' GC ATG TGA CAT GAC TTA ATA GTA CC 3' and Rev: 5' GGT TCT AAA ACA CTA CTA ACT GGA C 3' |
| *CbpSTKa* (419 bp, promoter) | Fwd: 5' GAT AGG GTT CGT TCA TCA TCC AC 3' and Rev: 5' ATA ACT CCT TGC TTA CAT GCG AC 3' |
| *CbpSTKb* (583 bp, 1st intron) | Fwd: 5' CAC TTG TTT GAT CGT CTC ATC ATC AAG 3' and Rev: 5' AAT AAG GGG AGA GAG AGA GAG AAA C 3' |

Primers used in the pyrosequencing method.

| | |
|---|---|
| *CbpAGa* | 5' TAA AAC AAG TTA AAC TAA AAC CAA ATC TTT 3' |
| *CbpAGb* | 5' ACA AGT ACG TTC CAT TAT TTT CTA TC 3' |
| *CbpSHP1a* | 5' AAA TAA TCA AGT TAA GGT ACA AAG ATA G 3' |
| *CbpSHP1b* | 5' TAG ACT CCT TGT AGT CCT AGC 3' |
| *CbpSHP2a* | 5' TAT GAT CTT ATT AGT CAA GTC TCA TAT 3' |
| *CbpSHP2b* | 5' GAA GTG AAA TTT GTA AAT ACT TTG CCA 3' |
| *CbpSTKa* | 5' AAT AAA CTC TTG TTA GTC ACT ATC GA 3' |
| *CbpSTKb* | 5' TCT GTG TAA TAA TGT TTC TTG TGT TG 3' |

Primers used in the test for a deletion in the different *SPE* lines.

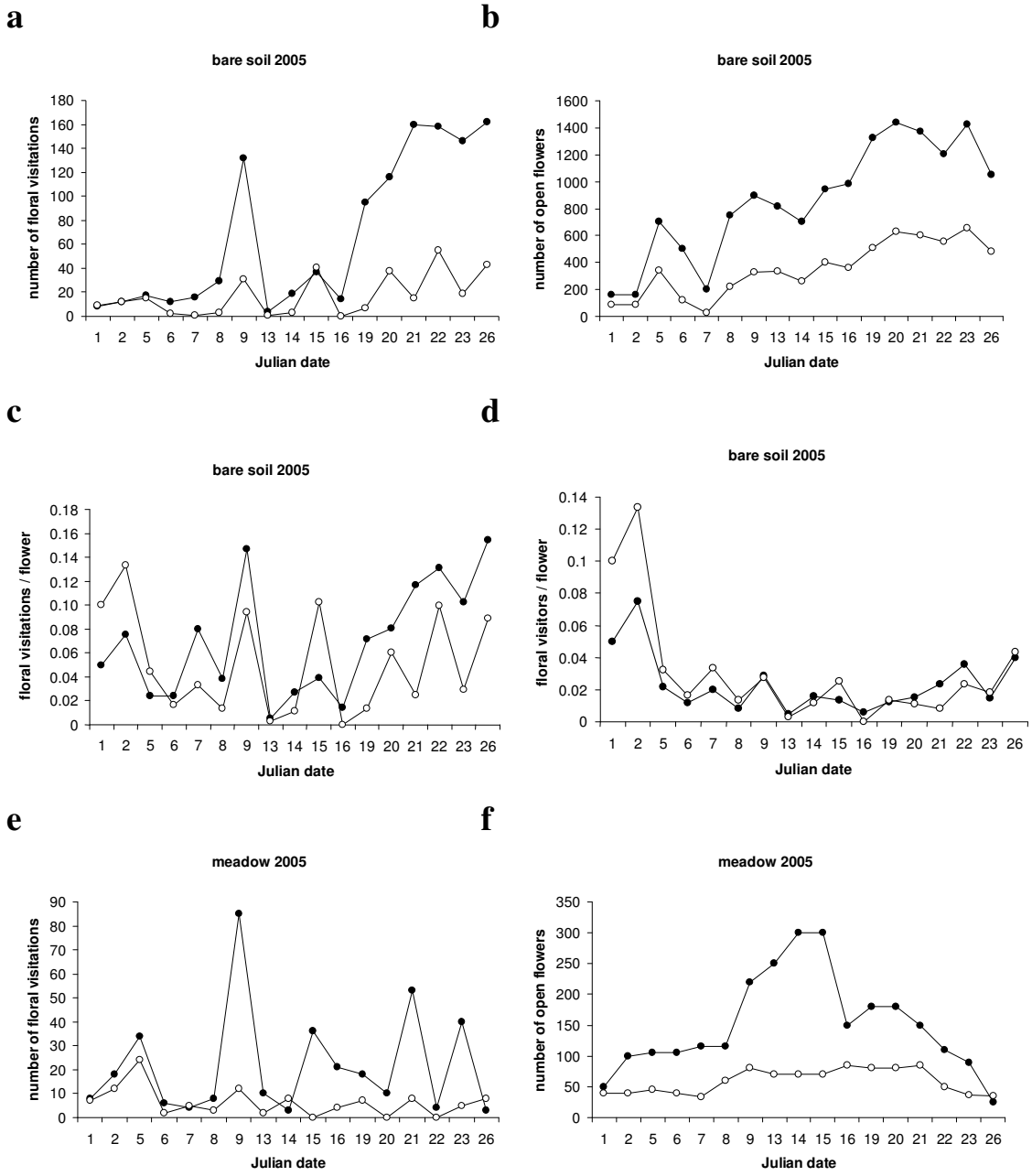
| | |
|---|---|
| *CbpAGa*_del_fwd (277-299bp) | 5´AGT TTT GAT CAT ACA ACA CTA CAC ATG TC 3´ |
| *CbpAGa*_del_rev (277-299bp) | 5´GAA ATC TCA AAT CTT TTA TGG TTG GAG ATG 3´ |

