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**Conséquences écologiques et évolutives du flux de gènes
entre Brassica napus transgénique et ses apparentés
sauvages**
Yongbo Liu

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Yongbo Liu. Conséquences écologiques et évolutives du flux de gènes entre Brassica napus transgénique et ses apparentés sauvages. Biologie végétale. Université de Bourgogne, 2010. Français. NNT : 2010DIJOS028 . tel-00584017

HAL Id: tel-00584017

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INRA



UNIVERSITE DE BOURGOGNE

THÈSE

Pour obtenir le grade de

Docteur de l'Université de Bourgogne

Discipline : Sciences vie

par

Yongbo LIU

Soutenue le 29 octobre 2010

CONSEQUENCES ÉCOLOGIQUES ET ÉVOLUTIVES DU FLUX
DE GÈNES ENTRE *BRASSICA NAPUS* TRANSGÉNIQUE ET SES
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有志者，事竟成

Quand on veut, on peut

Where there is a will, there is a way

REMERCIEMENTS

My heartfelt thanks go to my supervisor Dr. Henri Darmency (INRA, Dijon) for his gentle encouragement, enthusiasm, and insightful guidance throughout my Ph.D. studies at BGA INRA, Dijon. I am also extremely fortunate to have other two supervisors Professor Keping Ma and Dr. Wei Wei, Institute of Botany, the Chinese Academy of Sciences (IBCAS) during the past six years. I give my special thanks to Prof. Ma who enrolled me as a Ph.D. student to continue my favorite science research after finishing my master study. My sincere thanks also go to Dr. Wei who gave me the chance to come to study in France for his tireless assistance and constant support in China.

I would also like to thank all the members of my thesis jury, Joelle Ronfort, François Felber, François Bretagnolle, Jane Lecomte, Neal Stewart Jr. for their critical reading and valuable suggestions on my thesis.

My sincere thanks go to the secretaries in Biologie et Gestion des Adventices, INRA (Claudine Chotel, Emmanuelle Ferrand and Sandrine Géslain), and in the University of Burgundy (Daouya Commaret and Josette Théry), as well as those in IBCAS (Jianfeng Zhao, Haiyan Wu and Wenjuan Zhang) who played fundamental roles in the success of these projects. I wish give my special thanks to Denise Sauvadet (Responsable du Service de Relations Internationales, CNOUS, Dijon) for her kind help in my scholarship in France.

I wish to thank all colleagues in the research group of BGA, INRA for their ideas, suggestions, and assistance: Xavier Reboud, Fabrice Dessaint, Nathalie Colach, Jacques Gasquez, Bruno Chauvel, Louis Assémat, Nathalie Grandgirard, François Bretagnolle, Nicolas Munier-Jolain, Sandrine Petit, Christian Gauvrit, Sabrina Gaba, Delphine Moreau, Jean-Philippe Guillemin, Catherine Cardinal-legrand, Stéphanie Gibot-leclerc, Sylvie Granger,

Jacques Caneill, Béryll Laitung, Valérie Le Corre, Bernard Nicolardot, Jean-Michel Thomas, Maurice Tremoy and many others.

My special thanks also go to Alain Fleury who worked together with me in greenhouse and field, and Annick Matejcek who helped me in laboratory, Dominique Meunier, Florence Strbik and François Dugue who helped me in field experiments. I would like to thank Émilie Cadet, Gilles Louviot, Séverine Michel, Karelle Boucansaud, Arnaud Coffin, Hugues Busset, and many others for their support in manipulating experiments.

I appreciate the PhD or post-doctor researchers who worked diligently in BGA: Dominique Jacquin, Helmut Meiss, Richard Gunton, Solène Bellanger, Delphine Mézière, Stéphane Cordeau, Cécile Petit, Yann Tricault, Antoine Gardarin, Aline Boursoult, Aimé Dongmo, Mélanie Le Guilloux, Young Jin Chun, Benjamin Borgy, Benjamin Gard, Coraline Caullet, Clément Tschudy and many others.

I wish to express my gratitude to all my friends in France, Dr. Zhanwu Dai, Sebastien Guyot, Yacine Merabtine, Claire Simon, Ann-Katrin Embries, Fanny Chouette, Hamidou Diallo, as well as Tianlu Zheng, Sailing Yuan, Rujin Zhang, Yuanyuan Luo, Huaiqian Li, Bin Fan, Xiangdong Meng, Ting Zhang, Yucong Duan, Jiali Ruan, Beibei Qi, and many others who helped me in some experiments and in my daily life, making my stay in France more colorful and wonderful. I appreciate Dominique Jacquin families who generously shared their farmland, time, and local knowledge.

I am very grateful to the members of the Ma lab, Dr. Shouren Zhang, Dr. Kequan Pei, Dr. Mei Yu, Dr. Xiangcheng Mi, Dr. Yu Liang, Dr. Xiaojun Du, Dr. Haibao Ren, Dr. Li Zhu, Dr. Jiangshan Lai, Dr. Tiemei Chen, Hu Yuan, Shunzhong Wang, Yanhong Bin, Zhixi Tang, Kun Di, Baocheng Shen, Yingting Le, Jingjing Dong, Chaoyang Wu, Lei Lei, Pengfei Zhang, Qian Luo and many others for their assistance in experiments and important discussions in

forming many of the ideas I present and constructive criticisms of the work in early and late stage.

Many thanks go to my master supervisor Qibin Zhang who enrolled me as a master to start my study and taught me how to write English articles. I also would like to thank the members of the Zhang lab, Hongyan Qiu, Lixin Lv, Gaiai Guo, Caiyun Liu, Chunming Shi, Zongshan Li, Xiaochun Wang, Pei Xing and others for their help in my study.

I am very grateful to my friends in China, Zhongkui Luo, Weiqing Wang, Zhenchang Liang, Guangkun Yin, Tingting Meng, Juan Wang, Fan Bai, Jianyin Hu, Chuanglong Li, Liyan Zhang, Jin Shi, Sha Chen, Feng Wang, Liang Chen, and Qinglu Yuan, Xijiang Ouyang, Yu Wei, as well as Xiaohong Yuan, Jianfen Li, Xiahui Peng, Yurong Wang, Yiping Fu, Kun Chen, Honghua Xiao, Ji She, Jiaoyan Zhu and many many others for their constant assistance in my study and daily life. I'd like to thank Jinlong Zhang, Lixin Lv and Guoke Chen in dealing with many kinds of administrative issues for my studying aboard.

The work presented here was financially supported by several projects of the Natural Science Foundation of China (grant no. 30970432 and 30670316) and by a PhD joint fellowship between China and France (CNOUS, No. 20072315).

Finally, special thanks go to my family: my dad Yunzhong Liu, my mom Guitao Yi, my old sisters Yanling Liu and Yanjiao Liu, my little brother Xiaobo Liu, brothers-in-law, nephews and nieces. My parents still work hard and save money on food and expenses for my study. Without your support, I could not have realized this goal. Thank you for the freedom to pursue my own path - such a rare luxury for poor families in the world.

Thank you, thank you, thank you!

Yongbo LIU

刘勇波

Résumé

Les conséquences des flux de gènes et de l'introggression entre les cultures transgéniques et leurs apparentés sauvages sont encore au cœur des débats associés à la commercialisation des plantes génétiquement modifiées. J'ai développé mon étude sur les conséquences écologiques et évolutives du flux de gènes entre le colza (*Brassica napus*) et ses apparentés, la moutarde brune sauvage (*B. juncea*) et la ravenelle (*Raphanus raphanistrum*), en réalisant une série d'expériences en serre, au jardin et au champ à Beijing et à Dijon.

En premier, j'ai présenté une revue synthétique de la littérature publiée sur les flux de gènes et ses effets sur la fitness chez les Brassicées. En second, j'ai cherché à mettre en évidence le rôle de la taille des semences hybrides entre du colza transgénique Bt et la moutarde. La petite taille des semences a réduit les capacités de croissance et de reproduction, mais l'effet sur la fitness était variable en fonction des fonds génétiques ou spécifiques. Les rétrocroisements sur le colza étaient plus faciles et productifs que pour les autres types de descendants. La plupart de ces plantes avait une morphologie de colza. Liée à la résistance à l'herbicide, cette caractéristique pourrait permettre aux descendants de survivre dans les champs et de disséminer les transgènes aux repousses et aux autres colzas, ce qui serait peut être plus gênant que de voir l'introggression réelle dans le génome du parent sauvage. Troisièmement, j'ai simulé le phénomène d'herbivorie chez la moutarde pour étudier la compétition entre des plantes résistantes et des plantes sensibles indépendamment des problèmes de fitness des hybrides interspécifiques. Les plantes résistantes ont un avantage compétitif évident sous la pression d'herbivorie, et cet avantage est exacerbé sous des conditions difficiles telles que de faibles ressources du milieu et l'intensité de l'herbivorie. L'utilisation d'insectes pour attaquer des populations mixtes composées de rétrocroisements sensibles et Bt-résistants aux insectes a confirmé ce résultat et a montré que le transgène n'avait pas de coût en l'absence d'insectes. La productivité totale des populations a augmenté avec la proportion de plantes résistantes. Quatrièmement, des populations de ravenelles ont été échantillonnées dans quatre régions éloignées entre elles, dont une ayant une longue histoire de coexistence avec le colza et donc ayant plus de chance d'avoir été soumise à l'hybridation interspécifique avec le colza. J'ai interprété la divergence des traits et leur polymorphisme dans le cadre d'une hypothèse d'introggression stabilisée en opposition au simple hasard, bien que les différences avec les autres populations n'étaient pas assez marquées pour faire sortir ces populations du domaine de variation décrit pour les ravenelles.

Ces études soulignent plusieurs facteurs qui peuvent accroître le risque des flux de transgènes et l'introggression entre les cultures génétiquement modifiées et leurs apparentés sauvages, et cela doit être pris en compte dans les procédures d'évaluation des risques de l'usage de ces plantes. A savoir : la morphologie cultivée qui rend confuse l'identification des introgressants dans le cadre de la bio-surveillance, les petites semences hybrides avec une dormance et une dispersion supérieures, et l'intensité de l'herbivorie et de la compétition qui exacerbe l'avantage adaptatif des plantes transgéniques résistantes aux insectes. Cependant, l'hypothèse de la formation de « super mauvaises herbes » ne semble pas justifiée.

Mots clés: colza (*Brassica napus*), moutarde sauvage (*Brassica juncea*), ravenelle (*Raphanus raphanistrum*), compétition, valeur adaptative, organisme génétiquement modifié (OGM), flux de gènes, introgression, traits morphologiques, dynamique des populations, transgene.

Abstract

In the framework of commercial release for transgenic crops with novel traits, consequences of gene flow and introgression are still one main concern. I explored the ecological and evolutionary consequences of gene flow between oilseed rape (*Brassica napus*) and its wild relatives, brown mustard (*B. juncea*) and wild radish (*Raphanus raphanistrum*), through several experiments carried out in greenhouse, common garden and field in Beijing and Dijon.

First, I revised a comprehensive review of the literature about gene flow and its effect on plant fitness in the Brassicaceae. Second, I investigated the effects on gene flow of seed size of hybrids between Bt-transgenic oilseed rape and mustard. Small seed size significantly reduced plant growth and reproduction, but its influence on plant fitness varied among genetic backgrounds. Backcrosses to oilseed rape were easier and more productive than other types of progeny of hybrids. Most of these plants exhibited oilseed rape morphology. Together with herbicide-resistance, this trait could help the progeny to survive in the field and disseminate the transgene to volunteers and feral populations, which could be more troublesome than completing introgression into the genome of the wild parent species. Third, I simulated herbivory on mustard to study the competition between insect-resistant and susceptible plants independently to the fitness of the interspecific hybrid. Resistant plants held a competitive advantage under herbivory pressure, and this advantage was magnified in harsh conditions, such as low resources and high simulated herbivory pressure. The use of insects to attack mixed populations composed of transgenic Bt-resistant and susceptible backcrosses confirmed the same conclusion and provided evidence of no cost due to the transgene in the absence of insect. The overall population production increased with the increasing proportion of insect-resistant plants in the presence of insects. Fourth, wild radish populations were sampled from four geographically distant regions, of which one region had a long history of oilseed rape cultivation, and, therefore, higher chance to have been submitted to interspecific hybridization with the crop. Traits divergence and polymorphism in the putative introgressed populations could be supported as alternate hypothesis to random variation, although the differences were not marked enough to place these populations out of the range of variation described in wild radish.

These studies pointed out different factors that could enhance the risk of transgenic flow and introgression from transgenic crops to wild relatives, and they must be taken into account in the risk assessment of the use of GM crops: crop traits to identify the hybrid progeny and perform monitoring, small seed size to account for seed dispersal and dormancy, and intensity of herbivory and competition that magnify the fitness advantage of insect-resistant transgenic plants. However, the impact of introgression to create super-weeds was not supported.

Key words: oilseed rape (*Brassica napus*), wild mustard (*Brassica juncea*), wild radish (*Raphanus raphanistrum*), competition, fitness, genetically modified (GM), gene flow, introgression, morphological traits, population dynamics, seed production, transgene

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Introduction générale

INTRODUCTION GENERALE

Dans le cadre de la commercialisation mondiale des cultures transgéniques obtenues par génie biotechnologique, l'une des réserves le plus souvent soulevées concerne le flux de gènes entre la culture et ses apparentés sauvages. Ce phénomène est dû aux échanges polliniques entre ces espèces et à la diffusion ultérieure des transgènes par le pollen et les semences des hybrides. Les descendants transgéniques, hybrides et générations rétrocroisées, peuvent perdurer dans les champs et d'autres habitats, causer des problèmes agricoles et dans le milieu naturel, et même représenter une nouvelle source de flux de transgène pour d'autres organismes (Hall et al. 2000; Simard et al. 2002; Jorgensen et al. 2007; Colbach et al. 2008; Warwick et al. 2008).

En fait, aussi bien les flux de gènes que les introgressions entre espèces sont des phénomènes naturels qui surviennent spontanément et jouent un rôle important dans l'évolution des plantes et des espèces cultivées. Beaucoup d'espèces végétales sont d'origine allopolyploïde, et les cultures d'aujourd'hui ont souvent connu un épisode de flux de gène et d'introgression dans leur passé, ce qui a façonné leurs forme et leur devenir. Cependant, dans le cas des transgènes, le flux de gènes apporte des caractéristiques jouant sur la fitness qui n'étaient pas présentes dans l'espèce sauvage auparavant, et cela peut augmenter sa vigueur ou sa faculté de colonisation ou au contraire avoir des conséquences nuisibles à sa survie. On peut craindre que ces gènes perdurent pour plusieurs générations dans les populations sauvages, et effectivement des descendants d'hybrides interspécifiques et des populations sub-spontanées de repousses des cultures transgéniques ont été trouvés dans les champs (Jorgensen et al. 2007; Méssean et al. 2007; D'Hertefeldt et al. 2008). Ces gènes qui ont des effets neutres ou bénéfiques sur la fitness et ne sont pas liés à des caractéristiques cultivées non-adaptatives ont le potentiel pour persister. Aussi, nous devons nous interroger sur les conséquences écologiques et évolutives de ce phénomène aussi bien à court qu'à long terme.

A court terme, le flux de gènes forme des hybrides et des descendants par rétrocroisement disposant de nouvelles caractéristiques telles que la résistance aux herbicides, aux insectes, aux virus et aux maladies. Ces nouvelles caractéristiques vont affecter la croissance et la reproduction des hybrides interspécifiques (Ellstrand et al. 1999;

Snow et al. 2003; Halfhill et al. 2005; Campbell et al. 2006). Lorsque des plantes résistantes colonisent une population sensible, ou lorsque le transgène donnant la résistance est transmis à des populations sauvages, la compétition entre les plantes résistantes et sensibles va affecter la biologie de la population. A cause de l'avantage lié à la résistance, les plantes résistantes vont s'approprier une partie supplémentaire des ressources locales, entraînant ainsi un développement réduit des plantes sensibles. La relation entre les deux types évoluera avec leur proportion car le paysage compétitif de chaque individu va évoluer en conséquence. Ceci varie bien sûr avec le niveau des ressources, les pressions de sélection exercées par les herbicides, les insectes ou les maladies.

Sur le long terme, la persistance des transgènes dans une population sauvage pourrait conduire à des modifications de leur structure et de leur dynamique et altérer leur potentialité d'évolution. La coexistence ou non des types résistant et sensible pourrait entraîner une meilleure exploitation des ressources du milieu, ou au contraire être nuisible à la productivité de la population. On peut aussi se demander si des caractéristiques morphologiques importantes pour l'écologie des espèces peuvent être affectées concernant la reproduction (dates de floraison, pollinisation, forme et attractivité des fleurs), la survie (type de fruit, conditions de germination). L'étude de tels effets sur le long terme est très difficile pour des raisons de limitation dans le temps et d'absence de matériel sauvage transgénique approprié. La modélisation serait une alternative à l'expérimentation, mais il faut alimenter le modèle avec des paramètres dont on ne dispose pas. En revanche, la recherche de populations anciennement introgressées par des gènes de plantes cultivées pourrait nous permettre une telle analyse.

Dans notre travail, nous avons essayé d'aborder ces questions par les deux extrémités du scénario de l'introgression de caractéristiques d'une culture transgénique dans une espèce sauvage apparentée : l'implantation initiale, et la recherche des conséquences finales. Nous avons choisi le colza, *Brassica napus*, comme exemple de culture transgénique car il produit beaucoup de pollen et son hybridation avec plusieurs Brassicées sauvage est connue, et des résistances à un herbicide et aux insectes étaient disponibles. Le choix de *Brassica juncea* comme cible sauvage a été motivé par sa présence à l'état de population sauvage en Chine, et donc d'un besoin de connaissance à son sujet. Après un

chapitre 1 dressant l'état des connaissances sur les flux de gènes chez le colza, nous avons abordé les questions suivantes que nous avons présentées sous la forme d'articles :

1) Est-ce que la petite taille des semences hybrides représente un obstacle au flux de gène (Chapitre 2.2) ?

2) De quelle manière le croisement *B. napus* x *B. juncea* permet-il de diffuser et d'implanter les transgènes dans les champs (Chapitre 2.3) ?

3) Quelle est le bénéfice de la résistance aux insectes pour l'individu et pour la population : en simulant l'herbivorie (Chapitre 3.2 et 3.3) ou en expérimentation avec les insectes (Chapitre 3.4)

4) Est-ce que des traces d'introgessions anciennes peuvent être détectées chez des populations sauvages et quelles conséquences (Chapitre 4)

Chapter 1

Revue bibliographique sur les conséquences des flux de gènes entre le colza et ses apparentés sauvages

CHAPTER 1

LITERATURE REVIEW ON THE CONSEQUENCES OF GENE FLOW FROM OILSEED RAPE TO ITS RELATIVES

Gene flow and introgression between transgenic crops and wild relatives is considered one of undesirable consequences of commercial release of genetically modified (GM) / transgenic crops conferring herbicide, insect or virus-resistance genes. Commercialized GM crops involving many important resources species, such as soybean, maize, cotton, oilseed rape, squash, papaya, alfalfa and sugarbeet, are cultivated worldwide (James 2009). Most of these transgenic species could hybridize with their wild relatives (Ellstrand et al. 1999; Armstrong et al. 2005).

Hybridization is a prerequisite for gene flow, and gene flow is a prerequisite for introgression. But, not all hybridization could induce gene flow. Hybrids can form when certain conditions are achieved, including sympatry with a compatible relative (weedy, wild or crop), overlapping flowering phenology, pollen and seed dispersal, successful fertilization and viable offspring. Successful gene flow does not necessarily result in gene introgression. The introgression of transgenes into the receiving species or population depends mainly on the barriers to hybridization and introgression, the evolution and fate of inter-specific hybrids, their fitness, and the potential costs and benefits of transgenes in the offspring under variable habitat conditions.

Inter-specific gene flow via hybridization and subsequent introgression plays a biologically important role in the acquisition of genetic adaptations through natural plant populations. It has been recognized that introgression can occur frequently and may produce viable, even highly-adapted, progeny (e.g., Arnold 1997, 2006; Rieseberg et al. 1999), although evolutionary scientists early focused on the important role of rare events of introgression for the evolution of many plant groups (e.g., Lotsy 1916; Anderson 1949; Stebbins 1959; Grant 1981).

We present here a literature survey on the processes and consequences of gene flow and introgression between oilseed rape and its wild relatives. Such a topic has been ignored before the release of GM varieties, but many studies have documented this issue to date.

1.1 Hybridizations between *Brassica napus* and its wild relatives

In order to understand the possible risks of gene flow from *Brassica napus* to wild relatives, it is first important to understand the relationship between the species of its primary gene pool amongst others belonging to the Brassicaceae, consisting of over 3000 species in 370 genera. Three species, *B. napus* (AACC, $2n = 4x=38$), *B. juncea* (AABB, $2n = 36$), and *B. carinata* (BBCC, $2n = 34$), are allotetraploids derived from three diploid species *B. nigra* (AA, $2n = 16$), *B. rapa* (CC, $2n = 20$), and *B. oleracea* (BB, $2n = 18$) (Fig. 1.1).