**Supplemental Table 6.** GenBank/EMBL accession numbers of isolated genes.

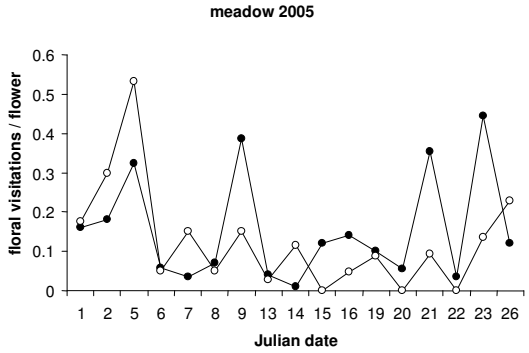
| Gene name | Sequence character | GenBank/EMBL Accession number |
|---|---|---|
| *CbpH4* | Partial cds, part of 3´UTR | EU551759 |
| *CbpAP1a* | Partial cds & 3´UTR | EU551760 |
| *CbpAP1b* | Partial cds & 3´UTR | EU551761 |
| *CbpPIa* | Partial cds & 3´UTR | EU551762 |
| *CbpPIb* | Partial cds & 3´UTR | EU551763 |
| *CbpAP3a* | Partial cds & 3´UTR | EU551764 |
| *CbpAP3b* | Partial cds & 3´UTR | EU551765 |
| *CbpSTKa* | 5´UTR, partial cds | EU551766 |
| *CbpSTKb,* | 5´UTR, complete cds, 3´UTR | EU551767 |
| *CbpSHP2a* | 5´UTR, complete cds, 3´UTR | EU551768 |
| *CbpSHP2b* | 5´UTR, complete cds, 3´UTR | EU551769 |
| *CbpSHP1a*, | 5´UTR, complete cds, 3´UTR | EU551770 |
| *CbpSHP1b*, | 5´UTR, complete cds, 3´UTR | EU551771 |
| *CbpAGa*, | 5´UTR, complete cds, 3´UTR | EU551772 |
| *CbpAGb*, | 5´UTR, complete cds, 3´UTR | EU551773 |
| *CbpAGa_Spe* | Partial genomic DNA | EU662251 |
| *CbpAGa_wt* | Partial genomic DNA | EU662252 |
| *CbpAGb_Spe* | Partial genomic DNA | EU662253 |
| *CbpAGb_wt* | Partial genomic DNA | EU662254 |
| *CbpSHP1a_Spe* | Partial genomic DNA | EU662255 |
| *CbpSHP1a_wt* | Partial genomic DNA | EU662256 |
| *CbpSHP1b_Spe* | Partial genomic DNA | EU662257 |
| *CbpSHP1b_wt* | Partial genomic DNA | EU662258 |
| *CbpSHP2a_Spe* | Partial genomic DNA | EU662259 |
| *CbpSHP2a_wt* | Partial genomic DNA | EU662260 |
| *CbpSHP2b_Spe* | Partial genomic DNA | EU662261 |
| *CbpSHP2b_wt* | Partial genomic DNA | EU662262 |
| *CbpSTKa_Spe* | Partial genomic DNA | EU662263 |
| *CbpSTKa_wt* | Partial genomic DNA | EU662264 |
| *CbpSTKb_Spe* | Partial genomic DNA | EU662265 |
| *CbpSTKb_wt* | Partial genomic DNA | EU662266 |

## 10. Zusatzmaterial zu Manuskript III

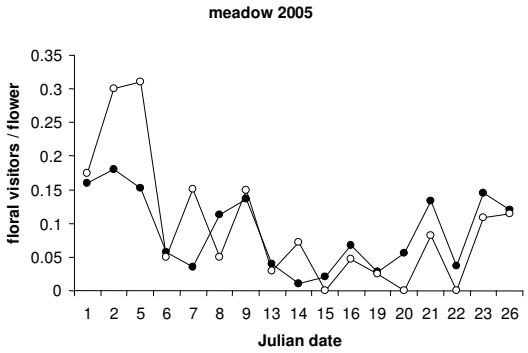
**Suppl. Fig. 1** Detailed observations of the bare soil (**a-d**) and meadow habitat (**e-h**) 2005, two different bare soil habitats (**i-p**) 2006 and the bare soil habitat (**q-t**) 2007 in Jena and 2006 in Halle (**u, v**) of 1947-wt (black circles) and 1947-*Spe* (open circles) plant plots per daily observational interval.
Number of floral visitations (**a, e, i, m, q, u**), open flowers (**b, f, j, n, r**) as well as the frequencies of floral visitations (**c, g, k, o, s, v**) and floral visitors (**d, h, l, p, t**) per flower. (Julian date 1 = 26 May 2005 (**a-h**); 19 June 2006 (**i-p**); 13 June 2007 (**q-t**) and 1 Juli 2006 (**u, v**))



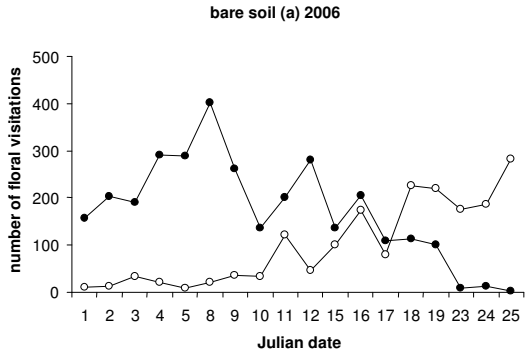
**g**


meadow 2005
floral visitations / flower
0.6
0.5
0.4
0.3
0.2
0.1
0
1 2 5 6 7 8 9 13 14 15 16 19 20 21 22 23 26
Julian date

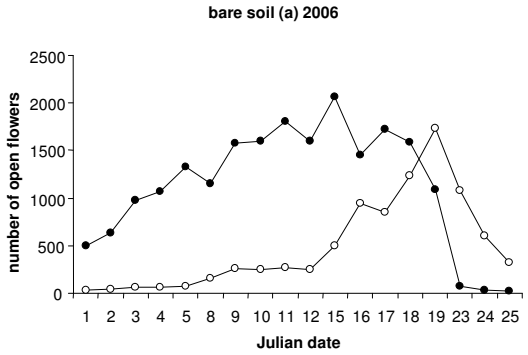
**h**


meadow 2005
floral visitors / flower
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1 2 5 6 7 8 9 13 14 15 16 19 20 21 22 23 26
Julian date