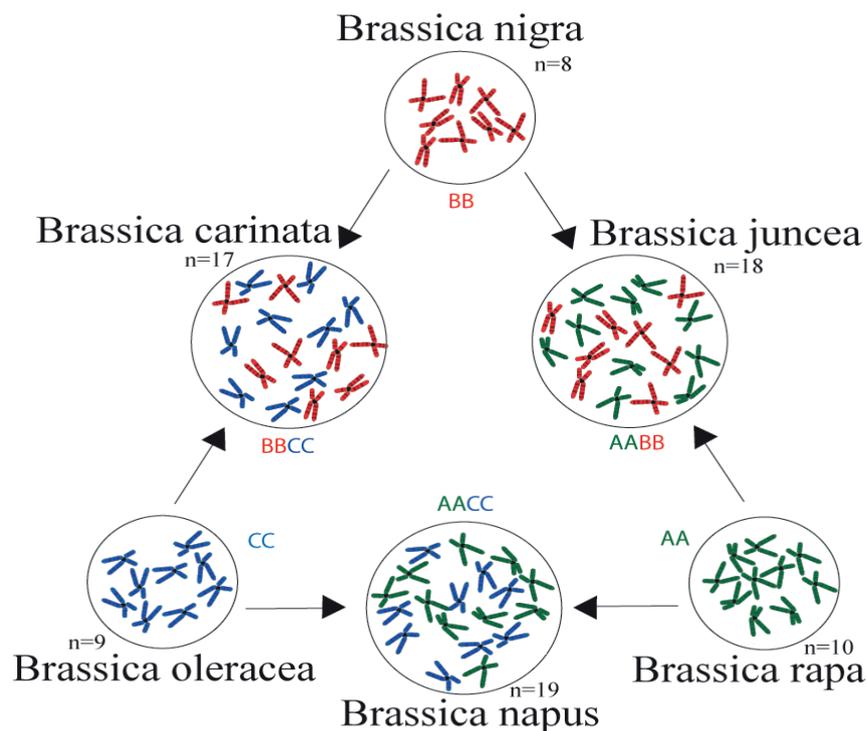


Fig. 1.1. “U-triangle” representing the genomic relationships between Brassica species (redrawn from U 1935)

Almost all hybridization among *B. napus* and the five other species could occur, despite it presented varying difficulty. The hybridization is influenced by external factors, including wind direction and speed, insect movement (Scheffler et al. 1993), locations and flowers positions at plants (Becker et al. 1992). Natural crosses can frequently occur among the three oilseed Brassicas, *B. napus*, *B. rapa* and *B. juncea* (Bing et al. 1991, 1996a; Leckie et al. 1993; Jorgensen and Andersen 1994), and transgene flow from *B. napus* into *B. rapa* (Bing et al. 1991, Leckie et al. 1993) or *B. juncea* (Bing et al. 1991, Frello et al. 1995) may occur through natural pollination. Although the frequency of successful crosses between

oilseed rape and *B. oleracea* is very low, hybridization could be achieved by hand pollination. In the United Kingdom, spontaneous hybrids between *B. napus* and *B. oleracea* were observed in the *B. oleracea* wild population, using flow cytometry and crop-specific microsatellite markers (Ford et al. 2006). No natural crosses are reported between the three species and *B. nigra* (Bing et al. 1996a), but male sterile *B. napus* produce spontaneous hybrids when placed in a *B. nigra* stand (Wei and Darmency 2008), although there is no indication about the reciprocal cross. Hybrids formed between *B. rapa* and *B. nigra* was obtained by ovule culture (Bing et al. 1996b). Hybridization of *B. napus* × *B. carinata* and *B. juncea* × *B. carinata* have been achieved by hand pollination (Chang et al. 2007). Hybrids between *B. rapa* and *B. carinata* were only obtained when *B. carinata* was used as the female parent (Choudhary et al. 2000).

No natural crosses occurred between the three species and *Sinapis arvensis* (Bing et al. 1996a, Lefol et al. 1996), indicating that direct transgene escape from *B. napus* to *S. arvensis* appears very unlikely (Moyes et al. 2002). Spontaneous hybridization between *B. napus* and wild radish (*Raphanus raphanistrum*, Rr, 2n=18) could occur in the nature, and some hybrids were reported on male sterile *B. napus* as mother in Canada and Denmark (Eber et al. 1994; Baranger et al. 1995; Ammitzboll and Jorgensen 2006) and a low frequency on *R. raphanistrum* as mother in Australia and France (Darmency et al. 1998; Chèvre et al., 2000; Rieger et al. 2001). The frequency of hybridization depended on environmental conditions, oilseed rape variety, wild radish populations and their density (Eber et al. 1994; Baranger et al. 1995; Darmency et al. 1998; Chèvre et al. 1996, 2000; Rieger et al. 2001; Ammitzboll and Jorgensen 2006). Genetic polymorphism for pollen germination and ovule fertilisation also represented variation of pre-zygotic barriers to interspecific hybridisation (Guéritaine and Darmency 2001).

Several overviews on hybridization between *B. napus* and its wild relatives and associated species are available in Rieger et al. (1999), FitzJohn et al. (2007), Andersson and de Vicente (2010).

1.2 Dispersal of transgenes

Extensive transgene flow between oilseed rape varieties has been detected in Canada resulting in the generation of volunteers that contain three types of herbicide resistance (Hall et al. 2000; Beckie et al. 2003; Knispel et al. 2008). The flow of transgenes could have occurred within a

given field (sympatry) or across fields through long distance dispersal of pollen can be transmitted either through pollens or seeds from crops to wild relatives.

1.2.1 Seed dispersal

Seed banks of transgenic crops with dormant seeds can build up naturally in the soil. Seed loss at harvest, shallow cultivation and ploughing timing have been identified as key factors to prevent incorporation of seeds into the seedbank (Begg et al. 2006). Oilseed rape (*B. napus*) possesses characteristics that favour the formation and persistence of a seed bank, i.e. pod shattering and inducible secondary dormancy. Volunteer recruitment from oilseed rape field was observed, suggesting seeds could be dormant in tilled as well as no-tilled fields (Simard et al. 2002). Besides the actual production area of transgenic crops and the locations where crops are grown, humans can also be a vector for unwanted gene flow during the transport, processing, and exchange of transgenic seeds. Several GM oilseed rape plants were detected at major ports and along roadsides in Japan, and these plants probably resulted from imported GM oilseed rape seeds because these seeds were not commercially cultivated (Saji et al. 2005; Aono et al. 2006). Similarly, in Korea, a feral plant of GM maize (*Zea mays*) was detected along a roadside near a Korean seaport (Kim et al. 2006). Volunteers arise from the seed bank in subsequent years, which can serve as a source or bridge for pollen flow.

1.2.2 Pollen dispersal

Oilseed rape is self-fertile, with pollen movement by both wind and insects (Williams et al. 1987). Interplant outcrossing rates range from 12 to 47% (Becker et al. 1992; Lavigne et al. 1998). Pollen-mediated gene flow in oilseed rape is affected by a variety of factors, including flowering time, genotypes, wind speed and direction, distance between donor and recipient populations. Pollens of oilseed rape were observed 1.5km from source field, and they were sufficient in number (22 pollen grains m³) to allow seed set (Timmoms et al. 1995). Gene flow for transgenic oilseed rape could occur at several kilometers from pollen resource (Table 1.1; Rieger et al. 2002; Cai et al. 2008). However, approximately half of the pollen produced by an individual plant fell within 3 m, and dispersal from whole plots instead of individual plants would have underestimated the proportion of pollen (Lavigne et al. 1998).

However, the majority of GM crossing fertilization occurs less than 10m (Husken and Dietz-Pfeilstetter 2007), with a decline over 50m. Generally, the degree of gene flow between

populations is reduced as the distance between the pollen source and the recipient population increases (Beckie et al. 2003). However, some results also have found long-distance pollen dispersal was random. The rate of long-distance pollen dispersal from 33 to 2000 m did not present a gradual decrease, and wind significantly affects the pollen dispersal direction and distance (Cai et al. 2008). Similarly, a random distribution with isolated pollination events was detected by Rieger et al. (2002), and this could be explained by the multiple pollinating agents (wind and insects) of oilseed rape and the large size of the source. In addition, Cresswell (1997) found that pollinator-mediated gene flow was unrelated to plant spatial heterogeneity, and the effects of plant aggregation on gene flow will depend on the spatial scale of the inter-patch distances.

Table 1.1 Pollen-mediated gene flow (GF) occurred at the maximum distance from pollen resources of GM oilseed rape (*B. napus*)

References	Max. distance studied (m)	Max. distance occurred GF (m)	Gene flow rate (%)	GM
Scheffler et al. 1995	400	400	0.004	glufosinate
Beckie et al 2003	800	400	0.04-0.05	glyphosate, glufosinate
Cai et al. 2008	2000	2000	<0.015	glufosinate
Rieger et al. 2002	5000	3000	0-0.15	ALS herbicide resistant

1.2.3 Persistence and dispersal of transgenic feral plants and volunteers in field

The pollen source is not always a full field of oilseed rape, but sometimes they are isolated or patchy plants not grown by farmers. Volunteers or feral plants of transgenic oilseed rape (*B. napus*) can often be observed in subsequent seasons in and/or out of cultivated fields (Table 1.2; Hall et al. 2000; Simard et al. 2002; Warwick et al. 2008), and these transgenic plants either arise from seed bank because of pod shattering (Simard et al. 2002). Outside cultivated areas, in roadside and railway, the occurrence frequency with which feral transgenic plants was similar to the proportion of the area cultivated transgenic plants in successive years, and 66% of the sampled feral plants were transgenic (Yoshimura et al. 2006). These results suggest the persistence and dispersal of transgenic oilseed rape in and outside of cultivated sites, thus making volunteer populations seem to be an important source of intraspecific gene flow (Jorgensen et al. 2007; Colbach et al. 2008).

Jorgensen et al. (2007) showed that 6-32% volunteers of other varieties belonged to varieties cultivated 4-17 years ago. In Canada, F1 and backcross hybrid generations between of herbicide-resistant *Brassica napus* and *B. rapa* were detected over six years in the field even in absence of the glyphosate selection pressure (Warwick et al. 2008). Herbicide-tolerant seedlings of oilseed rape emerged ten years after a field trial conducted in 1995, which confirmed the long-term persistence of transgenic seeds in the seed bank in Sweden (D’Hertefeldt et al. 2008), and multi-herbicide-resistant oilseed rape volunteers were found five to eight years after the last GM cultivar was grown in a multi-years farm-scale study in France (Méssean et al., 2007).

Table 1.2. Long-term persistence of volunteers and feral plants in and/ or outside of cultivated fields

References	Years	Sites	Types	GM	GM rate
Jorgensen et al. 2007	4-17	oilseed rape fields	Oilseed rape varieties	Non	6-32%
D’Hertefeldt et al. 2008	10	oilseed rape fields	Herbicide-tolerant seedlings from seed bank	Yes	39%
Warwick et al. 2008	6	oilseed rape fields	Herbicide-tolerant hybrids between <i>B.napus</i> and <i>B. rapa</i>	Yes	2.5%
Méssean et al., 2007	5-8	Convention oilseed rape fields	Herbicide-tolerant volunteers	yes	0-18%

1.3 Consequences of the hybridization

Halfhill et al. (2005) found that hybridization, with or without *Bt*-transgene introgression, resulted in less vegetative growth and seed production than *B. rapa* and less competitive populations. Di et al. (2009) found that transgenic hybrids between *Bt*-transgenic *B. napus* and *B. juncea* had intermediate composite fitness compared to their parents without herbivory.

Generally, the hybrids show intermediate morphologic characteristics compared to their parents (e.g. leaf hairness of hybrids formed between *B. napus* and *Hirschfeldia incan* by Lefol et al. 1996; intermediate leaf and flower morphology of hybrids formed between *B. carinata* and *B. rapa* by Choudhary et al. 2000). The cytoplasm of crops or wilds introgress

into hybrids would affect the morphology of the progeny (e.g. Lefol et al. 1996; Zhang et al. 2002; Guéritaine et al. 2002; Chang et al. 2007). For example, after seven backcrossing of *B. carinata* to *B. napus* and *B. juncea*, plants with the cytoplasm of *B. napus* flowered later, had shorter filaments and longer pistils, lower pollen amount, lower seed set, lower petal length and width and different petal color compared with plants with the cytoplasm of *B. juncea*, suggesting that the cytoplasm is involved in the development of flower organs (Chang et al. 2007).

In the case of *B. napus* x *R. raphanistrum* crosses, F1 hybrids formed between oilseed rape and wild radish are mainly allotriploids and present low fertility (3.2 seeds/plant for Darmency et al. 1998; 11 seeds/plant for Chèvre et al. 1998). The F1 hybrids showed significantly reduced seedling emergence, emergence delay, lower survival rate, and decreased dry matter compared with their parents (Guéritaine et al. 2003). However, the progeny of F1 (F2, BC1) grew well and produced viable seeds (Darmency et al. 1998), and the fertility increased after repeated backcross to wild radish. In addition, the progeny having wild radish cytoplasm were more likely to propagate in agronomic and natural sites and had a fitness value 100 times more than that having oilseed rape cytoplasm (Guéritaine et al. 2002).

1.4 Consequences of the transgene

A transgene transferred from oilseed rape to wild relatives might be neutral, but it is likely to increase or decrease the fitness of receiving hybrid/backcross plants (Table 1.3). This depends on the transgenic character, its selection and costs, how and where the transgene is introgressed in the genome, and the population composition (Felber et al. 2007). In response to insect herbivory, the *Bt*-transgene for insect resistance was shown to confer high relative fitness in oilseed rape in several studies (Stewart et al. 1997; Ramachandran et al. 2000; Letourneau and Hagen 2009). In turn, no fitness cost in the absence of the selection pressure was detected in the Brassiceae (Mason et al. 2003; Ramachandran et al. 2000; Di et al., 2009) and in sunflower (Snow et al. 2003).

Table 1.3. The relative performance of transgenic crop-wild relative hybrids as compared to non-transgenic hybrids (modified according to Hails and Morley 2005)

Crop	Wild relative	Hybrid	Confirmation of hybrid status	Results	Ref
<i>Brassica napus</i> (glufosinate tolerant)	<i>B. rapa</i>	F1	PCR and herbicide spray	Male fitness was frequency and density dependent, with male fitness of hybrids (pollinating <i>B. rapa</i>) being low, even when female fitness was high	Pertle et al. (2002)
<i>B. napus</i> (with Bt transgene)	<i>B. rapa</i>	F1	Transgenic hybrids identified using a GFP marker	Biomass, flower production and fecundity were higher for transgenic hybrids compared to <i>B. rapa</i> in the presence of herbivores. Biomass and fecundity were lower in the absence of herbivores, but flower production remained higher	Vacher et al. (2004)
<i>B. napus</i> (with Bt transgene)	<i>B. rapa</i>	BC2	Transgenic hybrids identified using a green fluorescent protein marker	No difference in vegetative biomass was found between transgenic backcrosses and non-transgenic <i>B. rapa</i> and <i>B. napus</i> at low insect pressure	Mason et al. (2003)
<i>B. napus</i> (high laurate)	<i>B. rapa</i>	F1	Not tested, transgene insertion assumed owing to multiple inserts in parental plants	Modified hybrids showed lower seed dormancy than null-segregants and <i>B. rapa</i> . Germination and growth was similar for transgenic and non-transgenic hybrids and <i>B. rapa</i>	Linder and Schmitt (1995)
<i>B. napus</i> (high laurate)	<i>B. rapa</i>	F1	Not tested; transgene insertion assumed owing to multiple inserts in parental plants	Modified hybrids showed similar levels of dormancy to non-segregants but much lower dormancy than wild parent. High laurate hybrids germinated and grew as fast or faster than <i>B. rapa</i>	Linder et al. (1998)
<i>B. napus</i> (glufosinate tolerant)	<i>B. rapa</i>	BC3	PCR for transgene and glufosinate test	Pollen fertility, seed production and survival were found to be equal to <i>B. rapa</i> parents	Snow et al. (1999)
<i>B. napus</i> (glufosinate tolerant)	<i>B. rapa</i>	BC1	PCR for transgene and glufosinate test	Pollen fertility similar to <i>B. rapa</i>	Mikkelsen et al. (1996)

Snow et al. (1999) found no fitness cost associated with the expression of a glyphosate-resistance transgene after introgressing the transgene from oilseed rape to *B. rapa*. Moreover, Ammitzboll et al. (2005) found no difference in glyphosate transgene expression at mRNA level in hybrids compared to their transgenic oilseed rape parents, which indicates this particular transgene expression was not impacted by genotypic background. Of course, this might not be the case for other transgenes. After hybridization of herbicide-resistant *Brassica napus* (oilseed rape) and *Raphanus raphanistrum* (wild radish), 18% of the progeny obtained from resistant hybrids were resistant in each advanced generation (G8-G11) in the presence of herbicide selection pressure (Al Mouemar and Darmency 2004), which suggests the introgression of transgene in wild radish populations is unstable. Ford et al. (2006) have identified one triploid F1 hybrid, nine diploid and two near triploid introgressants with flow cytometry and crop-specific microsatellite markers in *B. oleracea* wild populations where *B. napus* and *B. oleracea* grew together, which suggests newly discovered capacity for spontaneous introgression into *B. oleracea*.

These results suggest that these transgenes could be introgressed in wild populations. For example, in Canada, one introgressed individual of *Brassica rapa* was detected as displaying resistance to glyphosate six years after the last GM oilseed rape (*B. napus*) was grown on the field even in absence of the glyphosate selection pressure (Warwick et al. 2008). In United States, Whitton et al. (1997) and Linder et al. (1998) found that introgression of cultivar loci was widespread in wild sunflower (*Helianthus annuus*) populations, and the sympatric wild populations have been replaced by advanced generation hybrids. Snow et al. (2010) found that crop-specific alleles persisted 10 years in four weedy populations of hybrids between wild radish (*Raphanus raphanistrum*) and *R. sativus*, suggesting that neutral or even detrimental genes can persist rather frequently in the wild (Table 1.4).

Table 1.4. Long-term introgression of crop alleles into weed populations

References	Years existed	Sites	Types	GM	GM rate (%)	
Whitton et al. 1997	>5	Wild sunflower (<i>Helianthus annuus</i>)	Cultivar-specific R markers	APD	No	42%
Hansen et al. 2001	11	a mixed weedy population of oilseed rape and B. rapa	species-specific AFLP-markers		No	50%
Snow et al. 2001	3	four artificially established populations	white flower color, a dominant, crop-specific allele		No	8% to 22%
Snow et al. 2010	10	hybrids populations of radish	crop-specific alleles of hybrids between <i>R. raphanistrum</i> and <i>R. sativus</i>		No	---

1.5 Consequences of introgression

Introgression after hybridization between GM crops and their wild relatives may result in increasing: (1) genetic variation that promotes adaptive evolution (Rattenbury 1962; Postma and Noordwijk 2005), (2) weediness of wild species (Darmency 1994; Pilson and Prendeville 2004), (3) competitiveness or colonization abilities through hybrid vigor or new traits (Ellstrand et al. 1999; Ramachandran et al. 2000), (4) invasiveness via enhanced fitness (Vacher et al. 2004), and (5) the ability to match new environment (Campbell et al. 2006; Whitney et al. 2006). While transgenes introgressed from GM crops to wild relatives, the effects of novel genes on receiving wild plants would be detected at several levels, including possible genetic assimilation of morphologic traits, and population dynamics.

1.5.1 Consequences of interspecific introgression on the morphological traits

As the introgression alters the genetic structure of receiving individuals, their forms and behavior types, such as plant size, leaf size, petal color, fruit/ seed size, seed dormancy, etc., might be altered. There could be a directly relationship to transgenes (pleiotropy) or indirectly via the successive evolution of advanced generations (for instance by gene hitch-hiking or dramatic abundance of hybrids protected by herbicide and insect resistance). Crops are often sexually compatible with their wild relatives and yet exhibit obviously different life history traits, thus crop-to-wild gene flow and introgression may serve as a model system to evaluate the rapid evolution of life history and other fitness-related traits due to the hybridization between crops and wild relatives. Indeed, in some studies, morphology is used as one of techniques to measure introgression (Table 1.5, Jarvis and Hodgkin 1999).

Table 1.5. Morphology was used to measure introgression in documented cases of natural hybridization and introgression of crops and wild relatives (One part of Table 1 in Jarvis and Hodgkin 1999)

Crop	References	Technique(s) used to measure introgression
Cabbages, mustards, rapes (<i>Brassic spp.</i>)	Jorgensen & Andersen (1994)	Morphology, cytology, isozymes, RAPDs
	Perrino & Hammer (1985)	Morphology
	Snogerup <i>et al.</i> (1990)	Morphology
	Stace (1991)	Morphology
	Worede (1986)	Morphology
Radish (<i>Raphanus sativus L.</i>)	Hammer & Perrino (1995)	Morphology
	Klinger <i>et al.</i> (1992)	Morphology, isozymes
	Klinger & Ellstrand (1994)	Morphology

The evolution of morphology traits in a population depends at least in part on introgression and natural selection. For example, petal color of wild radish, a recessive single-locus trait (Stanton *et al.* 1989), could be as an indirect evidence for crop-to-wild gene flow (Lee and Snow 1998; Snow *et al.* 2001). Wild radish (*Raphanus raphanistrum*) in California had yellow petal color, whereas cultivated radish (*R. sativus*) and their hybrids had white to pink petals (Lee and Snow 1998; Kercher and Conner 1996). Butterflies (*Pieris rapae*) and syrphid flies prefer visit yellow flowers (Stanton *et al.* 1989; Lee and Snow 1998), while bumble bees show a slight preference for white and pink colors (Lee and Snow 1998). Hence, pollinators could contribute to the loss of white petal allele when bumble bees were infrequent at fields, although Snow *et al.* (2010) found this allele have existed 10 year in artificial populations. In addition, Campbell *et al.* (2009) found that the white petal is linked to late flowering, a heritable trait. While selection acts against late-flowering plants that failed to set fruit or never bolted, this linkage also could lose the white petal allele. Lehtilä and Holmén Bränn (2007) found that the lines with larger flowers started to flower later and produced

more flowers and larger plant size than the lines with small flowers. Therefore, if white color petal allele is lost during the process of introgression when pollinators are selecting for yellow petals, larger flower and larger plant will be infrequent in a population. Based on this, if transgenes related with white petal are transferred from the crop to the wild plant, the risk of transgene introgression is low in fields where pollinators select for yellow petals, and inversely, it is high.

Thus, in turn, introgression of transgenes from crops to wild relatives also could be influenced by morphological traits. Seed size is another example. Gene flow and introgression might be hampered by small seed size of hybrids between crops and wild relatives (Wei and Darmency 2008) because certain studies indicated that interspecific hybrids are present only among seeds with a diameter less than 1.6 mm (eg. Eber et al. 1994; Baranger et al. 1995; Chèvre et al. 2000). For example, most crosses between oilseed rape (*Brassica napus*) and wild relatives only produced small seeds, such as *B. juncea* (Bing et al., 1996), *B. rapa* (Jørgensen and Andersen, 1994), *Hirschfeldia incana* and *R. raphanistrum* (Eber et al., 1994; Chadoeuf et al., 1998). Wei and Darmency (2008) employed male sterile *B. napus* and five wild relatives to obtain hybrids and found all seeds of four hybrids were small. Small seeds performed disadvantage because seed mass affects the emergence and initial seedling size (Aparicio et al. 2002; Westoby et al., 2002). This could decrease the plant fitness of small-seeded plants (Gardner and van derlip 1989; Verdu and Traveset, 2005), especially under particular hazard conditions (such as high density, shade, drought or herbivory).