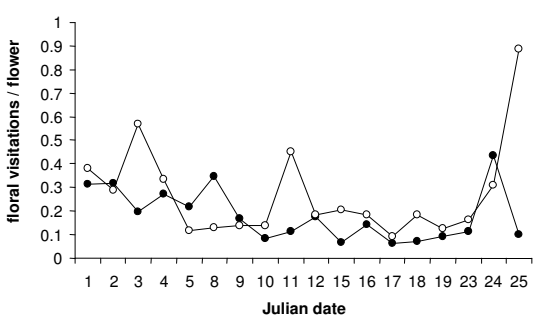
**i**


bare soil (a) 2006
number of floral visitations
500
400
300
200
100
0
1 2 3 4 5 8 9 10 11 12 15 16 17 18 19 23 24 25
Julian date

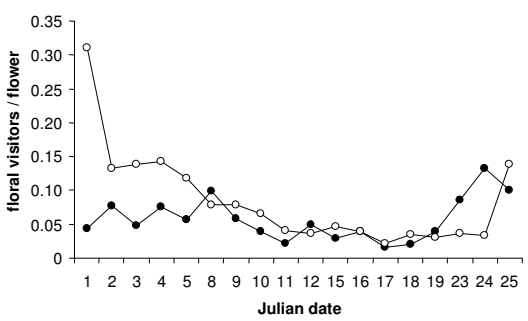
**j**


bare soil (a) 2006
number of open flowers
2500
2000
1500
1000
500
0
1 2 3 4 5 8 9 10 11 12 15 16 17 18 19 23 24 25
Julian date

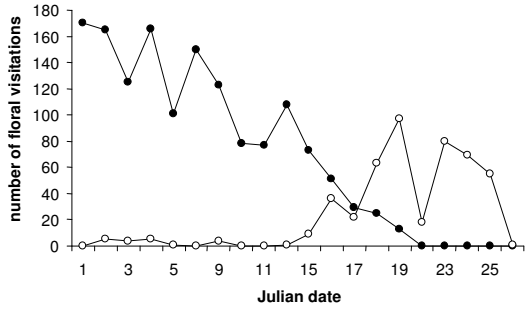
**k**


bare soil (a) 2006
floral visitations / flower
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1 2 3 4 5 8 9 10 11 12 15 16 17 18 19 23 24 25
Julian date

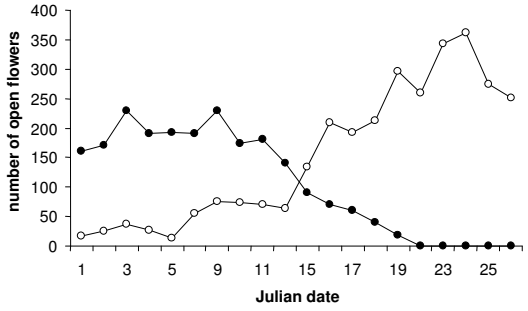
**l**

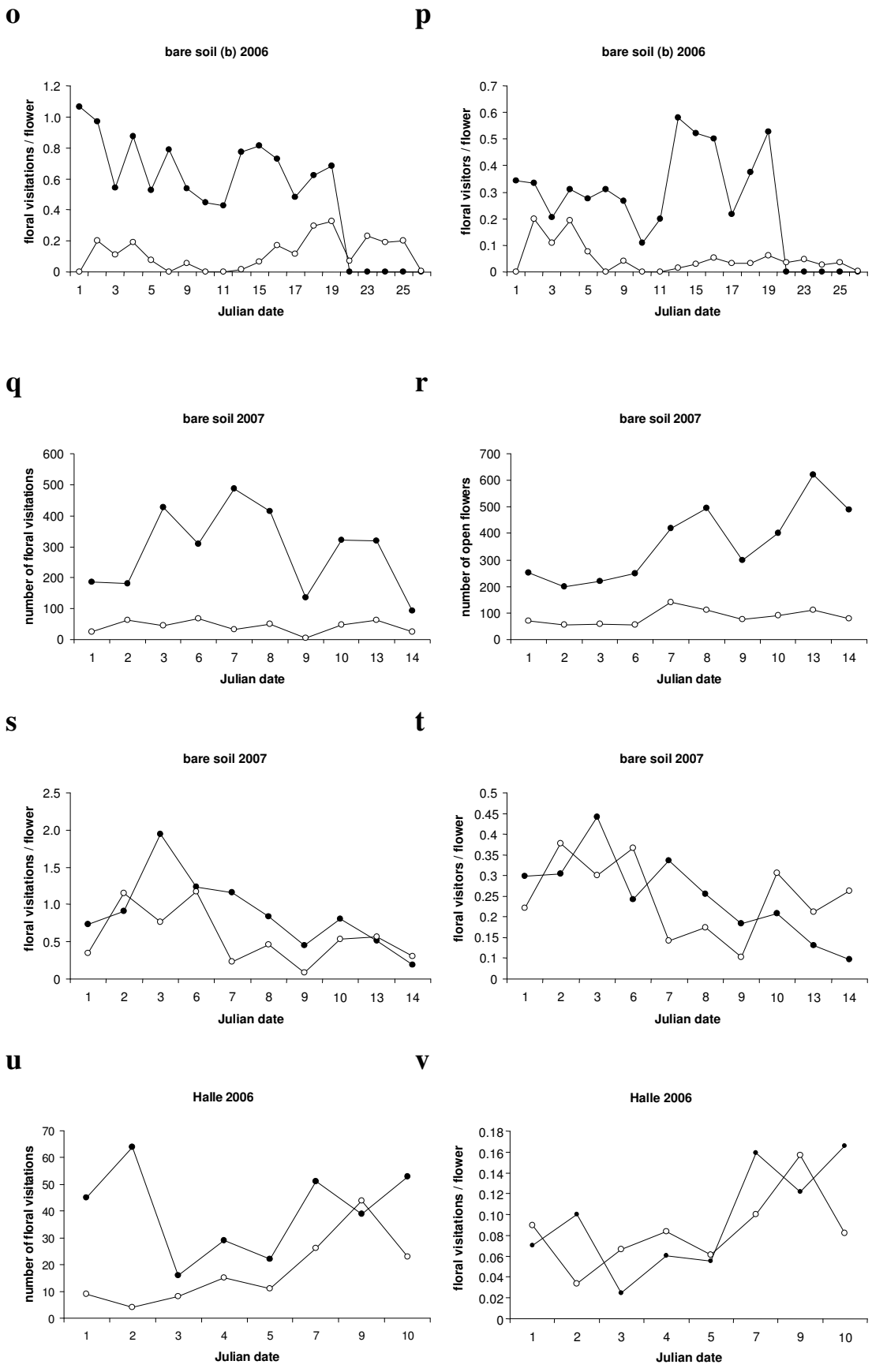

bare soil (a) 2006
floral visitors / flower
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
1 2 3 4 5 8 9 10 11 12 15 16 17 18 19 23 24 25
Julian date

**m**


bare soil (b) 2006
number of floral visitations
180
160
140
120
100
80
60
40
20
0
1 3 5 9 11 15 17 19 23 25
Julian date

**n**


bare soil (b) 2006
number of open flowers
400
350
300
250
200
150
100
50
0
1 3 5 9 11 15 17 19 23 25
Julian date




**Suppl. Table 1** Number of floral visitors of 1947-wt (Wt) and 1947-*Spe* (M) plant plots in the different habitats (a, a1, a2: bare soil, b: meadow) and years in Jena. Numbers correspond to

the sum of the detected floral visitors in the observational time intervals in the respective years

| year | habitat | plot | *Thysanoptera* | wild bees | *Apoidea* | *Coleoptera* | *Syrphidae* | *Brachycera* | *Heteroptera* | *Aranea* | ? |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2005 | a | Wt | 21 | 174 | 39 | 12 | 36 | 15 | 1 | 3 | 3 |
| | | M | 31 | 50 | 5 | 34 | 22 | 8 | 1 | 2 | 0 |
| | b | Wt | 2 | 65 | 2 | 30 | 26 | 45 | 0 | 1 | 6 |
| | | M | 5 | 16 | 0 | 19 | 14 | 35 | 0 | 1 | 2 |
| 2006 | a1 | Wt | 232 | 449 | 17 | 3 | 163 | 19 | 1 | 1 | 6 |
| | | M | 90 | 170 | 12 | 17 | 81 | 13 | 5 | 3 | 3 |
| | a2 | Wt | - | 498 | 35 | 6 | 82 | 14 | 1 | 2 | 3 |
| | | M | - | 51 | 5 | 2 | 35 | 3 | 0 | 3 | 1 |
| 2007 | a | Wt | - | 196 | 15 | 9 | 374 | 18 | 11 | 3 | 2 |
| | | M | - | 11 | 6 | 11 | 109 | 7 | 4 | 5 | 1 |
| | b | Wt | - | 96 | 18 | 12 | 301 | 10 | 16 | 3 | 6 |
| | | M | - | 17 | 7 | 9 | 152 | 4 | 10 | 3 | 0 |