1.5.2 Consequences of interspecific introgression on population dynamics

Gene flow and introgression plays a crucial role in the evolution of natural population (Postma and Noordwijk 2005; Lenormand 2002), because gene flow could counteract the negative effects of genetic drift, inbreeding (Ebert et al. 2002), genetic differentiation (Postma and Noordwijk 2005), and local selection (Lenormand 2002), on genetic variation and thus on the evolution of local adaptations. Over short time-scales, invading hybrid populations may experience strong selection (Sakai et al., 2001; Allendorf & Lundquist, 2003). The evolutionary trajectory of weed populations may persistently be altered by the long-term introgression of crop alleles into weed populations, such as with sunflower (Whitton et al., 1997), oilseed rape (Hansen et al., 2001) and radish (Snow et al., 2001). The interaction

between natural selection and hybridization may significantly alter the population dynamics of the introgressed taxa, and therein alter patterns of invasion and establishment (Lenormand 2002).

In general, crop-wild hybrid populations are expected to be more diverse than the non-hybridized parental taxa. Subsequently, hybridized populations would differ from their parental taxa or more similar to them under natural selection acts, and this process also depends on whether phenotypes created via their genetic variation are beneficial or deleterious to fitness. If the phenotype is advantageous, hybridization is expected to result in long-term introgression. However, studies on whether genotypes of hybridized weeds tend to converge on or diverge from those of their weedy ancestors, and at what rate, are scant (Hegde et al., 2006; Whitney et al., 2006). Gene flow and introgression plays a key role in maintaining cohesion among geographically separated populations in some species, such as wild radish (Kercher and Conner 1996), maize (Ross-Ibarra et al. 2009), and rice (Zheng and Ge 2010). Divergence could occur when natural selection is sufficiently strong to overcome gene flow or when gene flow is limited (Ross-Ibarra et al. 2009; Zheng and Ge 2010). Alternatively, gene flow and introgression from crops might result in no correlation between genetic differences and geographic distance of crops (Tokunaga and Ohnishi 1992; Kercher and Conner 1996).

Haygood et al. (2003) found that genetic assimilation involving thresholds and hysteresis through performing mathematical models of a wild population recurrently receiving pollen from an introgressed crop, and that a small increase in immigration can lead to fixation of a disfavoured crop gene and even drastic shrinkage of the wild population because demographic swamping could give rise to 'migrational meltdown'. Several studies have found that hybrids might replace their parents after crop gene introgression into wild relatives (Linder et al. 1998; Compell et al. 2006), and even resulted in the extinction of their progenitors (Hegde et al. 2006). Campbell et al. (2006) showed that hybrid radish population after crop gene introgression had greater fecundity and survival than wild populations. Hedge et al. (2006) found that hybrid derived descendants replaced the two local *Raphanus* species using morphological and allozyme evidences in California, and that California wild radish separated from both of its parents as a new evolutionary entity, suggesting the aggressive colonizing behavior of the hybrids probably not resulting from genetic variability of the population per se but from the combination of parental traits.

Moreover, beside the introgression of transgenes affecting the population dynamics of receiving plants, it also could impact the target or non-target predator populations. For example, a negative correlation was found between moth densities of *Helicoverpa armigera* and the planting years of *Bt*-cotton in both high and low *Bt* density areas, which indicates the reduction of moth population density of *H. armigera* with the introduction of *Bt* cotton (Gao et al. 2010). The variation of targeted insects will in turn affect the dynamics of transgenic and non-transgenic plants because insect pressure affects the plant relative fitness and competitive capability (Ramachandran et al. 2000; Moon et al. 2007). Thus, the interaction between transgenic and non-transgenic plants and enemy-insects is also an important factor that affects the consequences of introgressions from transgenic crops to wild relatives.

1.5.3 An applied example focusing on the insect-resistant and susceptible plants in plant biology and ecology

Herbivory could lead to some phenotypic effects (e. g. chemical and morphological defense), because plants respond to herbivory after damaging through producing chemicals, like glucosinolates (Agrawal et al. 1999), or changes in secondary chemistry (Baldwin 1994, Karban and Baldwin 1997), result in the variation of morphologic characteristics, such as trichome density and number (Baur et al. 1991; Agrawal 1999), leaf toughness (Kudo 1996), flower attributes (such as petal length and width) (Cresswell et al. 2001). For example, damaged wild radish produced 55% higher concentrations of glucosinolates compared to controls with the herbivore, *Pieris rapae* (Agrawal et al. 2002), and increased density and total number of setose trichomes on newly formed leaves of previously damaged plants (Agrawal 1999). Cresswell et al. (2001) found that the petal length and width and long stamen length was shorter in defoliated *B. napus* than those in controls. In addition, Lehtilä and Strauss (1997) showed that leaf damage could reduce the attractiveness to pollinators because of the reduction in the number and size of flowers of wild radish during the first week observation. Based on this, under herbivory, insect-susceptible wild plants might perform this response, whereas insect-resistant transgenic plants would not be induced to respond. Hence, this might lead to a divergence in morphological traits of hybrids compared to their parents in the presence of herbivory.

In general, herbivory had a negative effect on plant fitness (Escarre et al. 1999; Rogers and Siemann 2002), but certain studies showed no effects because of the compensatory growth (Strauss and Agrawal 1999; Gadd et al. 2001; Hawkes and Sullivan 2001; Boalt and Lehtila 2007). In addition, as herbivore attack following artificial leaf damage decreased with increasing initial leaf damage, plants induced by early-season herbivory had higher relative fitness than un-induced plants (Agrawal 1998; 1999). Thus, the effect of herbivory on plant growth and reproduction is uncommon, and it depends on lots of factors such as plant species (Rogers and Siemann 2002), resource level (Hawkes and Sullivan 2001; Rogers and Siemann 2002), damage time (Agrawal 1998; 1999), and herbivores (Schooler et al. 2006).

A crop-developed *Bacillus thuringiensis* (Bt) transgene is one of the most important transgenes employed in GM crops, because the insecticidal proteins produced by Bt are toxic to major pests of many of the world's most important crops such as cotton, rice, corn, oilseed rape, and sunflower. The gene flow and introgression from Bt crops to wild relatives is likely to result in a decrease in herbivore damage, therein hybrids produced greater plant fitness (survivor, growth and fecundity) compared with an equivalent wild population that is not protected by Bt based resistance trait (Stewart et al. 1996; Ramachandran et al. 2000; Snow et al. 2003; Vacher et al. 2004). Moreover, no fitness cost of expressing Bt-transgene was detected in the Brassicaceae (Mason et al. 2003; Moon et al. 2007) and in sunflower (Snow et al. 2003), although Vacher et al. (2004) found a lower seed output in Bt hybrids than wild plants in the absence of herbivores.

However, the plant fitness consequences of insect-resistant Bt-transgene on wild plants are herbivore dependent, and most studies showed a fitness increase when plants were protected from Bt-susceptible herbivores. Stewart et al. (1996) demonstrated a significant increase in biomass and seed yield in transgenic oilseed rape exposed to diamondback moth larvae compared to nontransgenic plants in field trials. Snow et al. (2003) found 55% more seed set on male-sterile, transgenic Bt sunflower than for a non-transgenic controls. Moon et al. (2007) showed significantly higher biomass and seed yield in greenhouse conditions with herbivore additions, using transgenic Bt wild *B. rapa* and *B. rapa* × *B. napus* hybrids (with and without the Bt transgene). However, insect-resistant trait did not result in a fitness increase in the absence of or presence of low herbivory (Ramachandran et al. 2000; Moon et al. 2007). Sutherland et al. (2006) found that simulated herbivory (only cotyledons were mechanically damaged) did not have a plant fitness effect for *B. napus* × *B. rapa* hybrids and

their parental plants. Moon et al. (2007) showed that there was no effect of Bt-transgene in field trials with low ambient herbivory levels. Taken together, these studies suggest that *Bt*-transgene produces an increased plant fitness consequence under moderate to high herbivore damage, but not for low or no herbivory pressure.

As the fitness advantage of insect-resistant (*Bt*-transgene) is detected, especially under high herbivory, the resistant plants are expected to suppress the growth of susceptible ones in mixed populations. Accordingly, the competition interaction between resistant and susceptible plants will determine in part the population dynamics. Ramachandran et al. (2000) found the insect-resistant transgenic plants were superior competitors in mixed stands with susceptible individuals. However, the dynamic of mixed populations including resistant and susceptible plants might be a complex process, because it depends on certain factors, such as intensity of herbivory, resource level and relative abundance of resistant plants (Verkaar 1987). As the invasion and colonization of resistant plants (transgenic hybrids or crops), the competition between the two classes (resistant vs. susceptible) decreased, but the competition among resistant plants (intra-class competition) increased. Therefore, the fate of wild relatives depends at least in part on the competition interaction between resistant and susceptible plants. The population will be either in equilibrium where resistant and susceptible plants coexist in a stable proportion or susceptible plants being replaced by resistant plants that boost the population demography. These data gaps face us, although it is crucial to understand the ecological and evolutionary consequences in the process of introgression from crops to wild relative populations.

Chapter 2

Conditions d'implantation des hybrides interspécifiques

CHAPTER 2

CONDITIONS D'IMPLANTATION DES HYBRIDES INTERSPECIFIQUES

2.1 Introduction

La plupart des travaux sur les flux de gènes entre une plante cultivée et un apparenté sauvage s'est concentrée sur l'introgession d'un ou de quelques gènes, leur effet sur la fitness et leur impact sur la biologie de l'espèce sauvage. Les conditions de l'implantation de s hybrides interspécifiques ont reçu moins d'attention.

En particulier, des études antérieures ayant mis en évidence que la plupart des hybrides interspécifiques chez les Brassicées étaient produit sous formes de petites semences (Eber et al. 1994; Chadoeuf et al. 1998; Weiland Darmency 2008), et puisque les petites semences donnent des plantes généralement désavantagées dans leur implantation juvénile dans la nature (Aparicio *et al.* 2002; Westoby *et al.* 2002), le flux de gènes pourrait être limité dès le début par cette corrélation. Dans la première partie de ce chapitre, nous nous demandons si les plantes issues de petites semences chez *B. juncea* ont effectivement une fitness réduite et si les hybrides transgéniques ou non entre *B. juncea* et le colza, *B. napus*, montrent les mêmes effets (**Article 1**).

La floraison des hybrides dans les champs cultivés dans ou à proximité du colza peut ensuite conduire à des rétrocroisements, non pas avec l'espèce sauvage (le seul cas étudié jusqu'à maintenant), mais avec la plante cultivée. Ceci peut conduire à l'introgession de caractéristiques sauvages dans les populations de repousses ou spontanées (ou « férales »). Le phénomène est généralement sous-estimé et peu travaillé car le type cultivé et les formes s'y r approchant sont considérés comme mal adaptés aux habitats sauvages (Crawley et al. 1993). Les champs et les terrains incultes représentent cependant un habitat approprié où la diversité nouvelle de ces plantes rétrocroisées peut leur donner à la fois des avantages d'adventices et de potentialités d'adaptation que n'ont pas les populations de repousses. La deuxième partie de ce chapitre aborde cette possibilité à la suite du croisement entre le colza, *B. napus*, et la moutarde brune *B. juncea* (**Article 2**).

2.2 Article 1: Les effets de la taille des semences sur la fitness des plantes et son rôle sur le flux de transgènes entre le colza (*Brassica napus*) et la moutarde brune sauvage (*B. juncea*)

Des hybrides transgéniques F₁ (trF1) ont été obtenus entre la moutarde sauvage (*Brassica juncea*) et un colza transgénique « Bt » (*B. napus*, cv. Westar) ainsi que des hybrides F₁ non transgéniques (ntrF1) avec le parent conventionnel (cv. Westar). Dans le but de détecter si la taille de la semence hybride représentait un obstacle dans le flux de gènes, les semences des deux hybrides F₁ et de leurs parents ont été séparées en trois catégories : grosse, moyenne et petite. Les trois catégories de moutarde ont été semées à trois densités à Beijing afin de tester l'interaction avec l'intensité de compétition. Dans un autre essai, les trois catégories de chacun des quatre types de plante, moutarde, colza Bt, trF1 et ntrF1, ont été semées en mélange à deux densités. Date de floraison, nombre et poids des semences et biomasse ont été mesurés pour chaque plante.

La densité des plantes a significativement affecté la biomasse et la production de semences. La taille des semences a aussi eu une influence sur la croissance et la reproduction, mais avec des variations selon le type de plante, la densité et le type de voisinage. Les plantes de colza Bt issues de petites semences ont produit moins de biomasse et de semences que celles issues de grosses semences. Elles ont seulement fleuri plus tard pour la moutarde et ntrF1, sans autre différence avec celles issues des grosses semences. Les plantes de petites semences et grosses semences de trF1 n'ont pas de différence pour toutes les issues.

Ces résultats montrent que le flux de gènes ne devrait pas être entravé par la production de petites semences hybrides du fait d'une fitness amoindrie. Au contraire, les petites semences sont plus facilement triées par les moissonneuses et éjectées sur le sol que les grosses semences (de type colza) lors de la récolte au champ. Elles sont ensuite enfouies dans le sol ou facilement dispersées à la surface et représentent un risque majeur d'infestation comme repousse les années suivantes.

ARTICLE 1

The effect of seed size on plant fitness with implication for transgene flow between oilseed rape (*Brassica napus*) and wild brown mustard (*Brassica juncea*)

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Abstract

Recent work has advanced the hypothesis that gene flow could be hampered by small seed size of hybrids formed between crop cultivars and their wild relatives as small seeds may be disadvantaged in their early establishment to plants in nature. Herein we report plant fitness of three seed-size categories in hybrids formed between transgenic oilseed rape (*Brassica napus*) and wild *B. juncea* as well as in their parents. Plant density is considered as an important factor. Plant density and seed size significantly affected plant growth and reproduction. The influence of seed size on plant fitness varied among genetic backgrounds and was affected by plant density and competing neighbors. Small-seeded transgenic oilseed rape plants had lower fitness than larger-seeded plants. Plants from small-sized seeds had delayed flowering but did not have different biomass and seed output in non-transgenic F₁ and wild *B. juncea*, and small-seeded plants had the same fitness as large-seeded ones in transgenic F₁. These results imply that further gene flow could not be reduced by the production of small-sized seeds in transgenic hybrids in field. Indeed, considering competition factors and also that small seeds can persist in soil and/or disperse more easily compared to larger seeds, biosafety and gene flow could actually be more problematic in small-seeded transgenic hybrids.

Key words: commercial release, competition, gene flow, plant density, seed size, transgenic crops

Introduction

One potential risk that is associated with commercial release of transgenic crops, most notably canola and cotton, is transgene flow between crops and their wild relatives (Pilson & Prendeville 2004). Ellstrand *et al.* (1999) reviewed that 12 out of the world's 13 most important crops could hybridize with wild relatives, suggesting transgenes could escape via spontaneous hybridization with and introgression to wild relatives, even though this full list has not been realized (Stewart *et al.* 2003). The escape of transgenes into wild populations could enhance the weediness or the invasion of new habitats, since the transgenes provide adaptive traits such as resistance to pests, herbicides, diseases and various environmental stresses (Darmency 1994). Volunteers and hybrids were observed in agroecosystems together with sown crops and in their neighborhoods (Knispel *et al.* 2008; Warwick *et al.* 2008), and one of the causes was the occurrence of gene flow via pollen dispersal (Hall *et al.* 2000).

Generally, gene flow can occur mainly via pollen and seed. Although more attention has been paid to the potential of pollen dispersal, the intentional movement of seed during harvesting, transportation and commerce may be of greater importance for the long-distance dispersal of transgenes. Seed loss can occur because of the pods shattering before- and/or post-harvest operation. The smaller the seed, the greater the risk of gene flow is through dispersal mechanism by wind, animals or human actions. Actually, most hybrids between oilseed rape (OSR, *Brassica napus*) and wild relatives produce a plethora of small seeds (Eber *et al.* 1994; Chadoeuf *et al.* 1998; Wei & Darmency 2008). Some studies showed that interspecific hybrids are present only among seeds with a diameter less than 1.6 mm (Eber *et al.* 1994; Baranger *et al.* 1995; Chèvre *et al.* 2000). For example, most crosses between OSR and wild relatives such as *B. juncea* (Bing *et al.* 1996), *B. rapa* (Jørgensen & Andersen 1994), *Hirschfeldia incana* and *Raphanus raphanistrum* (Eber *et al.* 1994; Chadoeuf *et al.* 1998) only produced small seeds. Wei and Darmency (2008) found all hybrid seeds formed between a male sterile OSR cultivar and four wild species were small. It was suggested that small seeds would result in a fitness disadvantage since seed mass would affect the emergence and initial seedling size (Aparicio *et al.* 2002; Westoby *et al.* 2002). Subsequent decrease of plant fitness of small-seeded plants might be exacerbated under stressful conditions such as high density, shade, drought or herbivory (Gardner & Vanderlip 1989; Verdu & Traveset 2005). Thus, the hypothesis was proposed that gene flow might be hampered by small seed size of hybrids between transgenic crops and wild relatives (Wei & Darmency 2008). In that paper

plant growth was evaluated without taking into consideration the competition among plants of different seed sizes. The effect of seed size was only observed in the field at early establishment under direct-sowing of seeds.

Transgenic OSR and wild *B. juncea* and their hybrids were employed in this study to test different performance between small-seeded hybrids and larger-seeded ones under competition with each other. OSR is an annual crop species, preferentially self-compatible, but outcrossing ranges from 12 to 47% (Becker *et al.* 1992). Both insect and wind-pollination occurs and plants have the potential to establish outside cultivation (Timmons *et al.* 1995). Besides the dispersal of abundant pollen (Lavigne *et al.* 1998), pods shattering before, or spilling during, harvest encourage seeds to enter into the seed bank. Secondary dormancy results in the potential survival in seed banks for years (Gulden *et al.* 2003). These seeds potentially give rise to volunteers in the subsequent years that are either a source or a recipient of transgene introgression events, facilitating transgene escape (Pivard *et al.* 2008). *B. juncea*, an allotetraploid wild relative species, is frequently found as a weed and a ruderal component of roadsides and waste places in China. Hybrids and subsequent backcross generations between *B. napus* and wild *B. juncea* were successfully obtained by open pollination (Frello *et al.* 1995; Bing *et al.* 1996; Song *et al.* 2007; Liu *et al.* 2010). The relatively high compatibility between *B. napus* and wild *B. juncea* raises the biosafety concern of transgene introgression.

In this study, we focus on components of plant fitness of different seed size categories in the agro-ecosystem and their interaction with transgene introgression. The work was conducted in condition of plant competition at different plant densities.

Materials and methods

Plants

Wild brown mustard (*Brassica juncea* var. *gracilis*, $2n = 36$, AABB), transgenic oilseed rape (*B. napus* cv. Westar, $2n = 38$, AACC) and the non-transgenic OSR parent (Westar) were grown. Seeds of wild brown mustard originated from a local field collection, provided by Nanjing Agricultural University, China. *Brassica napus* cv. 'Westar', a spring type OSR, was transformed with the pSAM12 plasmid containing genetically linked GFP and Bt Cry1Ac cassettes regulated by independent CaMV 35S promoters (Halfhill *et al.* 2001). The third generation of the progenies of transgenic event 1 (GT) was used in this study (Di *et al.* 2009).

Transgenic F₁ hybrids (trF₁) were formed between wild mustard (as female) and transgenic OSR (GT). No n-transgenic F₁ hybrids (ntrF₁) were obtained by crossing wild mustard (as female) with the non-transgenic parent (Westar).

The seeds of GT were sieved into three categories by seed diameter: larger than 1.6 mm (L), smaller than 1.2 mm (S), and medium-sized between 1.2 and 1.6 mm (M). These categories were the same as those used in previous studies on the hybridization of *B. napus* with five wild relatives (Wei & Darmency 2008). The three seed categories of mustard were: larger than 1.2mm, smaller than 1.0mm and between 1.2-1.0 mm. Because of the seeds of trF₁ and ntrF₁ were smaller than 1.2 mm, they were separated by 1.0 and 0.9 mm.

Monoculture experiment

The three seed categories of mustard were directly sown in the field on March 19, 2008. The experimental design consisted in three blocks, each having three plots with a different plant density: 400 plants /m² (high), 100 plants /m² (medium) and 25 plants /m² (low), i.e. the distance between sowing positions was arranged in three levels: 5 cm, 10 cm and 20 cm, respectively. Each plot had 60 sowing positions in 5 rows and 12 columns. Twenty positions per plot were taken at random for one seed category and sowed with five seeds each position. Emerged seedlings were counted, and then randomly thinned to one plant per position.

Multi-culture experiment

In order to study the interspecific competition, the four types of plants, *B. juncea* (mustard), transgenic *B. napus* (GT), transgenic F₁ (trF₁), and non-transgenic F₁ (ntrF₁), were co-cultured together. Seeds of the three categories were directly sown in the field on March 31, 2008. There were three blocks in this experiment and each block contained two plots of plant density: 100 plants /m² and 25 plants /m², i.e. the distance between plants at these two densities was 10 cm and 20 cm, respectively. Five seeds of every plant type per seed category combination were sown at 5 positions in each plot, with 60 positions in 10 rows and 12 columns per plot. Emerged seedlings were recorded, and then randomly thinned to one plant per position as in the monoculture trial above.

Measurements

These two experiments were kept weed-free by hand weeding, and every plant was labeled. Field management of the plants was performed identically to normal OSR cultivation. Open pollination was permitted during the flowering period.

The number of flowers was recorded in the monoculture trial. Flowering date, seed number, seed weight and total dried aboveground biomass were measured at harvest for every plant in the two experiments. Thousand seeds weight ($1000 \times \text{seed weight} / \text{seed number}$) was calculated. At harvest, the seeds of each plant type were sieved into three categories by seed diameter as described above. The number and weight of every seed category were recorded. The percentage of seed number for each seed category per plant was calculated. The seed production per unit biomass ($\text{Seed number} / \text{biomass}$) was calculated as reproductive allocation.