**Suppl. Table 2** Identified species of the most representative floral visitors (superfamilies *Apoidea* and *Syrphoidea*) on 1947-wt and 1947-*Spe* plants in the Botanical Garden Jena; * indicates wild bees. Listed species were found on both floral phenotypes

| | |
|---|---|
| Syrphoidea | ***Sphaerophoria* sp.** |
| | ***Sphaerophoria scripta*** |
| | ***Episyrphus balteatus*** |
| | ***Syritta pipiens*** |
| | ***Melanostoma mellinum*** |
| | ***Eupeodes corollae*** |
| | ***Scaeva pyrastri*** |
| | ***Epistrophe melanostoma*** |
| | ***Epistrophe nitidicollis*** |
| | ***Paragus* (*Pandylophtalmus*) *haemorrhous*** |
| | ***Paragus* (*Pandylophtalmus*) sp.** |
| | ***Eumerus tuberculatus*** |
| | ***Pipizella viduata*** |
| | ***Pipizella* sp.** |
| Apoidea | ***Hylaeus signatus* *** |
| | ***Hylaeus pictipes* *** |
| | ***Megachile willughbiella* *** |
| | ***Andrena minutuloides* *** |
| | ***Andrena minutula* *** |
| | ***Lasioglossum morio* *** |
| | ***Lasioglossum pauxillum* *** |
| | ***Cerceris rybyensis*** |
| | ***Ectemnius dives*** |

**Suppl. Table 3** Floral visitors of *Capsella bursa-pastoris* collected in the natural habitat Gau-Odernheim during observation time and in the area indicated in Material and Methods

| **Order** | **Family** | **Genus** | **# Individuals on** | |
|---|---|---|---|---|
| | | | *Spe* | wild-type |
| **Coleoptera** | Cantharidae | *Cantharis* | 1 | 1 |
| | Crysomelidae | *Phyllotreta* | 1 | - |
| | undefined | | 3 | 1 |
| **Diptera** | Anthomyiidae | Anthomyiinae | - | 2 |
| | Conopidae | *Tecophora* | - | 1 |
| | Bibionidae | *Bibio* | 2 | 2 |
| | Syrphidae | *Melanostoma* | 1 | - |
| | | *Platycheirus* | 1 | 1 |
| | | *Sphaerophoria* | 6 | 10 |
| | Platystomatidae | *Platystoma* | - | 3 |
| | Sacrophagidae | | 1 | - |
| | Tachinidae | *Tachina* | 1 | 1 |
| **Hymenoptera** | Apidae | *Andrena* | 1 | 9 |
| | | *Halictus* | 3 | 4 |
| | | *Lasioglossum* | 2 | 8 |

## 11. Danksagung

Die vorliegende Arbeit wurde am Lehrstuhl für Genetik unter Anleitung von Prof. Dr. G. Theißen angefertigt.

**Mein herzlichster Dank gilt:**

Prof. Dr. G. Theißen für die Möglichkeit, in seinem DFG-Projekt arbeiten zu dürfen, für die damit verbundene Erlangung unentbehrlicher Kenntnisse über viele molekularbiologische Methoden, das unendliche Training und die Geduld im wissenschaftlichen Schreiben, die Erweiterung meines Wissensschatzes nicht nur auf dem Gebiet der Pflanzengenetik, für die wissenschaftliche Motivation und Diskussionen, die vielmals mit Witz und Humor unterlegt waren.

Prof. Dr. M. Platzer für die Möglichkeit in den Laboren des Fritz-Lippmann-Instituts (FLI) für Altersforschung in Jena die Pyrosequenzierung mit allen dazugehörigen Vorarbeiten durchführen zu können, zusammen mit der unverzichtbaren Hilfe von Marco Groth und anderen Mitarbeitern der Arbeitsgruppe sowohl bei der Durchführung als auch Auswertung der Analysen.