Statistic analysis

A four-way fixed split-plot ANOVA model ($Y \sim D * C + B + E$) for monoculture experiment including plant density as the main plot (D), seed category (C), block (B), and edge effect (E), and a five-way fixed split-plot ANOVA model ($Y \sim D * C * T + B + E$) was employed (T, plant type). All data were log transformed for the normality of the residuals. In the monoculture experiment, as edge effect was significant, the plants in the plot border were omitted. In the multi-culture experiment, all plants in the plot border and center were included because the absence of significant edge effect. Similarly, some non-significant interaction effects were included in the residual. The resulting four and three-way ANOVA, respectively, were used to compare the differences among the four plant types as tested for emergence rate, days to flowering, biomass, seed number and weight, reproductive allocation, thousand seeds weight and the descriptors of the three seed categories produced, using data of all seed-sized plants in both high and low densities. Tukey's honestly significant difference (Tukey's HSD) test was employed for multiple comparison. All statistic analysis was conducted in R software (<http://www.r-project.org/>).

Results

Monoculture experiment

Effects of plant density Plant density significantly affected the performances of the plants: increased density decreased number of flowers, biomass, seed number and weight and percentage of small seeds, and increased thousand seeds weight and the percentage of large and medium seeds (Table 2.1, Fig. 2.1). Emergence rate, days to flowering and reproductive allocation were not affected by plant density (Table 2.1).

Performances of plants from different seed categories Seed category significantly affected the days to flowering, number of flowers, thousand-seed weight and percentage of medium and small seeds (Table 2.1, Fig. 2.1). There were no differences among different seed categories for emergence rate, biomass, seed number, reproductive allocation, seed weight and percentage of large seeds (Table 1, Fig. 2.1). Large-seeded plants flowered earlier than small-seeded plants at all the three densities (Fig. 2.1). Small-seeded plants produced less flowers at low density and more small seeds at low and medium density than large-seeded plants, but there was no difference at high density. Thousand seeds weight in small-seeded plants was lower than that in large-seeded plants only at low density (Fig. 2.1).

There was no interaction between density and seed category effects for all the fitness components in this experiment (Table 2.1).

Multi-culture experiment

When considering all the conditions, plant density significantly affected the emergence rate, days to flowering, biomass, seed number and weight, and percentage of large and medium seeds, and did not affect reproductive allocation, thousand-seed weight and percentage of small seeds (Table 2.2). Seed category significantly affected the days to flowering, biomass, seed number and weight, thousand seeds weight and percentage of small seeds, and it did not affect the emergence rate, reproductive allocation and percentage of large and medium seeds (Table 2.2).

There were no interactions between density and seed category for all fitness components except of the reproductive allocation (Table 2.2). Days to flowering, seed weight, thousand-seed weight and percentage of large seeds had interaction between density and plant type. Days to flowering, seed number, reproductive allocation and thousand seeds weight had interaction between seed category and plant type, but it could be due to different categorization of the different plant types (i.e. seed size criteria were different). No

interactions were found among the three factors, plant density, seed category and plant type, for all measured fitness components (Table 2.2).

Effect of plant density on performances of plants from different seed categories For *trF₁*, increased density only delayed flowering of medium-seeded plants ($F_{1,41}=5.7$, $P=0.022$) and increased thousand seeds weight of small-seeded plants ($F_{1,35}=6.1$, $P=0.018$) (Fig. 2.2). Flowering date ($F_{1,39}=5.9$, $P=0.02$ for large-seeded plants; $F_{1,38}=5.2$, $P=0.028$ for medium; $F_{1,34}=10.5$, $P=0.002$ for small) and biomass ($F_{1,40}=15.01$, $P=0.003$ for large-seeded plants; $F_{1,38}=4.3$, $P=0.045$ for medium; $F_{1,37}=7.2$, $P=0.01$ for small) in *nrF₁* were significantly affected by plant density for all three seed categories. Seed number of small-seeded plants in *nrF₁* at low density was higher than at high density ($F_{1,37}=7.9$, $P=0.007$), but there were no differences between plant densities for large- and medium-seeded plants in *nrF₁*. Thousand-seed weight was not affected for all seed categories in *nrF₁* (Fig. 2.2).

Large-, medium- and small-seeded plants of mustard produced higher biomass and more seeds ($P<0.05$) in low density than in high density. Plant density did not affect the flowering date of large- and small-seeded plants but affected medium-seeded plants ($F_{1,40}=7.7$, $P=0.049$). Thousand-seed weight in all seed categories of mustard in low density was not different with in high density ($P>0.05$, Fig. 2.2).

Days to flowering, biomass, seed number and thousand-seed number of large- and medium-seeded plants of GT was not affected by plant density ($P>0.05$). Increased plant density delayed flowering time ($F_{1,36}=25.1$, $P=0.000$), decreased biomass ($F_{1,36}=6.7$, $P=0.014$) and seed number ($F_{1,36}=15.9$, $P=0.000$) for small-seeded plants of GT (Fig. 2.2).

Performances of different seed categories Small-seeded plants of *trF₁* revealed no difference in fitness components compared to medium- and large-seeded plants in both high and low density populations (Fig. 2.2). The three seed categories of *nrF₁* produced no different seed number and biomass at both high and low density levels, but small-seeded plants delayed flowering at high density and produced lower thousand-seed weight than large-seeded plants at low density (Fig. 2.2). Small-seeded plants of wild mustard produced no different biomass, seed number and thousand-seed weight compared with the other two seed categories, and they flowered later at both high and low density (Fig. 2.2).

The three seed categories of GT performed differently at the two densities (Fig. 2.2). At high density, small-seeded plants flowering later and produced less biomass and seeds than

large-seeded plants did, and there was no difference for thousand-seed weight (Fig. 2.2). At low density, small-seeded plants of GT produced lower biomass and thousand-seed weight than large-seeded plants, but lowering time and seed number was not different. The percentage of large, medium and small seeds produced was not different among the three seed categories for all four plant types at both high and low density.

Fitness comparison of four plant types All fitness components, except of the emergence rate, were significantly different in the four plant types: trF₁, ntrF₁, mustard and GT (Tables 2.2, 2.3). Wild mustard flowered the earliest, trF₁ and GT the latest, and ntrF₁ was intermediate. Transgenic F₁ and ntrF₁ produced higher biomass, lower seed number and weight and reproductive allocation than mustard and GT, with lowest biomass and highest seed number and allocation in mustard and highest seed weight in GT (Table 2.3). Thousand-seed weight of trF₁ and ntrF₁ was higher than that of mustard but lower than that of GT. The percentage of large seeds, medium seeds and small seeds produced by the four plant types was significantly different, and trF₁ and ntrF₁ produced 77% and 72% large seeds, 54% for GT and 1% for wild mustard (Table 2.3).

At small seed size level, the seed number and seed weight of small-seeded plants of trF₁ were not significantly different compared with their parents, mustard and GT, in high density population, and they were lower than their parents at low density level.

Discussion

Growth and reproduction of various plant types

Fitness of hybrids between transgenic crops and wild relatives influences the ecological consequences of transgene flow from GM crops to their wild relatives (Ellstrand *et al.* 1999; Pilson & Prendeville 2004; Vacher *et al.* 2004). Several fitness components of two interspecific hybrids (trF₁ and ntrF₁) and the parent plants--transgenic oilseed rape (GT) and *B. juncea* (mustard), are described at all growth stages from seedling emergence to seed production in the current study. The hybrids showed higher vegetative fitness but lower reproductive fitness than their parents, which is consistent with the results of Di *et al.* (2009). There are some cases in which wild-crop hybrids may be as fit (Lefol *et al.* 1995; Arriola & Ellstrand 1997), less fit (Hauser *et al.* 1998; Gueritain *et al.* 2002; Allainguillaume *et al.*

2006) or more fit than their parents (Klinger & Ellstrand 1994; Arnold & Hodges 1995; Linder & Schmitt 1995). Thus, the correlation in plant fitness between interspecific hybrids and their parents should be studied on a case-by-case basis (Arnold & Hodges 1995).

Influences of seed size on plant fitness

The flowering time and number of flowers of wild mustard was affected by seed category in the monoculture experiment of *B. juncea*, but the final seed set was not influenced by seed size. It is expected that small-seeded plants have lower plant fitness than large-seeded plants (Ahmed & Zuberi 1973; Westoby *et al.* 2002; Moles & Westoby 2004), because it is generally accepted that the most obvious influence of seed mass is on the seedling emergence and development; smaller seeds contain less energy stores for germination and early seedling growth (Major 1977; Shanmuganathan and Benjamin LR, 1992; Aparico *et al.* 2002; Wei & Darmency 2008). As the strong relationship between seed size and morphological traits of the seed and the embryo considered (Aparico *et al.* 2002; Diggle *et al.*, 2010), seed size obviously affects the initial size of the seedling and the provisions available during early seedling life. For instance, in *R. raphanistrum*, larger seeds generally produce larger plants with more flowers than do smaller seeds (Stanton 1985, Choe *et al.* 1988). However, after the early seedling stage, plant growth and development rates are similar regardless of seed size, and the principal energy sources become photosynthates from emerged leaves (Choe *et al.* 1988; Peterson *et al.* 1989; Aparico *et al.* 2002). These studies have suggested that the disadvantage of small seeds vary with genetic background and environmental stresses such as limited resources, high plant density, or herbivory.

Influences of seed size on plant fitness affected by plant density

The influence of seed size on plant fitness is affected by population density and the competition of neighboring plants for limited resources. Wei and Darmency (2008) found that seed size effect was observed in a direct sowing experiment but not in a transplant experiment. They suggested that the homogeneous environment in greenhouse, previous to the transplantation in the field, had complemented the disadvantage of small seed at early growth stage. In the monoculture trial, small-seeded plants of mustard produced less flowers and more small seeds than large-seeded plants at low density, but that was not the case at high density. In the multi-culture experiment, small-seeded ntrF1, mustard, and GT plants had delayed flowering compared with large-seeded plants at high density, but this effect was

apparent only with mustard at low density. Similarly, small seed size decreased the seed number of GT at high density but not at low density. Plant density could cause variation of the influences of seed size on plant growth and reproduction (Gardner & Vanderlip 1989). Obviously, the strong competition at high density decreased plant fitness, although the effect varied through different plant types and seed categories. GT was the plant type more liable to be affected by plant density and seed category, less for ntrF₁ and mustard, while trF₁ was not affected.

Influences of seed size on plant fitness varied with genetic background

Seed size did not impact the growth and reproduction of trF₁, and it also did not affect the biomass and seed output in ntrF₁ and wild mustard, with the exception of flowering time. However, seed size significantly affected the fitness of GT. The small-seeded plants of transgenic F₁ did not show lower fitness compared to larger-seeded plants of transgenic F₁. This is perhaps due to the date of the measurements, which were taken at maturity. Since hybrids produce few seeds, their growth can surpass that of mustard and OSR, with high biomass and high per-seed weight, thus hindering any initial difference at the early stage of life. This is not the case for mustard and OSR that produced copious amounts of seed and thus ending the vegetative stage as compared with hybrids.

There were no significant differences between trF₁ and ntrF₁ in seed set, which indicates costs associated with the transgene were probably negligible here. Fitness cost of transgene expression was observed to be associated with herbivore resistance in some reports (Bergelson & Purrington 1996; Strauss *et al.* 2002). Fitness costs depend on not only genetic background in which resistance traits occur, but also on the environment in which the comparisons between resistant and susceptible plants are made and on the ecological context in which costs are measured (Bergelson & Purrington 1996). In this study, transgenic F₁- and non-transgenic F₁- plants were obtained with the same genetic background. Mason *et al.* (2003) found no fitness cost of *Bt*-transgene in transgenic *B. napus* under low and high insect pressure. Snow *et al.* (2003) also found that *Bt* transgene was not associated with a fitness cost in sunflower under insect pressure, and transgenic plants produced more seeds since they experienced reduced herbivory. Moreover, small-seeded plants of transgenic F₁ plants had high vegetative performance, which suggests *Bt* resistance plant might exhibit stronger competitive capacity and would counterbalance the disadvantage of small seed size, since

they bear beneficial transgenes that might provide adaptive advantages (Darmency 1994). Ramachandran *et al.* (2000) found that transgenic plants were more competitive under insect pressure. Vacher *et al.* (2004) showed higher fitness of *Bt* transgenic hybrids than wild plants under insect pressure. In addition, both F₁ hybrids produced the highest percentage of large seeds, although the difference was not drastic, which could make the second generation with more chance of establishment than the small-seeded F₁ generation.

Implications for gene flow between transgenic *B. napus* and wild *B. juncea*

Seed size could affect the emergence and initial seedlings (Major 1977; Aparico *et al.* 2002), which further enhance plant fitness (Verdu & Traveset 2005). GT showed significantly seed size effects, and small-seeded plants demonstrated lower fitness than large-seeded plants. However, small-seeded of transgenic F₁ did not show plant fitness difference compared to larger-seeded plants of transgenic F₁. Small seeds are more easily sieved out by harvesters and fall onto the soil, and further buried in the seed bank of soil. The complex correlation between seed size and persistence in the soil is unclear. However, interspecific hybrid seeds survived as well as seeds of oilseed rape in the soil (Chadoeuf *et al.* 1998). In addition, the small seed dispersed more easily through wind and animals, such that the survival of small-sized seeds of weeds could be higher in conventional tillage systems and arable habitats (Ghersa & Martinez-Ghersa, 2000). They might represent the main risk of transgene escape in the field, and their small size is not a counterbalancing force exposing them to higher competition from the neighborhood.

Acknowledgements

This work is supported by two projects of the Natural Science Foundation of China (grant no. 30970432 and 30670316) and enabled by a USDA Biotechnology Risk Assessment Grant. This work was also supported by a PhD joint fellowship between China and France (CNOUS, No. 20072315).

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Table 2.1. F-values from a four-way fixed split-plot ANOVA on the fitness of mustard in the monoculture experiment.

	Density (D)	Seed category (C)	Block	D*C
Emergence rate	0.11 ^{ns}	0.48 ^{ns}	1.68 ^{ns}	2.33 ^{ns}
Days to flowering	1.59 ^{ns}	35.57 ^{***}	1.09 ^{ns}	1.44 ^{ns}
No. of flowers	10.87 ^{***}	8.75 ^{***}	1.10 ^{ns}	1.13 ^{ns}
Biomass	85.28 ^{***}	2.36 ^{ns}	2.57 ^{ns}	1.95 ^{ns}
Seed number	69.59 ^{***}	1.77 ^{ns}	0.98 ^{ns}	1.16 ^{ns}
Reproductive allocation	1.30 ^{ns}	0.98 ^{ns}	0.12 ^{ns}	0.90 ^{ns}
Seed weight	52.03 ^{***}	2.97 ^{ns}	6.30 ^{**}	1.89 ^{ns}
Thousand seeds weight	5.29 ^{**}	9.47 ^{***}	0.42 ^{ns}	0.23 ^{ns}
% of large seeds	3.15 [*]	0.53 ^{ns}	0.37 ^{ns}	0.66 ^{ns}
% of medium seeds	25.89 ^{***}	12.52 ^{***}	0.61 ^{ns}	1.30 ^{ns}
% of small seeds	13.48 ^{***}	16.84 ^{***}	0.52 ^{ns}	0.68 ^{ns}

*, p<0.05; **P<0.01; ***, P<0.001, ns, not significant, P>0.05.

Table 2.2. F-values from a five-way fixed split-plot ANOVA on the fitness of trF1, ntrF1, mustard and GT in the multi-culture experiment.

	Density (D)	Seed category (C)	Plant type (P)	Block	D*C	D*P	C*P
Emergence rate	4.69*	2.65 ^{ns}	2.49 ^{ns}	1.42 ^{ns}	0.33 ^{ns}	1.09 ^{ns}	1.08 ^{ns}
Days to flowering	8.78**	6.80**	241.02***	0.71 ^{ns}	0.93 ^{ns}	6.53***	2.72*
Biomass	27.98***	13.67***	35.52***	1.40 ^{ns}	0.4 ^{ns}	0.51 ^{ns}	2.06 ^{ns}
Seed number	10.41***	4.99**	32.69***	0.20 ^{ns}	2.82 ^{ns}	1.3 ^{ns}	2.78*
Reproductive allocation	2.45 ^{ns}	0.67 ^{ns}	197.83***	0.34 ^{ns}	3.94*	1.9 ^{ns}	2.88**
Seed weight	98.37***	6.87**	99.19***	3.07 ^{ns}	0.14 ^{ns}	3.78*	1.8 ^{ns}
Thousand-seed weight	0.07 ^{ns}	13.98***	283.22***	0.25 ^{ns}	1.79 ^{ns}	7.68***	2.70*
% of large seeds	19.29***	0.39 ^{ns}	32.58***	0.05 ^{ns}	1.02 ^{ns}	6.42***	0.28 ^{ns}
% of m edium seeds	19.84***	2.53 ^{ns}	6.45***	12.05**	1.16 ^{ns}	1.28 ^{ns}	0.68 ^{ns}
% of small seeds	0.01 ^{ns}	3.34*	118.12***	0.21 ^{ns}	0.30 ^{ns}	3.57 ^{ns}	1.94 ^{ns}

*, p<0.05; **P<0.01; ***, P<0.001, ns, not significant, P>0.05.

Table 2.3. Mean (SE) of plant characteristics for four plant types in multi-culture trial as analyzed by one-way ANOVA and Tukey's HSD

	trF ₁	ntrF ₁	mustard	GT
Emergence rate	0.70 (0.03) ^a	0.71 (0.03) ^a	0.63 (0.03) ^a	0.49 (0.03) ^a
Days to flowering	60 (0.30) ^a	58 (0.29) ^b	52(0.29) ^c	61(0.32) ^a
Biomass	22.19 (1.66) ^a	19.29 (1.46) ^a	5.52 (0.51) ^c	12.54 (1.04) ^b
Seed number	340 (35) ^b	231(20) ^b	1799(185) ^a	1901(181) ^a
Reproductive allocation	13.13 (1.28) ^c	14.65 (2.81) ^c	427.62 (93.8) ^a	128.51 (6.21) ^b
Seed weight	0.38 (0.05) ^c	0.22 (0.02) ^d	1.73 (0.20) ^b	4.29 (0.39) ^a
Thousand-seed weight	1.03 (0.03) ^b	0.96 (0.02) ^b	0.84 (0.02) ^c	2.22 (0.06) ^a
% of large seeds	0.77 (0.01) ^a	0.72 (0.02) ^{ab}	0.01 (0.00) ^c	0.54 (0.03) ^b
% of medium seeds	0.09 (0.01) ^b	0.09 (0.01) ^b	0.10 (0.01) ^b	0.32 (0.02) ^a
% of small seeds	0.14 (0.01) ^b	0.19 (0.02) ^b	0.89 (0.01) ^a	0.14 (0.02) ^c

trF₁: transgenic hybrids formed between transgenic oilseed rape (GT) and *Brassica juncea* (mustard); ntrF₁: non-transgenic hybrids formed between non-transgenic oilseed rape 'Westar' and *B. juncea* (mustard); mustard: wild mustard (*B. juncea*); GT: transgenic oilseed rape. Different letters within a column indicate significantly different value at p<0.05.

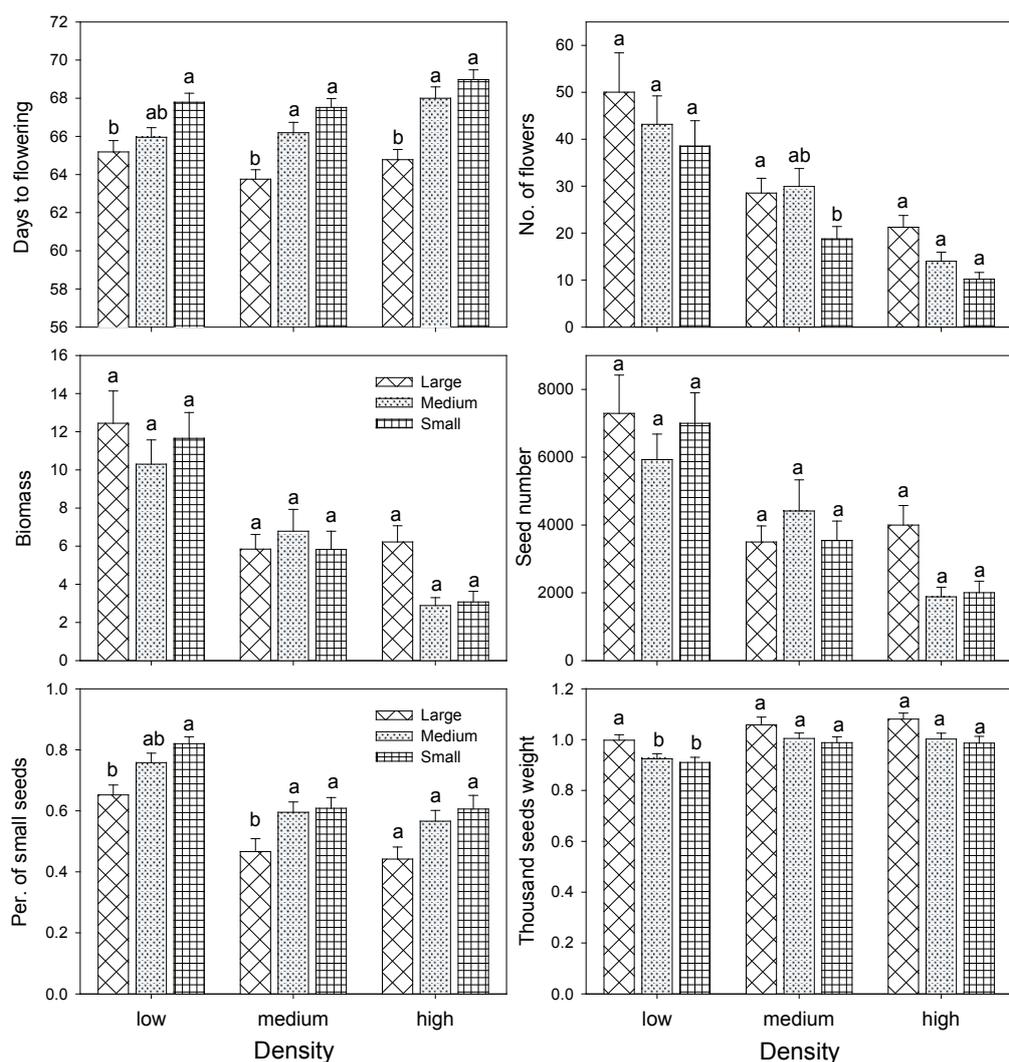


Fig. 2.1. ANOVA and Tukey's HSD test results of comparing the mean values of plant characteristics among three seed categories of wild *B. juncea* at three plant densities in monoculture trial. Each group bars represent three seed categories (large, medium and small, from left to right) for a given plant density. Different letters within a density level indicate significantly different values from Tukey's HSD test at $p < 0.05$.