Prof. Dr. B. Neuffer für die Beantwortung vieler *Capsella*-spezifischer Fragen, für die Zusammenarbeit und Ideen im *Capsella*-Projekt und die herzliche Gastfreundschaft, ebenso die ihrer Mitarbeiter, beim Wissensaustausch in Osnabrück

Dr. M.H. Hoffmann für die Erläuterungen des Freilandprojektes sowie die Realisierung der Beobachtungen im Botanischen Garten Halle.

Christian Abel vom MPI für Chemische Ökologie in Jena für die rege Bereitschaft und Durchführung der Analyse an den Blütenduftstoffen.

Christiane Ritz für spannende Diskussionen, ihre Ratschläge und Aufmunterungen, sowohl im Labor als auch privater Natur, und natürlich ihrem Mann Markus, der mir eine große Hilfe in statistischen Fragen und Durchführung der Analysen war, sowie für seine hilfreiche Mitarbeit am Manuskript III.

F. Burger und E. Stolle für die schnelle und selbstverständliche Bestimmung der Schwebfliegen sowie Bienen und Wespen.

Domenika Schnabelrauch vom MPI für Chemische Ökologie in Jena für die unendlichen Sequenzierungsarbeiten, die manchmal auch kurzfristig und schnell gehen mussten, sowie technische Ratschläge und Hilfestellungen bei den Vorarbeiten.

Dem Leiter und Personal des Botanischen Gartens in Jena für die Bereitstellung der Flächen und ihre beständige Hilfsbereitschaft in Gärtnerfragen und bei der Pflanzenversorgung.

Allen Mitarbeitern des Lehrstuhls für das angenehme Arbeitsklima und für viele Hilfen und Anregungen und immer ein offenes Ohr.

Pia Nutt für die gute Zusammenarbeit und hilfreiche Diskussionen im *Capsella*-Projekt.

Ulrike Wrazidlo für Fragen und Hilfe im Labor und bei Pflanzenprobleme und Heidi Kreßler, die vor allem in der Endphase viele arbeiten im Gewächshaus und im Labor durchgeführt hat.

Sabine Schein, die gute Fee im Biologicum, für die tatkräftige Unterstützung in außerbiologischen Fragen.

Susanne Schilling und Lydia Gramzow für das Lesen und Korrigieren so mancher meiner Niederschriften.

Rainer Melzer und Maren Fräger für rege Diskussionen und Ratschläge sowie ihre freundschaftliche Art, die mich oftmals aufbaute, wenn mal nicht alles in bester Ordnung war, ob im Institut oder privat.

Der Deutschen Forschungsgemeinschaft für die Finanzierung des Forschungsprojektes “Analyse einer natürlich bestandsbildenden floralen homöotischen Mutante des Hirtentäschel (*Capsella bursa-pastoris*)” (TH 417/4-1) und somit für die Finanzierung meiner Stelle.

Schließlich meiner ganzen Familie für ihr geduldiges Zuhören und Aufmunterungen, wenn es mal nicht so gut lief und ihrer Unterstützung in Problemen, die neben der Doktorarbeit auftauchten und der nötigen Ablenkung durch meine beiden Mäuse Vivi und Kira, die mich auch in stressigen Zeiten ertragen mussten und haben.

## 12. Ehrenwörtliche Erklärung

Hiermit erkläre ich ehrenwörtlich, dass mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist und ich die vorliegende Arbeit selbst angefertigt habe. Alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen habe ich in meiner Arbeit angegeben. Bei der Auswertung des Materials sowie bei der Herstellung des Manuskriptes haben mich die in der Danksagung auf Seite 142/143 der Dissertation genannten Personen unterstützt. Ferner erkläre ich ehrenwörtlich, für die Anfertigung der Arbeit keinen Promotionsberater in Anspruch genommen zu haben, und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Dissertation habe ich bisher nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung vorgelegt. Auch habe ich weder diese Dissertation noch eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation eingereicht.

Ort, Datum Janine Ziermann

## 13. *Curriculum vitae*

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| Name | Janine Ziermann |
| Geburtsdatum | 14. 02. 1979 |
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**Schulbildung**

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| 1985 - 1992 | Wilhelm-Pieck-Oberschule Rudolstadt-Schwarza |
| 1992 - 1997 | Friedrich-Fröbel-Gymnasium Bad Blankenburg<br>Abschluss: Abitur (Note: 2,4) |

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| 1997 - 1998 | 2 Semester Gartenbau<br>Fachhochschule für Landschafts- und Gartenbau Erfurt |
| 1998 - 2005 | Biologie Studium (Biologie, Diplom) an der Friedrich-Schiller-Universität Jena<br><br>Diplomarbeit am Institut für Spezielle Botanik mit dem Titel : „Phylogenetische Untersuchungen der Subgenera *Tacsonia* und *Manicata* (Gattung *Passiflora*)“ (Betreuer: Prof. Dr. F. H. Hellwig, Dr. V. Wissemann) |
| 2005 | Diplomabschluß (Note: 1,5) |
| 2005 - 2009 | Promotionsstudium am Lehrstuhl für Genetik der Friedrich-Schiller-Universität Jena mit dem Thema: „Charakterisierung der natürlich bestandsbildenden floralen homöotischen Mutante *Stamenoid petals* (*Spe*) des Hirtentäschel (*Capsella bursa-pastoris*)“ |

**Berufstätigkeit**

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| 2000 - 2004 | verschiedene Anstellungen als Studentische Hilfskraft an der Pharmazeutisch-Biologischen Fakultät der FSU Jena und am Max-Planck-Institut für Geobotanik Jena |
| 2005 - 2008 | Wissenschaftliche Mitarbeiterin am Lehrstuhl für Genetik, FSU Jena im DFG Projekt “Analyse einer natürlich bestandsbildenden floralen homöotischen Mutante des Hirtentäschel (*Capsella bursa-pastoris*)” (TH 417/4-1) |
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| 2002 - 2003 | Austauschstudent an der Universidad Autónoma de Madrid (UAM) (Spanien) im Rahmen des ERASMUS Programms |
| 2003 | Austauschstudent an der Karl-Franzens-Universität Graz (KFU) (Österreich) im Rahmen des ERASMUS Programms |

Ort, Datum Janine Ziermann

# 14. Wissenschaftliche Beiträge

## Wissenschaftliche Publikationen

P. Nutt, J. Ziermann, M. Hintz, B. Neuffer and G. Theißen (2006): *Capsella* as a model system to study the evolutionary relevance of floral homeotic mutants. Pl. Syst. Evol. 259, pp 217-235 (Übersichtsartikel)

M. Hintz, C. Bartholmes, P. Nutt, J. Ziermann, S. Hameister, B. Neuffer and G. Theißen (2006): Catching a ´hopeful monster´: Shepherd´s purse (*Capsella bursa-pastoris*) as a model system to study the evolution of floral development. J. Experim. Bot. 57 (13), pp 3531-3542 (Übersichtsartikel)

J. Ziermann, M. Ritz, S. Hameister, C. Abel, M. H. Hoffmann, B. Neuffer and G. Theißen (2009): Floral visitation and reproductive traits of *Stamenoid petals*, a naturally occurring floral homeotic variant of *Capsella bursa-pastoris* (Brassicaceae). Planta 230, pp 1239-1249 (Originalarbeit)

## Wissenschaftliche Vorträge

"Mapping candidate genes in the floral homeotic mutant Spe of Capsella bursa-pastoris (shepherd's purse)" Mitteldeutsches Pflanzenphysiologie-Treffen and “Symposium on Plant signal transduction” (SFB 604), Jena, Germany (2008)

„Genetische Charakterisierung der Blütenmutante Spe von Capsella bursa-pastoris (Brassicaceae)“
21. Tagung "Molekularbiologie der Pflanzen", Dabringhausen, Germany (2008)

## Wissenschaftliche Kongressbeiträge in Form von Postern

S. Hameister, P. Nutt, J. Ziemann, G. Theißen, B. Neuffer: 6+4=10: Decandric forms of *Capsella bursa-pastoris* (L.) Medik., characterising a floral variety of shepherd's purse; 17th International Symposium "Biodiversity and Evolutionary Biology, Bonn, Germany (2006)