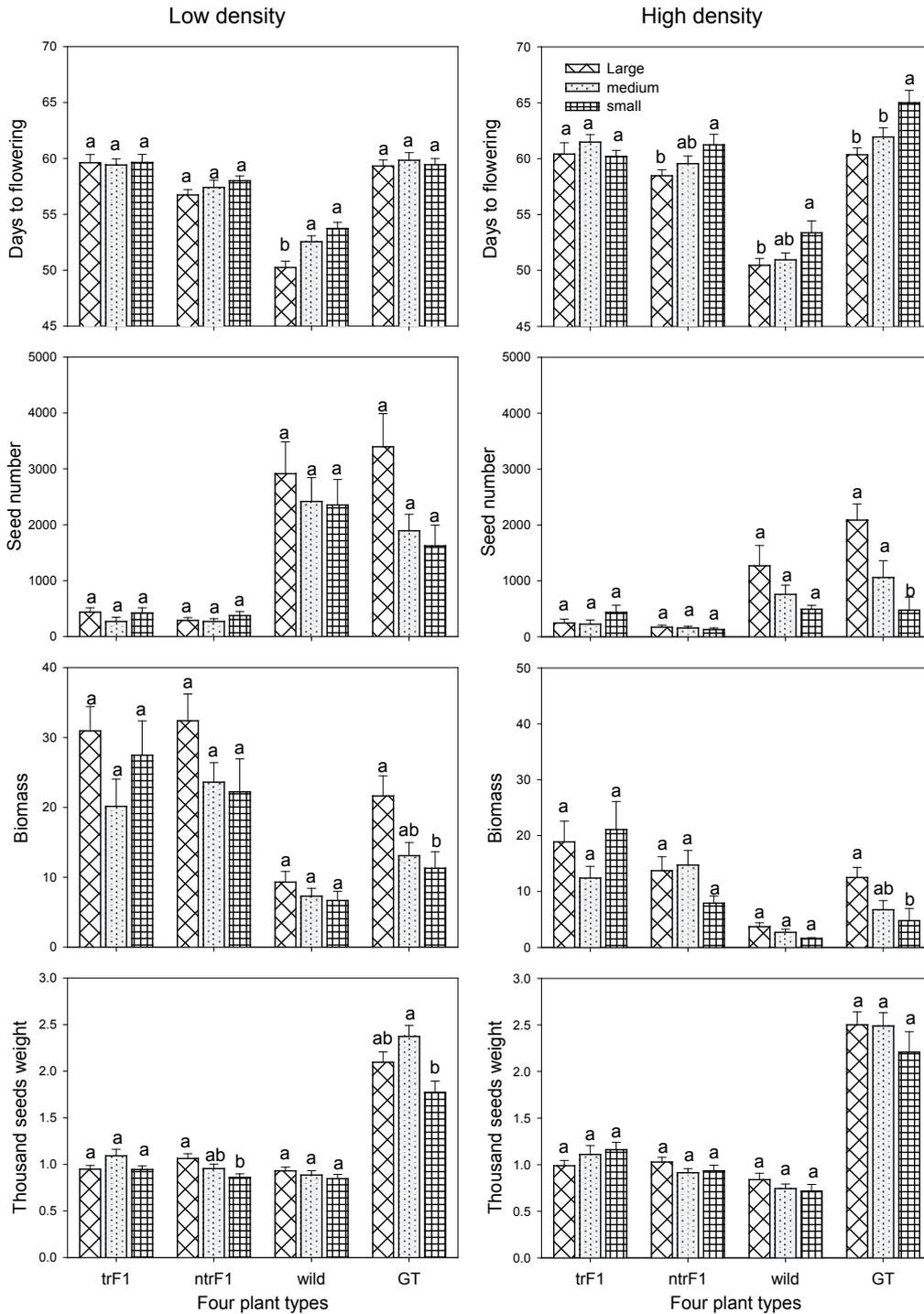


Fig. 2.2. ANOVA and Tukey's HSD test results of comparing the mean values of plant characteristics of four plant types, transgenic F1 (trF1), non-transgenic F1 (ntrF1), wild *B. juncea* (wild) and transgenic *B. napus* (GT), among three seed categories at low density (left column) and high density (right column) in multi-culture trial. Each group bars represent three seed categories, large, medium and small seeds, ranking from left to right. Different letters within a group bars (plant type level) indicate significantly different values from Tukey's HSD test at $p < 0.05$.

2.3 Article 2: Les rétrocroisements sur *Brassica napus* des hybrides entre *B. juncea* et *B. napus* comme source de nouvelles populations résistantes aux herbicides ressemblant aux repousses de colza

Dans le but de déterminer la voie préférentielle de transmission du génome des hybrides entre *B. juncea* et *B. napus*, des F₁ entre la moutarde et un colza cv. Brutor présentant un gène dominant de résistance au chlorsulfuron ont été pollinisés en rétrocroisement par le pollen des deux parents ou encore laissés en groupe pour produire des F₂. Parents, F₂ et BC₁ ont été plantés ensemble au champ à Dijon et caractérisés pour leur aspect et leur type de fleur, leur contenu en ADN (par cytométrie), leur fertilité pollinique (Alexander) et leur résistance à l'herbicide. Leur biomasse et leur production de semences ont été mesurées ainsi que leur proportion de descendants résistants à l'herbicide.

Nos résultats montrent que le rétrocroisement avec le pollen de *B. napus* a produit le plus de semences qui étaient plus grosses et plus viables que celui avec le pollen de *B. juncea*. Le contenu en ADN des BC₁ suivait une distribution Normale et l'hérédité du gène de résistance correspondait au rapport Mendélien attendu. Les BC₁ résistants avaient une plus forte biomasse et produisaient plus de semences que leurs équivalents sensibles et que les F₂, et ils avaient un aspect plus vigoureux.

Ces résultats permettent de penser que les BC₁ résistants produits à partir du pollen de *B. napus*, qui est abondant dans les champs, sont plus facile à produire et plus compétitifs que les autres BC₁ et les F₂ pour s'établir comme une fausse population de repousses dans les champs et les bordures ou les milieux incultes. L'implantation de ce type de descendance, proche de la morphologie du colza, est facilitée par une large variabilité génétique qui peut permettre une adaptation rapide aux contraintes du milieu, et elle serait plus rapide qu'une introgression véritable dans l'espèce sauvage. Elle est aussi favorisée par la résistance à l'herbicide dont elle représente une source secondaire de diffusion vers les moutardes sauvages et les colza non-transgéniques, ce qui pourrait être source de conflit dans le cadre des problèmes de co-existence entre cultures GM et non GM.

Cet article a été accepté dans la revue *Plant Science* sous le DOI *Plant Science*, doi:10.1016/j.plantsci.2010.07.005.

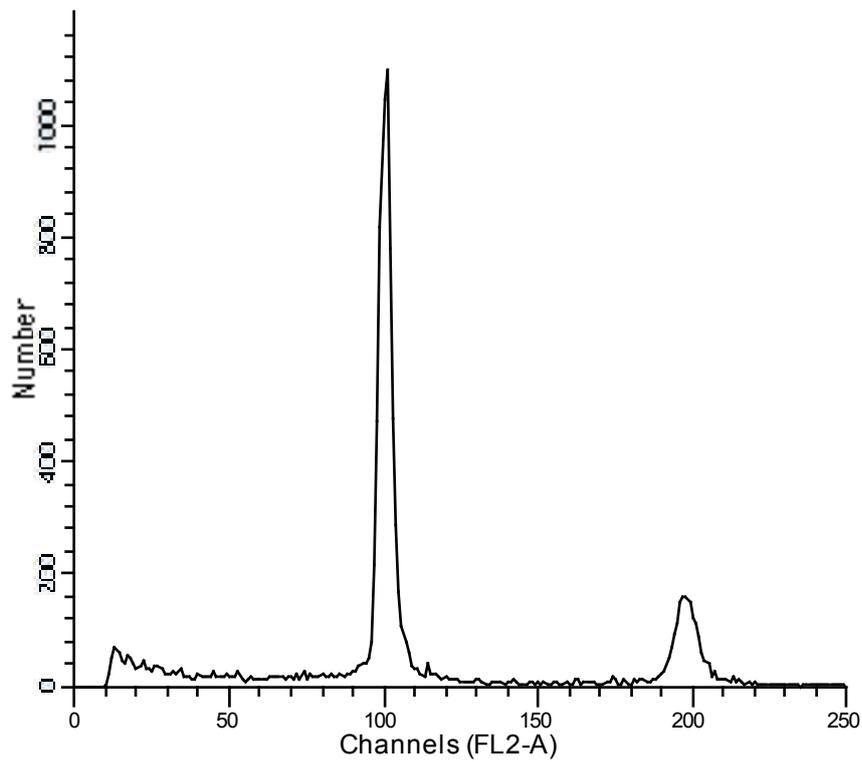


Photo.2.1. One example of flow cytometry photography on BC1 formed between *Brassica juncea* and *B. napus*

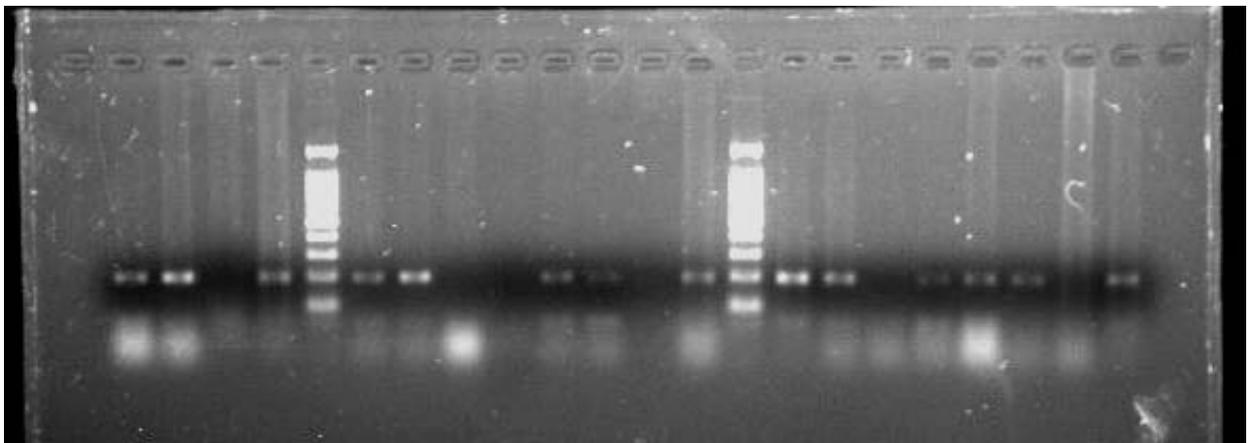


Photo. 2.2. PCR results of BC1 and F2 with 83B1 molecular marker (C-genome)



Photo.2.3. Spraying chlorsulfuron herbicide on seedlings of B. C1, F 2 and *B. napus* in greenhouse to check herbicide-resistance

ARTICLE 2

BACKCROSSES TO *BRASSICA NAPUS* OF HYBRIDS BETWEEN *B. JUNCEA* AND *B. NAPUS* AS A SOURCE OF NEW HERBICIDE-RESISTANT VOLUNTEER-LIKE FERAL POPULATIONS

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Plant Science (2010), 179: 459-465

Abstract

Introgression between genetically modified (GM) crops and wild relatives is considered to potentially modify the genetic background of the wild species. The emergence of volunteer-like feral populations through backcross of hybrids to the crop is also a concern. The progeny of spontaneous hybrids between mutant herbicide-resistant oilseed rape (*Brassica napus*) and wild *B. juncea* were obtained. Parents, F₂ and BC₁ to *B. napus* were planted together in the field so as to study their performance. The chromosome number of BC₁ followed a Normal distribution. Mendelian ratio of the herbicide-resistance gene was found. Herbicide-resistant BC₁ was associated with higher plant weight, seed weight and seed number than their susceptible counterparts. Most of them exhibited *B. napus* morphology and larger flowers. Pollen flow to susceptible plants within the mixed stand was observed. As a consequence, the resistant BC₁ produced with *B. napus* pollen could frequently occur and easily establish as a false feral crop population within fields and along roadsides. Their morphology was similar to *B. napus* volunteers but displayed additional genetic variability allowing further adaptation and propagation of the herbicide-resistance gene.

Keywords: feral crop population, mutant herbicide-resistant, introgression, pollen flow, seed production, oilseed rape

Introduction

In the framework of current research on the risk assessment of the commercial release of genetically modified (GM) crops, one of main concerns is whether transgenes could persist in the agro-ecosystem [1]. In the case of oilseed rape (*Brassica napus*), transgenes could escape through volunteer plants descended from seeds shattered before or spilled during harvest on previous years and that can survive for years into the soil seed bank [2] and affect both the habitat [3] and the quality of crop harvest [4]. They could also escape via spontaneous hybridization with and introgression to wild relatives [5, 6]. Volunteers and hybrids were observed in the agro-ecosystem field together with sown crops and in their neighborhoods [7, 8].

The introgression between a genetically modified crop and a wild relative is generally thought to potentially modify the genetic background of the wild species, which thus generates either concerns about its weediness and competitiveness in the agro-ecosystem or the risk of its extinction [6, 9, 10]. The backcrossing of hybrids with the crop parents could also occur, which could result in the introgression of wild characteristics into feral and volunteer populations. This process is generally underestimated and little investigated because the crop type is considered to be unfit to adapt to wild habitats [11]. However, the resulting plants are thought to be too close to volunteers and to deserve separate studies.

Gene flow between *B. napus* and *B. juncea* has received less attention compared to the *B. napus* / *B. rapa* case. *B. juncea*, a wild relative frequently found as a weed and a ruderal component of roadsides and waste places in China, is a tetraploid species like *B. napus* and preferentially self-pollinated with considerable outcrossing rate. Hybrids and subsequent backcross generations between *B. napus* and wild *B. juncea* were successfully obtained by open pollination [12-14]. The relatively high compatibility between *B. napus* and wild *B. juncea* raises the biosafety concern of transgene introgression. Di et al. [15] found that the hybrids formed between *B. napus* and wild *B. juncea* revealed maternal effects, high vegetative fitness and seed dormancy, which could increase the probability of the survival of hybrids after the occurrence of gene flow. However, Wei and Darmency [16] showed that gene flow could be hampered by low seed size of hybrids because of the low emergence, low survival rate and delayed flowering due to smaller seeds. Therefore, it is not clear under which form the progeny of interspecific hybrids between these two species could survive and

then transfer transgenes to further generations in the realistic agro-ecosystem where uncropped and cropped areas coexist.

In this study, we aim to investigate the behavior of the progenies of spontaneous hybrids between wild *B. juncea* and a herbicide-resistant germplasm of *B. napus* similar to those commercialized cultivars under the Clearfield® technology. We studied the formation of F₂ and BC₁ progenies, whether they may grow and reproduce under field conditions, and whether their performances are changed according to the presence of the herbicide-resistance gene. Instead of exploring the possibility of transgene introgression into the wild species, which has been already explored [17], this work focused on the likelihood of the emergence of backcrossed plants that could behave like *B. napus* volunteer-like plants.

Materials and methods

Hybrids

A wild accession of *Brassica juncea* (2n=36, AABB) from Turkey was obtained from the INRA laboratory collection of weeds (ref. X04-021) in Dijon, France. A mutant *B. napus* cv. Brutor, (2n=38, AACC), bearing a single-gene dominant resistance to chlorsulfuron, a sulfonylurea herbicide inhibiting the AcetoLactate Synthase (ALS), was provided by M. Renard [18]. These two species were grown in mixture in insect-proof cages continuously provided with houseflies, *Musca domestica*, in order to facilitate random pollination. The seeds of *B. juncea* were sown in a greenhouse at 22°C under natural light, and then two-leaved seedlings were sprayed with 2.5 g a.i. ha⁻¹ chlorsulfuron in 300 L of water (Glean, Du Pont de Nemours, France). Eleven out of 8,238 seedlings survived (0.13%), and these plants were confirmed to be interspecific hybrids since they showed molecular markers of both parents (not shown).

Progeny of hybrids

Branches of all the hybrid plants were cut and planted in the soil to multiply the hybrids. The hybrid cuttings were grown in the greenhouse and entirely covered by a pollen-proof net. Anthers of flowers of 24 cuttings from all eleven hybrids were removed and the stigmas

received pollens from wild *B. juncea* by hand to generate a BC₁ generation (BC₁J). On 22 other cuttings, emasculated flowers received pollens from a conventional herbicide-susceptible *B. napus* cv. Drakkar to obtain a BC₁ generation (BC₁N) segregating for herbicide resistance and susceptibility (although not yet identified at that stage). The rest of hybrid cuttings were kept isolated by a pollen-proof net and served to get a bulked F₂ generation (Fig. 2.3). Pollen fertility was checked by staining pollens of 5 flowers of each of 6 hybrids with Alexander solution. Number of pollinated flowers, seed number, per-seed weight and germination were recorded. Five hundred F₂ seeds, 100 BC₁N and 50 BC₁J seeds were sown in the greenhouse and sprayed with the herbicide Glean as described above, and then seedlings alive were counted.

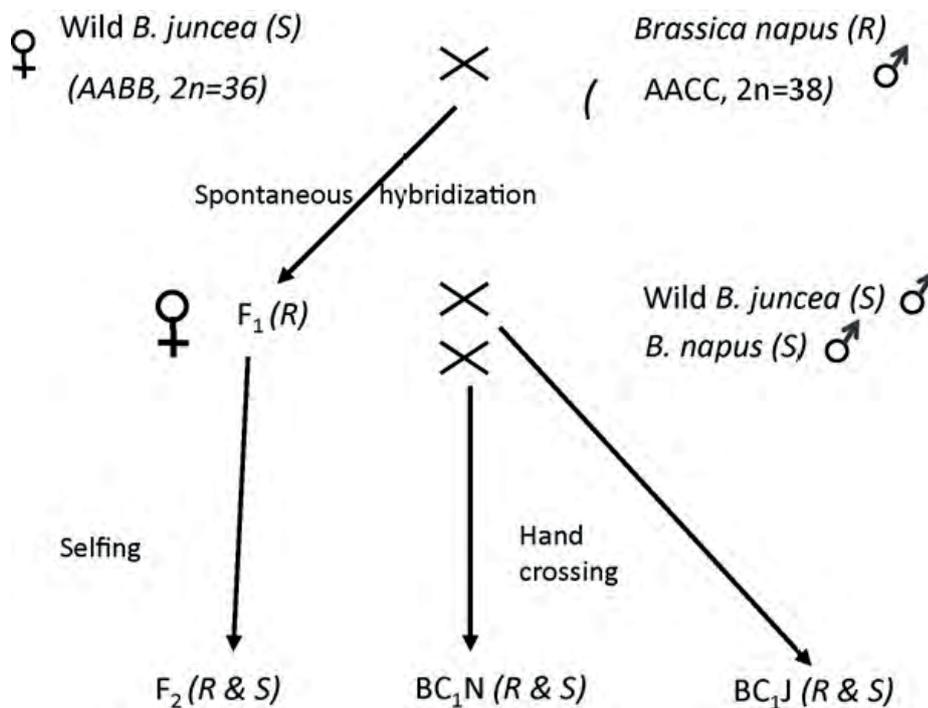


Fig. 2.3. Crossing chart indicating how the plant material was obtained and its phenotype for the herbicide-resistance (R or S)

Field trial

Seeds of conventional herbicide-susceptible *B. napus* cv. Drakkar, wild *B. juncea*, BC₁J, BC₁N, and F₂ were sown in Giffy 7 pots (Jiffy Prod. Int., Norway) in the greenhouse in order to promote simultaneous germination, and then the seedlings were transplanted into the field in Dijon on April 2007. In one experiment, *B. juncea*, BC₁J and F₂ were randomly planted in a bare field. In another place, *B. napus*, *B. juncea*, BC₁N and F₂ were planted randomly in the field with four blocks. Each block included three rows 30cm apart and 11m long, with one plant every 0.6 m. The blocks were enclosed in an insect-proof net (12 m wide /15 m large /2 m high, 2 mm mesh) to prevent insect attack, and houseflies were continuously provided in order to facilitate random pollination. Most *B. juncea* plants flowered very early in that experiment so that they were hardly comparable to the other plant types and were no more considered in the following study.

The *B. napus* versus *B. juncea* morphology (large versus small habit) was visually noted before flowering. The flower sizes of all BC₁N and F₂ plants were visually rated in two categories: as large as that of *B. napus* and small. In order to estimate the DNA content of the plants, whenever possible, a piece of fresh leaf was sampled to perform flow cytometry as described by Akinerden [19] and estimate (the G1 peak) with *B. juncea* as the control. When the first pods were about to open, the surviving plants were harvested. The vegetative parts were separated from the pods and dried in an oven for 48h at 80°C and weighted. Seed weight and seed number were measured. The weight of one thousand seeds was calculated and expressed as per-seed weight.

Up to 2000 seeds of every harvested plant were sown in the greenhouse and sprayed with chlorsulfuron as described above. In order to assign a phenotype to the BC₁N and F₂ plants, we considered that a resistant one would ideally produce 75 % of resistant offspring if entirely self-fertilized, while a susceptible one would produce no resistant seedlings. Because of possible biased segregations after the interspecific hybridization and the backcrosses on the one hand, and because of some potential cross-pollination that could have occurred in the field on the other hand, we interpreted a susceptible all the plants showing a progeny containing less than 5 % resistant seedlings, which could correspond to a maximum rate of fertilization by resistant pollen. Otherwise the plant was designated as resistant.

Statistical analysis

Mean values are given with their 95 % confidence limits. Chi-square analysis was employed to test herbicide-resistant rate of BC₁ and F₂ with the expected Mendelian segregation ratios. Analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test were carried out to compare plant weight, seed weight, seed number, per-seed weight and germination rate of the plants in the field trial. Bartlett's test was used for testing variance homogeneity and data were log-transformed to ensure a normal distribution. We followed a fixed ANOVA model including a block effect and plant type as the unique factor ($Y \sim X_p + X_b + e$ where Y, biological characteristics; X_p, plant type; X_b, block effect). Firstly, the three types, *B. napus*, BC₁N and F₂, were considered. Secondly, in order to check whether herbicide-resistance had any effect, BC₁N was separated into two phenotypes: resistant plants (BC₁NR) and susceptible plants (BC₁NS). F₂ was separated into herbicide-resistant (F₂R) and -susceptible F₂ plants (F₂S). Contrasts were used to analyze the effect of herbicide-resistance on growth and reproductive performances. Linear regression functions between seed number and biomass were constructed for the three plant types: *B. napus*, BC₁N and F₂. Chi-square analysis and Fisher's test were used to compare the difference associated with plant morphology and flower size among different phenotypes in BC₁N and F₂. Shapiro-Wilk normality test was employed to test the normal distribution of G1 peak values of flow cytometry. The statistical analysis was conducted using statistical software R.

Results

Hybrids and backcrosses production

The hybrids produced $34 \pm 3\%$ viable pollens. They produced fewer seeds per pollinated flower when backcrossed with *B. juncea* pollen than when backcrossed with *B. napus* pollen (5.9 and 12.4 %, respectively). Self-pollinated hybrids produced seeds (Table 2.4). The backcross with *B. napus* pollen produced heavier seeds, and they germinated better than the seeds produced with *B. juncea* pollen ($\text{Chi}^2 = 7.2$, significant at $p < 0.01$).

Table 2.4. Reproduction of hybrids according to the pollination mode (BC₁J and BC₁N: backcrosses to *B. juncea* and *B. napus*, respectively).

Type of progeny	No. of hybrids	No. of pollinated flowers	No. of seeds	Backcrossing rate (%)	Per-seed weight (mg)	Germination (%)
BC ₁ J	24	4159	247	5.9	3.2	30
BC ₁ N	22	3392	420	12.4	4.0	45
F ₂	32	/	238	/	3.3	39

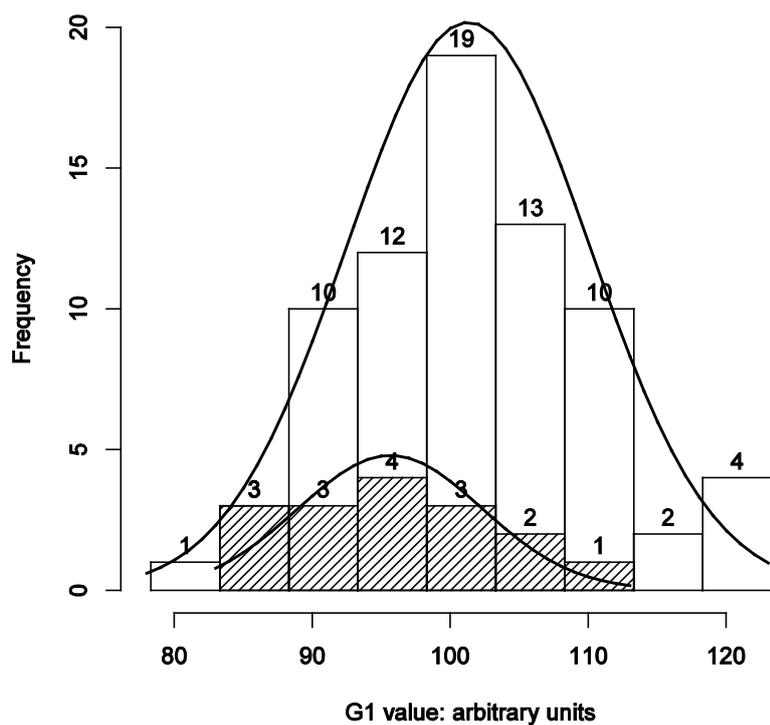


Fig. 2.4. Histogram of flow cytometry results and estimated Normal distribution curve of BC₁N (empty bars) and BC₁J (hatched bars).

Characteristics of the backcrosses

G1 peak values of flow cytometry results of BC₁N plants were Normally distributed: N (m = 101.3, σ = 8.7, p = 0.82) (Fig. 2.4). The G1 peak values of BC₁J plants also fitted a Normal law distribution, N (m = 95.6, σ = 6.7, p=0.36), but the mean value was significantly lower than that of BC₁N by 5.7 units (t = 2.9, P=0.007), very close to that of the control *B. juncea* parent (m = 93.7).

Both direct seedling treatment and phenotype inference from progeny analysis of the plants grown in the field experiments showed expected Mendelian ratios: 3: 1 resistant:susceptible in F₂, and 1: 1 for both BC₁J and BC₁N plants (Table 2.5). Some plants growing in the field and determined as susceptible produced at least one resistant descendant (Table 2.6): 13 BC₁NS individuals, later on referenced BC₁NS*, showed 0.8 ± 0.6 % resistance on average in their progeny (0.34 % for all the BC₁NS plants); the 4 F₂S plants produced 3.6 % resistant progeny; and 2 conventional herbicide-susceptible *B. napus* plants had 0.13 % resistant descendants (0.007 % for all the *B. napus* plants).

Table 2.5 Segregation of herbicide-resistant and susceptible plants in BC₁N and F₂, and Chi-square (χ^2) test against expected Mendelian segregation ratio (NS, not significant, p > 0.05)

Plant type	No. of plants	Resistant	Susceptible	Expected ratio	χ^2
Data of direct seedling treatment					
F ₂	166	120	46	3:1	0.65 ^{NS}
BC ₁ N	50	27	23	1:1	0.32 ^{NS}
BC ₁ J	16	8	8	1:1	0 ^{NS}
Data inferred from progeny analysis					
F ₂	25	21	4	3:1	1.08 ^{NS}
BC ₁ N	62	27	35	1:1	1.03 ^{NS}

Growth and reproduction in the field

The experiment with BC₁J and F₂ suffered a strong insect attack previous to the setup of the net; therefore, this trial had to be abandoned. In the experiment with BC₁N and oilseed rape, the seedling mortality and absence of flowering in the field were more frequent for the F₂ generation (10 out of 38) than for BC₁N (10 out of 72) and *B. napus* (9 out of 53). BC₁N plants weighted more than those of *B. napus*, and F₂ had intermediate weight (Table 2.7). Both BC₁N and F₂ plants showed lower seed weight, per-seed weight and seed germination percentage than *B. napus*. The F₂ produced less seeds than *B. napus*, and BC₁N had intermediate production (Table 2.7).

Table 2.6. Percentage of resistant plants in the progeny of susceptible *B. napus*, BC₁N and F₂ plants in the field experiment.

Plant type	Number of plants		Progeny		Percentage of resistant
	Total	With R progeny	Resistant	Susceptible	
BC ₁ NS	35	13 ^a	48	13925	0.34
F ₂ S	4	4	81	2254	3.6
<i>B. napus</i>	47	2	3	43574	0.007

^a identified as BC₁NS* in Fig. 2.6 and 2.7.

There were significant correlations between seed number and total aboveground biomass (plant weight plus seed weight) for the three plant types ($P < 0.001$, Fig. 2.5). The ranking of the slopes of the linear regression equations of seed number in terms of biomass, from the higher to the smaller value, was: *B. napus*, all phenotypes of BC₁N as intermediate, and then F₂. Seed number and biomass of F₂ showed greater variability than those of BC₁N, which were also more variable than those of *B. napus*, as indicated by the ranking of the coefficients of determination (R^2).

Table 2.7. Mean ($\pm 95\%$ CL) of plant weight, seed weight, seed number, per-seed weight and germination of F_2 , BC_1N and $B. napus$.

Plant type	Plant weight (g)	Total seed weight (g)	No. of seeds	Per-seed weight (mg)	Germination (%)
BC_1N	33.9 ± 7.8^a	2.10 ± 0.78^b	1084 ± 450^b	1.90 ± 0.11^b	60.2 ± 4.5^b
F_2	26.1 ± 8.6^{ab}	0.90 ± 0.35^b	438 ± 155^b	1.90 ± 0.17^b	67.9 ± 9.5^b
$B. napus$	17.0 ± 3.7^b	3.60 ± 0.87^a	1383 ± 342^a	2.60 ± 0.10^a	79.1 ± 2.5^a
$F_{2,130}$	5.41^{**}	15.81^{***}	10.57^{***}	33.53^{***}	8.86^{**}

F-values are from one-way ANOVA on individual traits (*, $P < 0.05$; **, $0.05 < P < 0.01$; $0.01 < ***P < 0.001$). Different superscript letters indicate significant differences between mean values within columns for each experiment according to Tukey's HSD ($\alpha = 0.05$).

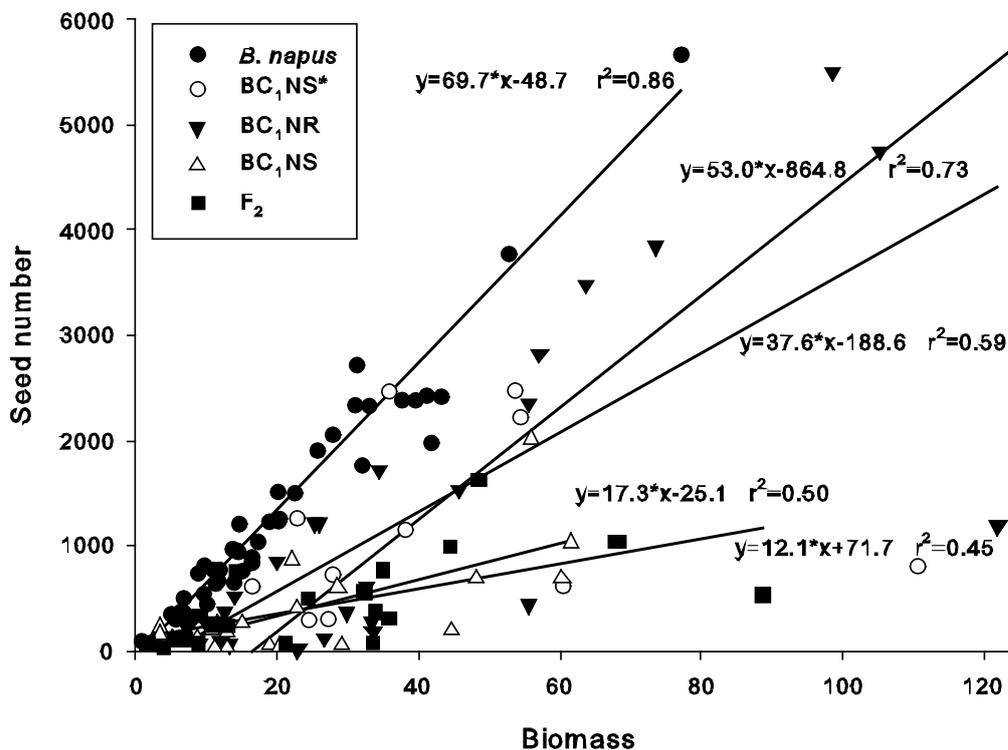


Fig.2.5. Linear regression between seed number and aboveground biomass (plant weight plus seed weight) of $B. napus$, BC_1N and F_2 .

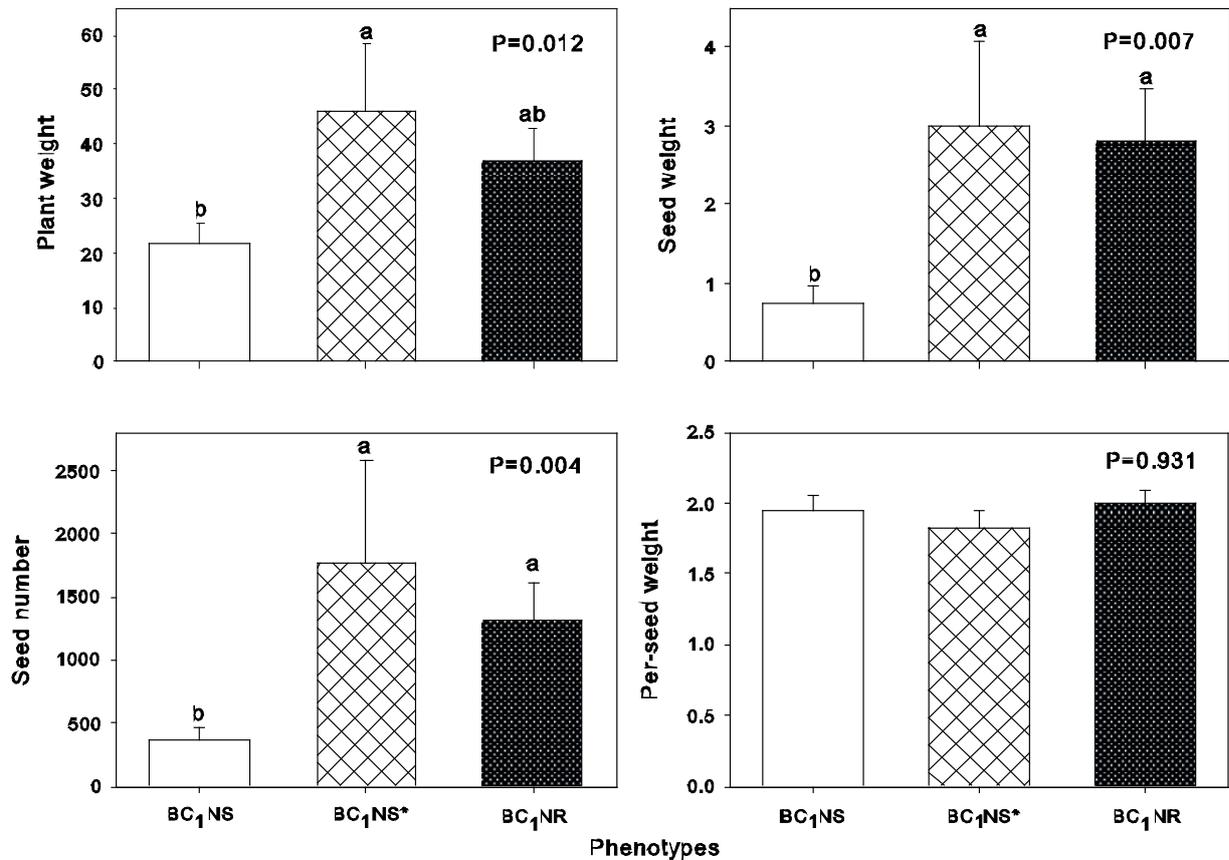


Fig.2.6. Plant weight, seed weight, seed number and per-seed weight for BC₁NR and BC₁NS in the field experiment. BC₁NS is separated into group BC₁NS that did not produce any R progeny and group BC₁NS* that produced a few R progeny (see Table 2.6). P-values are from one way ANOVA results. Vertical bars denote SE of the mean. Different letters indicate significant differences between mean values according to Tukey's HSD ($\alpha = 0.05$) and Contrast tests.

Correlation to discrete markers

BC₁NR and BC₁NS* plants produced higher plant weight, more seeds and seed weight than the other BC₁NS plants. The per-seed weight remained unchanged. There was no difference between BC₁NS* and BC₁NR plants (Fig. 2.6). The number of seeds in terms of biomass of the BC₁NS was as low as that of the F₂ plants (Fig. 2.5). Plants with *B. napus* morphology had higher plant weight, produced higher seed weight and seed number than plants with *B. juncea* morphology (data not shown). BC₁NS* and BC₁NR exhibited more

plants with *B. napus* morphology than the other BC₁NS (86 and 73% versus 39%, respectively; $\chi^2 = 31.2$, $p < 0.001$; Fig 2.7). No such a difference was found between F₂S and F₂R (Fig 2.6), but this was perhaps due to the low number of F₂S plants tested. The percentage of plant with large flowers increased significantly ($\chi^2 = 9.5$, $p = 0.027$) from BC₁NS (52%) to BC₁NS* (64%) and BC₁NR (73%). F₂R produced more large flowers than F₂S, 27 versus 0%, respectively ($\chi^2 = 31.2$, $p < 0.001$, Fig. 2.6).

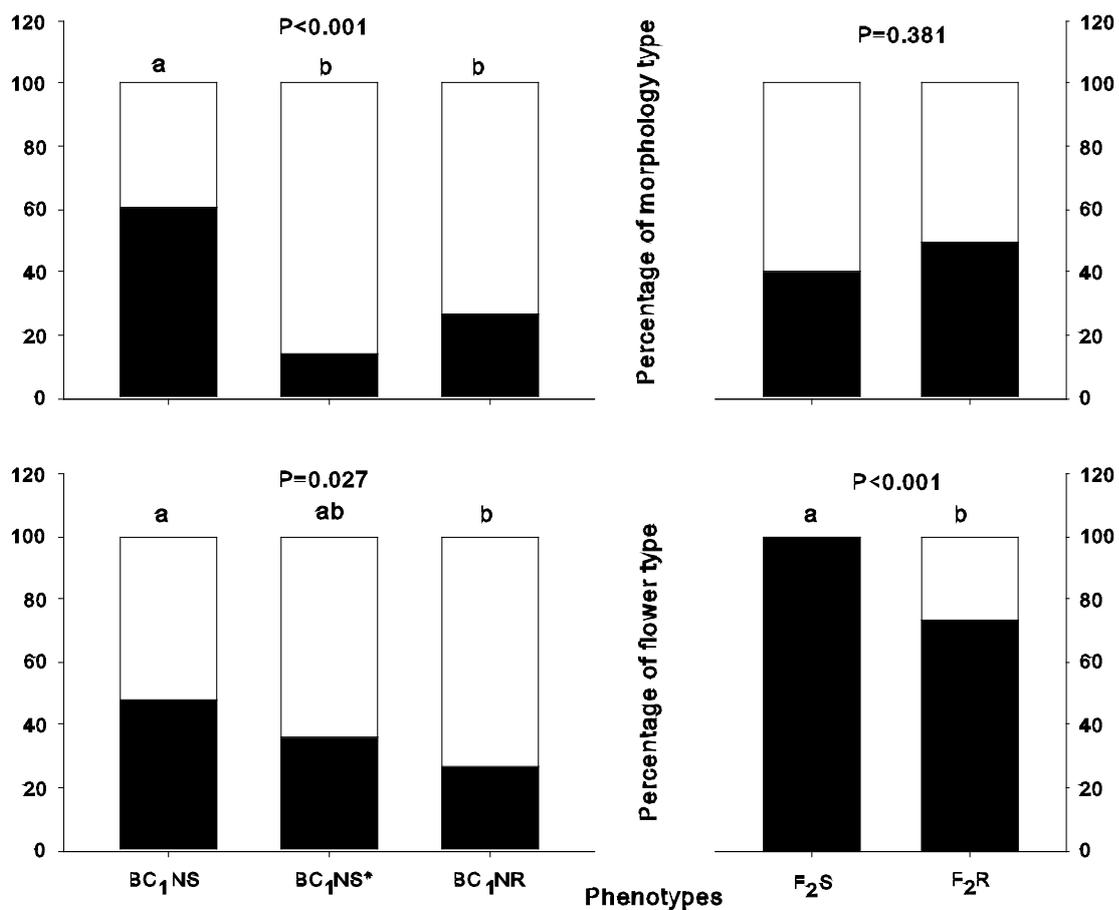


Fig. 2.7. Percentage of the two categories of morphology (top) and flower size (bottom) for resistant (R) and susceptible (S) phenotypes of F₂ (right) and BC₁N (left) in the field experiment. BC₁NS is separated into group BC₁NS that did not produce any R progeny and group BC₁NS* that produced a few R progeny (see Table 2.6). *B. juncea* morphology and small flower size are filled bars; *B. napus* morphology and large flower size are empty bars. Different letters indicate significant differences according to Fisher's test (P-values).

Discussion

Hybrids and backcrosses production

Hybrids produced 34 % viable pollens on average, a value similar to that found in other studies (24-28% in [11]; 32 -35% in [14]), thus allowing self-fertilization to produce F₂ generation, and possibly serving for outcrossing. However, most of the viable pollen in the pollen cloud in a mixed stand of *B. napus* and *B. juncea* in the fields comes from the two parent species, so that self-pollination should not be the most frequent pollination event. Hybrids F₁ receiving pollens from *B. napus* produced twice as many BC₁ seeds as those receiving *B. juncea* pollens. It is likely that pollens of *B. napus* possessed stronger fertilizing ability than *B. juncea* ones [20]. However, the rate of seed set observed here is much lower than that observed in a similar study, which indicates high variability according to genotypes and/or conditions [17].

BC₁ produced with *B. napus* (BC₁N) had larger seeds, and germinated better than the F₂ and BC₁ produced with *B. juncea* (BC₁J). These characteristics, together with a better ability to produce BC₁ with *B. napus*, could be useful in predicting a greater prevalence of these plants, especially at the places where abundant pollens of *B. napus* occur, i.e. close to oilseed rape fields. The difference of DNA content between the two types of BC₁ suggests a rapid differentiation within one generation, by around 2 chromosomes on average, but there was a large variability within each group. Cytological analysis of similar plant material indicated that B-chromosomes in the progeny of hybrids tend to be eliminated more rapidly than C-chromosomes [21], which again suggest a more rapid return to *B. napus*-like plants.

The expected Mendelian ratios obtained for the resistant phenotype show that this trait is easy to be transferred between the two species and stably inherited in subsequent generations of hybrids whichever genitor is used as female. Such a feature is not uncommon in the transmission of transgenes although they have no DNA counterpart in the recipient species [10, 12, 22], but distorted segregation was sometimes encountered in different lines [10] or for different transgenes [23]. In our study, the precise location on the A, B or C genome of the ALS gene conferring resistance gene was not known, but non-biased segregation would suggest an A-chromosome. This situation gives rise to the persistence of the herbicide resistance trait in the field because the hybrid gametes always display native or recombined A-chromosomes, which would be not the case if it were on a B or C chromosome.

Productivity of the resistant progeny

In our study, we evaluated productivity, measured as biomass and seed set parameters, without competition between co-cultivated genotypes, which could correspond to the conditions at the field border (i.e. the cultivated border line that is not sown). In most studies of transgene escape between *B. napus* and *B. rapa*, BC₁ and F₂ exhibited lower seed production than their parents [6, 9]. In our study, BC₁ and F₂ demonstrated significantly higher vegetative performance than *B. napus*, which could provide opportunity for quick establishment at the expense of volunteer and cropped oilseed rape. In contrast, BC₁ and F₂ produced less seeds than *B. napus*, and they had lower seed production efficiency per biomass unit than *B. napus*, (Fig 2.5), which could impact their reproductive success because more resources were needed to produce seeds. The difference of seed production between BC₁ and F₂ was bordering significance, because of the effect of the high variance found in BC₁, which probably denoted an effective difference. Especially, the productivity of BC₁ per biomass unit was clearly higher than that of F₂ (Fig. 2.5). Therefore, it is likely that BC₁ are able to establish in waste areas, field borders, and even arable fields, more easily and rapidly than F₂ plants. The low variability noted for *B. napus* in the regression function between seed number and biomass was of environmental nature only because the cv. Drakkar is a pure line, which suggests that the higher variability recorded in BC₁ and F₂ could be genetically determined. A similar case of higher variability of performances was also observed in *B. napus* and *B. rapa* hybrid progeny [8], which could be the source of further genetic adaptation and lead to successful volunteers or new weeds in both non-crop and crop habitats. Such a situation is highly favorable to the rapid evolution of adaptive traits to the agroecosystem [24]. In addition, half of the BC₁ exhibit the herbicide-resistance, which could help plants to survive in arable fields and roadsides if the corresponding herbicide were employed.

No difference was observed between resistant and susceptible BC₁ plants (BC₁NR versus BC₁NS + BC₁NS*) and between resistant and susceptible F₂ plants, which suggests that no detrimental effect existed for expressing the herbicide-resistance gene. Preliminary data of the comparison of the mutant line used here with its original cultivars showed that delayed flowering was responsible for 6-14% lower yield [18]. In two other cases only the fitness differential due to ALS-endowed resistance was studied with appropriate plant materials and

both showed fitness cost [25], but there are many ALS mutations endowing herbicide-resistance so that generalizations could not be made. In our experiment, the resistant BC₁ (BC₁NR) had higher frequency of *B. napus* morphology and larger flower size than susceptible BC₁ (BC₁NS). Plants with *B. napus* morphology are more robust, which could help BC₁NR plants to establish in a mixed stand. Similarly, larger flowers like oilseed rape flowers could attract more foraging insects, which is a gain a way to better disperse the *B. napus* characteristics – and the herbicide-resistance – in rural and roadside plant communities. As for the higher performances of BC₁NS* compared to BC₁NS, we suppose that the largest plants producing the highest number of flowers were more frequently visited by the houseflies carrying foreign pollen, which could result in the production of resistant seeds, in contrast to the smallest plants. This phenomenon could make the dispersal of the resistance gene more rapid again.

Finally, the presence of herbicide-resistant plants in the progeny of susceptible F₂, BC₁N and conventional *B. napus* indicated that the first generation progeny of the hybrid is capable of transmitting the herbicide-resistance gene to *B. napus* volunteers and feral populations, which provides an unexpected way to the transfer of transgenes via pollen dispersal from GM crops to non-GM crops [26]. It is also a source for further gene flow and introgression [8]. Our results show that the *B. napus*-like BC₁ plants produced with *B. napus* pollen are very likely to establish and persist in the fields and along roadsides as false feral crop population. They look like *B. napus* volunteers but display additional genetic variability that allow for further adaptation of the plants and propagate the herbicide-resistance gene. In particular, they could have inherited from the wild *B. juncea* long-term seed viability in the soil, which can make them even weedier [2]. This phenomenon should be considered in biosafety assessment of genetically modified oilseed rape with herbicide-resistance traits.

Acknowledgements

The authors thank Alain FLEURY and Zhixi TANG for their assistance in the experiments. This work was supported by a PhD joint fellowship between France (CNOUS, No. 20072315) and China (Natural Science Foundation, grant No. 30970432).

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Chapter 3

Effets de la resistance aux insectes sur la fitness

CHAPTER 3

EFFETS DE LA RESISTANCE AUX INSECTES SUR LA FITNESS

3.1 Introduction

Les gènes de résistance aux insectes développés à partir de *Bacillus thuringiensis* (Bt) et produisant une toxine représentent la famille de transgènes la plus répandue avec la résistance à l'herbicide RoundUp, ces deux types totalisant 134 millions d'hectares. Après le coton, la biotechnologie Bt a été approuvée en Chine pour le riz le 27 novembre 2009 (James, 2009). Concernant, le riz, le colza, voire le soja et des légumes, les flux de gènes entre les variétés Bt et leurs apparentés sauvages sont le sujet d'attentions particulières à cause des possibilités d'hybridation spontanée (Ellstrand et al. 1999; Armstrong et al. 2005).

L'introgession des transgènes Bt des cultures Bt dans les plantes sauvages peut conduire à une diminution de la pression de sélection des insectes herbivores sur les hybrides et leurs descendants leur conférant ainsi une fitness relative (survie, croissance et reproduction) supérieure aux individus sauvages typiques non protégé par une gène semblable (Stewart et al. 1996; Ramachandran et al. 2000; Snow et al. 2003; Vacher et al. 2004). L'avantage des plantes résistantes n'est cependant observé qu'en présence d'herbivorie, aucune pénalité de fitness n'apparaissant sans pression de sélection (Ramachandran et al. 2000; Vacher et al. 2004; Moon et al. 2007).

Si cet avantage est bien établi, la dynamique de l'invasion d'une population sauvage par le transgène et l'évolution des capacités relatives de compétition des plantes résistantes et sensibles n'a pas été souvent décrite. La démographie d'une population sauvage initialement sensible est susceptible de changer sous l'impact de la présence des plantes résistantes, et peut dépendre de l'interaction avec d'autres facteurs tels que l'intensité de l'herbivorie, le niveau de ressources du milieu et l'abondance relative des plantes résistantes.

Dans ce chapitre, nous étudierons donc l'effet sur la fitness de la résistance aux insectes chez *B. juncea*, aux niveaux individuel et de la population. Dans une expérience préliminaire en serre, nous avons défolié de différentes manières des plantes de moutarde afin de simuler différents niveaux de pression herbivore indépendamment des problèmes de fitness

liés aux hybrides interspécifiques (**Article 3**). Une expérience similaire mais au champ a ensuite été répétée deux années à Dijon afin de tester les effets de compétition intra-population ainsi que la productivité totale des populations sous différents pourcentages de plantes simulées résistantes ou sensibles (**Article 4**). Par ailleurs, nous avons effectivement testé l'effet du transgène Bt chez un colza transgénique et les hybrides transgéniques ou non en présence d'insectes dans une expérience à Beijing (**Article 5**).

3.2 Article 3 : Simulation de la résistance aux insectes dans l'étude du flux de gènes entre *Brassica napus* et *B. juncea* sauvage

Afin de simuler les performances de descendants transgéniques sauvages stabilisés, nous avons défolié des plantes de moutarde qui ont donc figuré les plantes sensibles alors que les plantes intactes figuraient les résistantes.

Différentes modalités de défoliation ont été pratiquées, surtout des feuilles entières, de une feuille sur deux à une sur quatre. Nous avons réparti ces plantes coupées (CP) et non coupées (NC) à différentes proportions dans des pots contenant 8 plantes : 0/8, 2/6, 4/4, 6/2 et 8/0. Lorsque les premières siliques menaçaient de s'ouvrir, l'expérience a été arrêtée et les parties aériennes de chaque plante ont été pesées, et le nombre de siliques et de semences comptés. Les semences ont été mises à germer en enceinte climatique (16h de jour à 25°C et 8h de nuit à 15°C) et arrosées avec une solution de gibbérelline lorsque le premier flux de germination s'est arrêté, 12 jours après semis, afin d'évaluer leur viabilité et leur dormance.

Une forte proportion de feuilles coupées a diminué les performances des plantes CP, mais l'ablation d'une feuille sur quatre n'a pas entraîné de différence avec les témoins NC. Les performances des plantes NC ont été généralement supérieures à celles de CP quand elles étaient en forte proportion (50 et 75% de NC), mais pas significativement différentes à faible proportion (25% de NC). Toutes les plantes CP et NC ont des performances décroissantes lorsque la proportion de NC augmente. Cependant, la biomasse totale par pot a augmenté avec la proportion de NC.

Ces résultats impliquent que l'intensité de l'herbivorie, ou corrélativement le niveau de résistance, et la compétition entre résistants et sensibles peuvent augmenter l'avantage adaptatif des résistants et donc augmenter le risque de fixation des transgènes s'ils venaient à envahir des populations sauvages de moutarde.

Cet article a été présenté lors du 13^{ème} Colloque International sur la Biologie des Adventices à Dijon en 2009, p144-153

ARTICLE 3

SIMULATION OF INSECT-RESISTANCE IN GENE FLOW STUDY BETWEEN *BRASSICA NAPUS* AND WILD *B. JUNCEA*

Y.B. LIU, H. DARMENCY, W. WEI

XIII^{ème} COLLOQUE INTERNATIONAL SUR LA BIOLOGIE DES MAUVAISES HERBES, DIJON. Association Française pour la Protection des Plantes, 144-153

SUMMARY:

Gene flow from transgenic crops could introduce novel traits into wild relatives, but the fate of transgenic hybrids is unknown within wild populations. We study here how an insect-resistance gene escaped from *Brassica napus* into *B. juncea* may affect plant fitness, competition and population dynamics of the wild relative. Insect herbivory was simulated by four different leaf-clipping treatments, while different ratios of clipped versus non-clipped plants provided different competition intensities. With increased proportions of clipped leaves, the intensity of simulated herbivory amplified the fitness advantage of the insect-resistant population. Competition magnified the fitness advantage of the insect-resistant plant, but as their frequency increases, neighbor competition limits their growth.

Key words: insect-resistance, plant fitness, genetically modified crops, competition

RÉSUMÉ :

SIMULATION DE LA RESISTANCE AUX INSECTES DANS UNE ETUDE DE FLUX DE GENES ENTRE *BRASSICA NAPUS* ET *B. JUNCEA* SAUVAGE

Le flux inéluctable de gènes des cultures transgéniques peut drainer de nouveaux traits chez les plantes sauvages, mais le devenir des hybrides transgéniques au sein de populations sauvages reste à préciser. Nous étudions ici comment un gène de résistance aux insectes transmis de *Brassica napus* à un *B. juncea* sauvage peut modifier la valeur adaptative, la

compétition et le devenir des populations. La résistance aux insectes et différents niveaux de compétition ont été simulés par quatre régimes d'ablation des feuilles et différentes proportions de plantes à feuilles coupées ou non. Plus l'ablation est fréquente, plus l'intensité de pression de sélection augmente et amplifie l'avantage des plantes résistantes. La compétition augmente encore l'avantage des résistants, mais lorsque leur fréquence augmente, la compétition limite leur expansion.

Mots-clés: résistance aux insectes, valeur adaptative, culture transgénique, compétition

INTRODUCTION

Genetically modified (GM) plants can hybridize spontaneously with wild or weedy relatives (Ellstrand et al. 1999), which has triggered concern about the fate of transgene escapes. Transgenes could spread and persist in stands of wild relatives through transgenic hybrids backcrossing with wild plants. Two important factors determining the likelihood of this occurrence are i) the initial invasion and persistence of the transgene in wild relatives stand and, ii) competition of transgenic hybrids with their neighbors (Hauser et al. 1998). Many transgenes inducing a commercially beneficial trait to crops, such as herbicide or insect resistance, may also bring benefits in weeds. In contrast, a fitness cost may be caused by the transgene expression in terms of resource allocation and also by interspecific hybridization. Several studies have detected fitness effects of the transgene when introduced into populations of wild relatives (Burke and Rieseberg 2003; Snow et al. 2003).

Herbivore pressure is obviously a factor affecting the fitness of weeds and wild plants. Insect-resistant hybrids may have enhanced fitness values in comparison to their wild relatives (Vacher et al. 2004; Moon et al. 2007; Letourneau and Hagen, 2009). Because of this higher fitness, resistant plants are expected to increase in populations where gene flow occurs. The rate of the frequency change would depend on the advantage provided by the transgene, but also on the growth conditions in the habitat, in particular intra-population competition. For example, plant density has recently been considered as an ecological factor interacting with fitness (Weis and Hochberg 2000; Vacher et al. 2004). More complex environments might increase the relative fitness of transgenic hybrids and backcross generations. However, we are not yet clear on how herbivore and competition pressure together affect the fitness performance of insect-resistant hybrids and wild relatives.

In this study, we report on a preliminary experiment to simulate the impacts of herbivore pressure on the fitness of stabilized hybrid progeny between transgenic *Brassica napus* and wild *B. juncea* under different competition intensities. *B. juncea* is known to be able to be fertilized by pollen of *B. napus* (Roy 1978; Frello et al. 1995) such that interspecific hybrids are likely to occur, which may in turn transfer the transgenic insect-resistance to the wild *B. juncea* populations through backcrossing. Indeed, the absence of fitness cost of the transgene might allow the transgene to persist in the field populations (Diet al., 2009). However, the speed at which the transgene would spread through the wild population is

difficult to predict. Similarly, it is not known if the transgenic plant could totally replace the wild type. Experiments are needed to answer these questions. However, it is risky to prepare such “wild transgenic” material, and even experiments with such material are forbidden in several countries. We therefore simulated the response to insect damage of the resistant and the wild plants by clipping leaves. Intact plants without clipped leaves represented the introgressed insect-resistant plants. With different proportions of leaves clipped, and different ratios of plants with clipped versus plants without clipped leaves in mixed populations, we investigated the effect of competition for limited resources on the respective reproductive potential of resistant and susceptible plants.

MATERIAL AND METHODS

Eight *Brassica juncea* seeds were planted at the periphery of 18*18*20 cm pots. In order to simulate the performance of transgenic insect-resistant and insect-susceptible wild populations, the experimental treatment consisted in clipping some of the leaves as they appeared on some plants, but not on others. Leaves were clipped if necessary from the four-leaf stage up to the opening of the first flower for each plant. When the plants were about 60 cm tall, four poles with ropes were used to maintain the plants within a given volume. The positions of pots were changed every week in order to minimize the edge effect. This experiment was carried out in a greenhouse at 12 and 22 °C for night and day.

Different percentages of NC plants

We created five treatments of varying proportions of plants with non-clipped leaves (NC): 0, 25, 50, 75 and 100%. One out of every two successive leaves was clipped in the clipped plants (CP). CP and NC plants were disposed at random in the pots according to the percentage.

Different proportions of clipped leaves

In another set of pots, we assayed four clipping treatments: one of every four leaves (1/4), one out of two (1/2), two successive leaves out of four (2/4) and three out of four (3/4). For these

four groups, 50 % of plants were clipped. CP and NC plants were alternately planted within each pot.

When the first siliques were matured completely (i.e. yellow in color), the siliques were counted on every plant, then all the plants were cut at the base and left to dry at room temperature, and they were threshed to collect the seeds. Stems and leaves and silique material, except seeds, were dried in an oven at 80 °C for 48 h, and final dry weight was measured. Seed number per plant was calculated. The averaged CP and NC data per plot were used in fixed-effects ANOVA, and we used a 0.05 probability level for statistical significance.

RESULTS

Effects of different percentages of NC plants

The ANOVA showed that clipping leaves had a significant effect on the three measures. NC plants produced more siliques, higher biomass and more seeds (Table 3.1) than CP. No difference was observed among treatments for the NC percentage, and there was no interaction (Table 3.1).

Among the different NC percentages, no difference was recorded for the three measures of NC: silique number ($df=3$, $F=1.32$, $P=0.30$), biomass ($F=1.20$, $P=0.35$), and seed number ($F=0.68$, $P=0.58$). For CP, different treatments showed different silique number ($df=3$, $F=5.58$, $P=0.012$) and biomass ($F=7.88$, $P=0.0036$), but not different seed number ($F=1.93$, $P=0.18$). CP plants in pure stands produced two times more than in 50% mixed stands.

When looking at the given NC percentage treatments, some significant differences appeared between NC and CP in spite of high variability. At the mixed stand of 75% NC, NC population produced more siliques ($df=1$, $F=8.11$, $P=0.029$) and higher biomass ($F=21.97$, $P=0.003$) than CP on average. At the 50% mixed stand, the differences reached a maximum: the average silique number (80 ± 7) was 2.9-fold more than CP (27 ± 4) ($F=8.11$, $P=0.029$); the biomass of NC (10.84 ± 0.36) was 3.2-fold more than CP (3.29 ± 0.19) ($F=21.97$, $P=0.003$); and seed number (460 ± 43) of NC was 3.3-fold more than CP (138 ± 16) ($F=11.92$, $P=0.014$). There was no difference at the 25 % NC treatment.

Table 3.1: ANOVA results of the effect of clipping (CP) versus not clipping (NC) leaves and the percentage of plants with non-clipped leaves (NC), showing the F test value and its probability P for each test.

Tableau 3.1: Résultats de l'ANOVA des effets de couper (CP) ou non (NC) les feuilles et du pourcentage de NC. F valeur du test et sa probabilité P.

	DF	Silique number		Biomass		Seed number	
		F	P	F	P	F	P
CP vs NC	1	19.62	<0.0002	53.75	<0.0001	14.67	<0.0008
NC %	4	0.85	0.718	0.98	0.438	0.29	0.879
Interaction	2	1.28	0.296	1.33	0.283	0.40	0.672

Plotting the data against NC % (Fig. 3.1) showed a general trend. Linear regressions were significant for biomass (F= 18.13, P=0.0008) and silique number (F= 9.37, P=0.0085) of CP (Table 3.2). Due to the high variability of the data recorded among pots, the other regressions were not significant. However, the regression (or the smooth of mean values) decreased as the percentage of NC increased for all the three characters and the two types of plant (Fig. 3.1), which is not random (P=0.016).

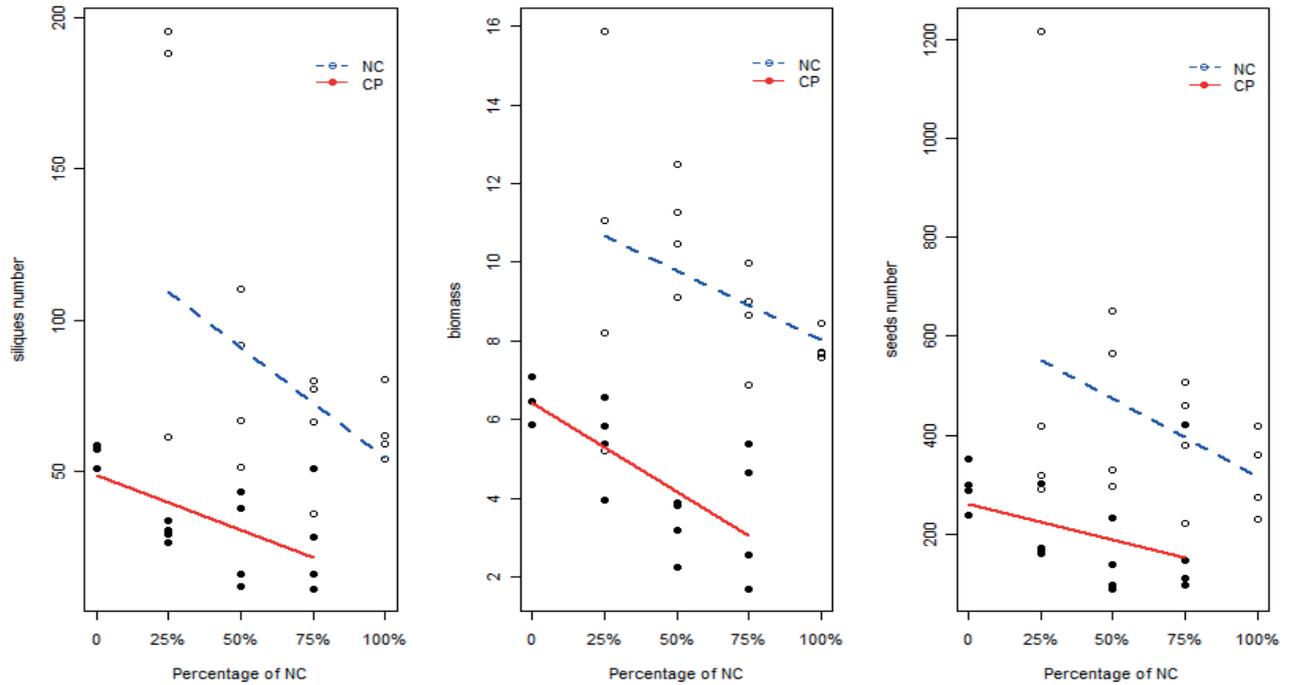


Fig. 3.1: Regression lines of siliques, biomass and seed numbers in terms of the percentage of non-clipped plants per pot (CP, dots and red line; NC, circles and dotted blue line)

Fig. 3.1: Les régressions linéaires de siliques, biomasse et semences d'après le pourcentage de NC chaque pot (CP, points et ligne rouge ; NC, cercle et ligne bleu)

Significant linear regression ($Y=6.14+0.32X$) was observed for biomass ($F=14.11$, $P=0.0015$) only, when the data of all eight plants per pot were summed. Total biomass per pot increased as the percentage of NC among pots increased.

Different proportions of clipped leaves

The ANOVA showed that clipping leaves, on average, had a significant effect on the three measures. NC produced more siliques, higher biomass and more seeds (Table 3.3) than CP. No difference occurred among clipping treatments for all plants. A significant interaction between treatments and clipping was observed for biomass only.

Table 3.2: Parameters of linear regression ($Y=A+B*X$) between silique number, biomass and seed number for different percentages of NC

Tableau 3.2: Paramètres des régressions linéaires ($Y=A+B*X$) du nombre de siliques, de la biomasse et du nombre de semences en fonction des différents pourcentages de NC

		A ± SE	B ± SE	R ²	F	P
Silique number	NC	36±26	18±9	0.202	3.55	0.080
	CP	3±10	9±3	0.401	9.37	0.0085
Biomass	NC	7.12±1.46	0.89±0.53	0.166	2.80	0.117
	CP	0.78±0.98	1.13±0.26	0.564	18.13	0.0008
Seed number	NC	235 ±141	79 ±51	0.144	2.35	0.147
	CP	79±78	36±21	0.172	2.91	0.110

Table 3.3: ANOVA results of the effect of clipping (CP) versus not clipping (NC) leaves and of the clipping treatment (proportion of clipped to non-clipped leaves).

Tableau 3.3: Résultats de l'ANOVA des effets de couper (CP) ou non (NC) les feuilles et du traitement de la coupe (la proportion de feuilles coupées et non-coupées)

	DF	Silique number		Biomass		Seed number	
		F	P	F	P	F	P
CP vs NC	1	19.01	<0.0002	54.26	<0.0001	15.17	<0.0006
Treatment	3	0.45	0.718	0.56	0.648	0.72	0.548
Interaction	3	2.67	0.070	8.63	<0.0004	2.02	0.137

No difference was recorded for the three measures of NC among treatments of different clipping proportions. For CP, different treatments showed different silique numbers ($df=3$, $F=4.78$, $P=0.020$), biomass ($F=8.91$, $P=0.0002$), and seed numbers ($F=5.86$, $P=0.0011$).

When one of four leaves (1/4) was clipped, CP and NC plants produced similar silique numbers, biomass and seed numbers (Fig. 3.2). Significant differences were observed when plants suffered severe leaf damage, when one of two (1/2), two successive leaves of four (2/4) and three of four (3/4) leaves were clipped. At the 3/4 clipping treatment, the average silique number of NC was almost 5-fold higher (78 ± 10) than CP (16 ± 2) ($df=1$, $F=9.42$, $P=0.022$), the biomass was almost 3-fold higher (9.72 ± 0.39 versus 3.38 ± 0.22) ($F=50.96$, $P=0.0004$), and seed number almost 6-fold higher (504 ± 84 versus 86 ± 12) ($F=6.12$, $P=0.048$). Therefore, silique number, biomass and seed number of CP plants decreased as the proportion of clipped leaves increased.

DISCUSSION

Simulated herbivory pressure enhances the fitness of insect-resistant populations

Experiments where simulated herbivory was employed to measure the differences between transgenic and wild relatives have already been carried out (Sutherland et al. 2006; Letourneau and Hagen 2009). Here, NC plants stand for a transgenic insect-resistant hybrid population and CP for a population of an insect-susceptible wild relative. As expected, clipping leaves had a negative impact on the performance of *Brassica juncea*: NC plants produced more siliques, higher biomass and more seeds than CP. Our results demonstrate that simulated herbivory pressure can enhance the fitness of an insect-resistant population.

Most studies have shown a statistically significant increase in seed output and final biomass when GM Bt plants were exposed to insect herbivores or to simulated herbivory, compared to non-GM plants. Ramachandran et al. (2000) reported such an effect in seed yield and biomass in transgenic oilseed rape infested by diamondback moth larvae in greenhouse and field experiments. Sutherland et al. (2006) found no significantly different fitness effect when only cotyledons were damaged mechanically of *B. napus* and *B. rapa* and their F1 hybrids, while a slight effect upon final biomass was recorded. In a comparison between transgenic Bt *B. rapa* and *B. rapa* \times *B. napus* introgressed hybrids in greenhouse and field experiments, Moon et al. (2007) demonstrated no significant difference in seed output and dry weight with low herbivore pressure. Letourneau and Hagen (2009) showed that seed output and silique number of *B. rapa* were significantly greater when plants were protected from

herbivores than when exposed to *Bt* susceptible herbivores. The decrease in herbivore damage expected after gene flow from *Bt* Brassica crops (*B. napus*) to *B. juncea* is likely to result in greater vegetative and reproductive performance, and an increase in plant fitness compared to a wild *B. juncea* population that does not contain the insect-resistance trait.

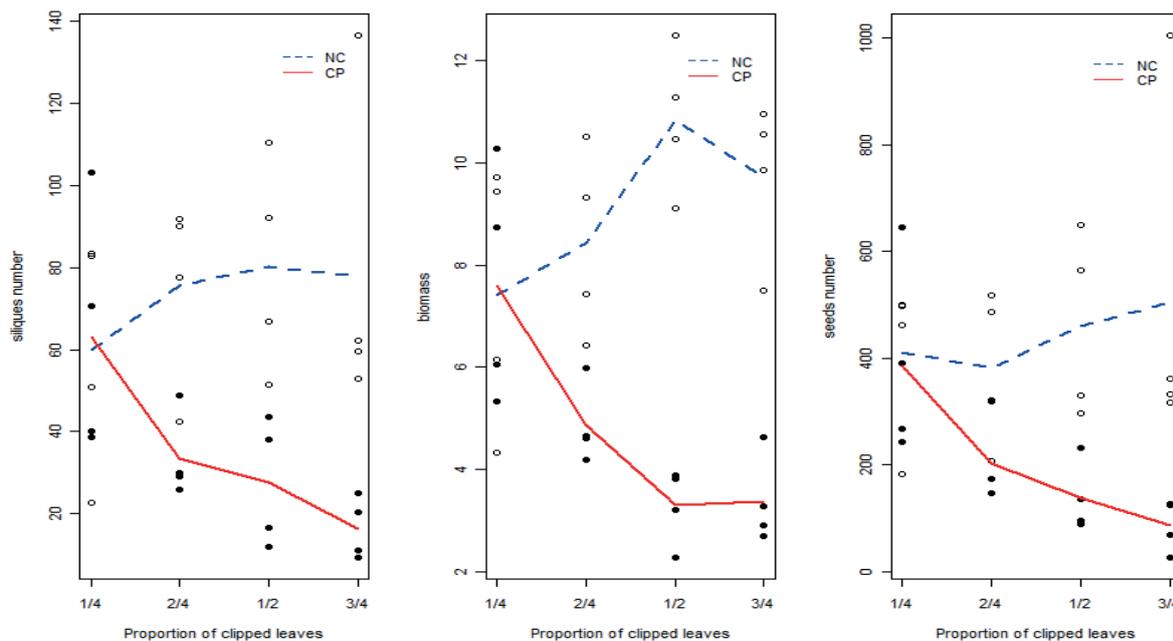


Fig. 3.2: Silique number, biomass and seed numbers of plants with (CP) or without (NC) clipped leaves according to the clipping treatment (CP, dots and red line; NC, circles and dotted blue line)

Fig. 3.2. Nombres de siliques, biomasse et nombre de semences des plantes avec (CP) ou sans (NC) feuilles coupées selon la proportion de feuilles coupées sur chaque plante (CP, points et ligne rouge ; NC, cercle et ligne bleu)

However, in the absence of herbivore pressure, transgenic plants might have lower fitness than wild plants because producing the Bt protein could be costly for the plant's physiology. Low seed production and low pollen fertility have been observed in hybrid material (Jorgensen and Andersen 1994; Chèvre et al. 1997; Hauser et al. 1998; Pertl et al. 2002), probably because of both the potential deleterious effects of hybridization and the cost of resistance expression. This question cannot be addressed in our experiment. In addition, before introgressed *B. juncea* is produced, the transgene must be transmitted through

interspecific hybridization, backcrossing and numerous generations where a hybrid fitness penalty could limit the spread of the resistance.

Intensity of simulated herbivory amplifies the fitness advantage of an insect-resistant population

The CP and NC plants did not show significantly different mean values when one of four leaves were clipped. As the proportion of clipped leaves increased, lower growth and reproduction were observed for CP plants. The absence of a few leaves did not affect the performance of *B. juncea*, while many clipped leaves obviously limited it. Three of four leaves clipped might be too serious. At the 3/4 clipping treatment, the average values of NC plants were about 5-fold higher than CP. Because clipping leaves limited the growth of CP plants, NC could utilize more of the resources in the pot. CP plants showed significantly different silique numbers and biomass among the treatments of four different proportions of clipped leaves.

Consequently, the difference between insect-resistant hybrids and related wild plants was amplified as the intensity of herbivore pressure increased because of the appropriation of their neighbors' resources. Most studies suggest that moderate to high herbivore or simulated damage produces an increase in plant fitness (Vacher et al. 2004; Sutherland et al. 2006; Moon et al. 2007). The high herbivore pressure might promote the invasion and persistence of transgenic (*Bt* transgenes) insect-resistant hybrids in mixed stands with a wild population. That might be one of the reasons why even very low levels of introgression and selection could lead to a high probability of fixing a transgene in a population (Meirmans et al. 2008).

Within-population competition magnifies the fitness advantage of insect-resistant plants

Among treatments of different NC percentages, CP plants showed significant differences for the measured traits, while no difference was recorded for NC. Competition pressure from NC plants significantly affected the development of CP plants, while CP plants did not affect NC plants in the first experiment. The more NC plants there were in the pot, the less fertile, heavy and reproductive the CP plants were. Because insect-resistant hybrids would suffer less damage, they would have access to resources that their insect-susceptible wild counterparts would have otherwise exploited. As a result, resistant plants not only escape herbivore attack, but they also capitalize on their neighbors' misfortune. Thus, the relative fitness of insect-

resistant hybrid populations might increase under herbivore pressure in mixed stands with a wild population. Weis and Hochberg (2000) demonstrated that the effect of herbivore pressure on the relative biomass advantage of resistant plants was magnified by plant density. Vacher et al. (2004) found that high-density patches of highly damaged wild plants are the most vulnerable to Bt-transgene invasion.

According to the tentative regression equations proposed in Fig 1 and Table 2, there is a possibility that the performances of NC plants decreased with increasing NC percentage. The lowest values appeared in the pure stands of NC plants. The most intense competition was in the pure stands of NC plants where there were no CP plants. This could be expected, as more healthy insect-resistant plants have to share the limited resources available in the pot. Because selection for the plants possessing the transgene would lead to populations consisting of 100 % insect-resistant plants, our data indicate that the overall reproductive output of the resistant population would be the same as a susceptible, insect-attacked population. Thus, when the resistant plants are in the minority, the fitness advantage for insect-resistant hybrids is obvious, but as the frequency of insect-resistant hybrids increases, neighbor competition limits their growth. Therefore, changing the population from susceptible to insect-resistant would not result in different demographic kinetics in resource-limited habitats.

CONCLUSION

In this study, we have not studied the fitness of the first hybrid generation after the interspecific hybridization between *B. napus* and *B. juncea*. Experiments on that topic show that transgenes are likely to persist in the wild populations (Di et al., 2009). In turn, we have simulated the fate of stabilized introgressants having the transgene for insect-resistance within a *B. juncea* population. The effect of simulated herbivory and competition on the vegetative and reproductive performances of transgenic plants and wild relatives was clear. Our results showed that clipping leaves significantly reduced silique numbers, final biomass and seed output of *B. juncea*. Clipping leaves had an increasingly negative influence on the survival of CP plants as the proportion of clipped leaves increased. However, the development of NC plants was not obviously impacted, irrespective of the proportion of clipped leaves of CP

plants. Competition affected the fitness of NC and CP plants. The stronger the competition was, the more obvious the fitness advantage of NC plants. Therefore, herbivore and competition pressure might enhance the fitness of transgenic hybrids in mixed stands with wild relatives. It is timely to pay enough attention to herbivore pressure that could increase the occurrence of gene flow between transgenic crops and wild relatives.

ACKNOWLEDGEMENTS

We thank Alain Fleury for help in the field and laboratory. This work was supported by a PhD scholarship from the French Embassy in China.

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Complément à l'article 3

Le taux de germination des semences issues des plantes CP a été inférieur à celui des plantes NC avant l'ajout de gibbérelline, mais pas après. Ceci suggère que l'herbivorie peut entraîner une fraction des semences à être plus dormante que pour une plante résistante. La germination a aussi été affectée par différentes proportions de plantes NC. Ces résultats n'ont pas été présentés dans l'Article 3 et figurent ici (Table 3.4; Fig. 3.3).

Table 3.4. *F*-values from two-way ANOVA results of seed germination of *B. juncea*

	DF	Before GA ₃	After GA ₃	Total
Treatment (T)	7	1.29 ^{ns}	3.70 ^{***}	2.69 [*]
Clipping (C)	1	6.99 ^{**}	2.97 ^{ns}	0.02 ^{ns}
Interaction T*C	5	1.16 ^{ns}	0.29 ^{ns}	0.62 ^{ns}

*, P<0.05; **, P<0.01; ***, P<0.001; ns, non significant

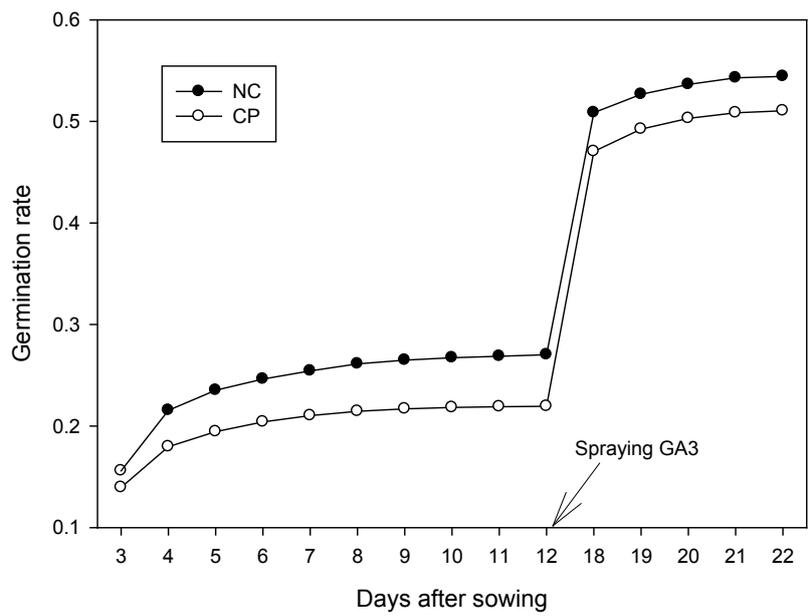


Fig 3.3. Germination rate of seed germination of *B. juncea* with days after sowing



Photo 3.1. Insect simulated experiment in a greenhouse

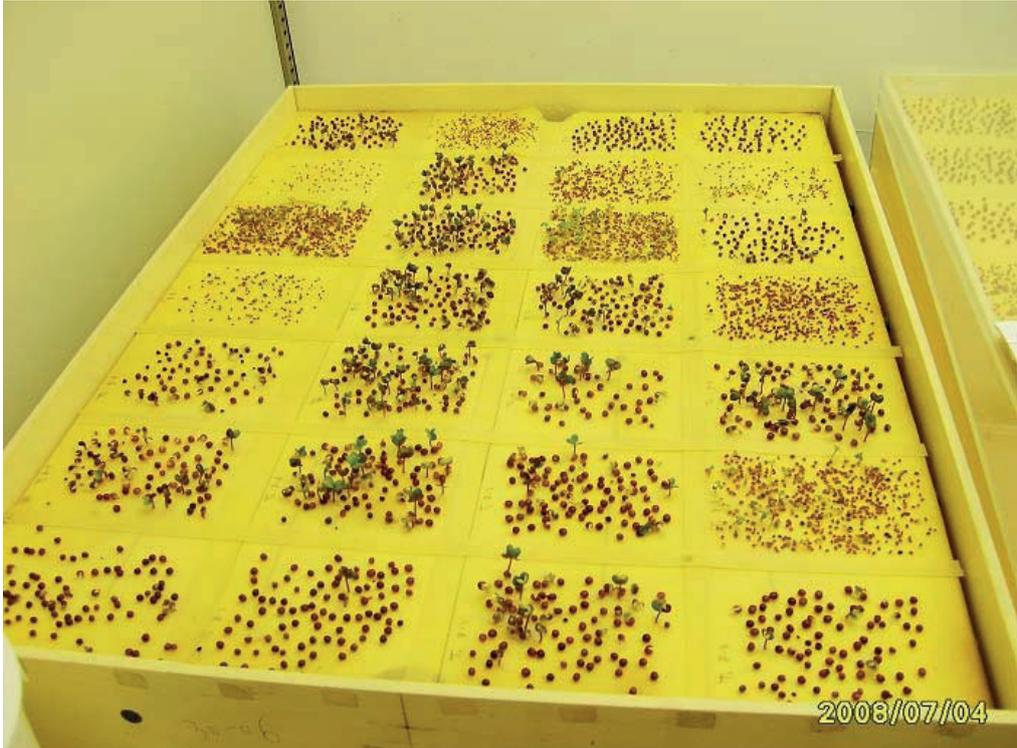


Photo 3.2. Seed germination in an artificial climate chamber

3.3 Article 4: Compétition entre plantes de *Brassica juncea* simulant la résistance ou la sensibilité aux insectes

La question et les procédures de l'étude sont les mêmes que pour l'article 3, sauf que les plantes ont été semées au champ. L'étude préliminaire en serre nous a conduit à ne couper qu'une feuille sur trois dans l'expérience de 2009 alors qu'il était un sur deux en 2008. Chaque proportion NC/CP a été répétée sur cinq placettes de 24 plantes dont nous avons distingué celles du centre de celles de la bordure (ces dernières étant supposées être moins soumises à la compétition).

La défoliation d'une feuille sur trois n'a pas été suffisante, à elle seule, pour modifier les performances des plantes CP, alors qu'une feuille sur deux en 2008 a eu un effet très marqué. Les effets combinés de la défoliation et de la compétition ont été additifs. Les plantes entières, NC, ont eu un avantage compétitif sur les plantes coupées CP surtout au centre des placettes où la compétition est plus forte, et leur avantage relatif augmentait avec leur proportion. L'investissement dans la reproduction n'était pas différent entre CP et NC, mais il augmentait avec la compétition intra-population, ce qui suggère que la résistance à l'herbivorie n'affecte pas l'utilisation des ressources, contrairement à la compétition. La production totale des placettes est restée constante, quelque soit la proportion de plantes NC et CP.

Ces résultats montrent que la résistance procure un avantage indiscutable de croissance et de reproduction sous forte pression herbivore, mais qu'une faible herbivorie peut induire des phénomènes de compensation entraînant peu de différences entre les résistants et les sensibles. La compétition accentue les différences, le rapport de reproduction entre résistant et sensible s'accroissant au fur et à mesure que la proportion de résistants augmente. Ces phénomènes rendent plus facile et inexorable la dispersion des transgènes de résistance aux insectes jusqu'à fixation dans les populations, mais la dynamique démographique des moutardes sauvages ne devrait pas en être changée, c'est-à-dire qu'elles ne devraient pas pouvoir envahir d'autres milieux sauf à agrandir leur niche en déplaçant d'autres espèces.

ARTICLE 4

SIMULATED INSECT-RESISTANT VERSUS SUSCEPTIBLE *BRASSICA JUNCEA* INTRA-POPULATION COMPETITION

Yongbo LIU, Wei WEI, KePing MA and Henri DARMENCY

Summary

- Few studies have focused on the effect of relative fitness on population dynamics when transgenes are transferred from a crop to its wild relatives, independent from the interspecific hybridization cost.
- We clipped leaves to simulate the performance of transgenic, insect-resistant plants of wild *Brassica juncea* in pure stands and in mixtures with non-clipped plants in field experiments over two field seasons.
- The total vegetative and reproductive production of mixed populations was the same as that of pure populations. The combined effects of defoliation and resource availability on the performance of *B. juncea* were additive. Healthy plants held a competitive advantage when in competition with damaged plants, and the relative advantage increased as the percentage of healthy plants increased. Investment in sexual reproduction did not differ between healthy and damaged plants, but it did decrease with decreased intra-population competition or decreased resource availability.
- These results suggest that if a transgene for insect-tolerance were to invade wild populations, high herbivory and low resource availability would facilitate the spread of resistant plants. However, a population shift from predominantly susceptible to predominantly insect-resistant individuals would not result in different demographic kinetics, i.e. the species would not become more invasive.

Key words: *Brassica juncea*, competition, invasion, population productivity, simulated herbivory

Introduction

The invasion of transgenic plants and transgenes into wild populations is a major biosafety issue linked to the release of genetically modified (GM) plants, most notably for oilseed rape, cotton, maize, sunflower, and soybean (Pilson, & Prendeville, 2004; Snow *et al.*, 2005). For many crops, spontaneous hybridization allows transgenes to spread to wild/weedy populations of related taxa that occur nearby (Ellstrand *et al.*, 1999). Numerous studies support the hypothesis that hybridization is a non-invasiveness stimulus (in review by Ellstrand & Schirenbeck, 2000), although plant adaptation does not guarantee subsequent invasion. Transgenic crops or hybrids and backcrosses can escape from the field and establish in other habitats as volunteer or feral plants via both pollen- and seed-mediated gene flow (Crawley & Brown, 1995; 2004; Hall *et al.*, 2000; Warwick *et al.*, 2003; Knispel *et al.*, 2008).

Long-term persistence of volunteer and feral plants, GM seeds, and crop alleles can occur in fields. In Canada, one introgressed, glyphosate-resistant individual of *Brassica rapa* was detected four years after the last GM oilseed rape (*B. napus*) was grown on the field, even in the absence of glyphosate selection pressure (Warwick *et al.*, 2008). Transgenic wild plants were found three years after accidental escape of pollen and seeds from transgenic creeping bentgrass (*Agrostis stolonifera*) in the USA (Zapiola *et al.*, 2008). Herbicide-tolerant oilseed rape seedlings emerged ten years after a field trial conducted in 1995, confirming the long-term persistence of transgenic seeds in the seed bank in Sweden (D'Hertefeldt *et al.*, 2008). Multi-herbicide-resistant oilseed rape volunteers were found five to eight years after the last GM cultivar was grown as part of a multi-year farm-scale study in France (Méssean *et al.*, 2007). In a recent study, Snow *et al.* (2010) found that crop-specific alleles persisted for ten years in four weedy populations of hybrids between wild radish (*Raphanus raphanistrum*) and *R. sativus*, suggesting that neutral or even detrimental genes can quite often persist in the wild. Though these studies show that transgenes can invade and persist in crop volunteers and wild/weedy populations, there are few studies of the dynamics of the invasion process and the impact on populations (Ramachandran *et al.*, 2000; Sutherland *et al.*, 2006; Damgaard & Kjaer, 2009).

Several studies have demonstrated that the *Bt*-transgene for insect resistance in oilseed rape confers high relative fitness in response to insect herbivory (Stewart *et al.*, 1997; Ramachandran *et al.*, 2000; Letourneau & Hagen, 2009). In the absence of selection pressure,

no fitness cost was detected in the Brassiceae (Ramachandran *et al.*, 2000; Mason *et al.*, 2003; Di *et al.*, 2009) or in sunflower (Snow *et al.*, 2003). The population dynamics of introgression could simply depend on the reproduction system, the rate of population turnover, the cost of being a n interspecific hybrid, or the benefit of being resistant. However, in habitats with limited resources, intraspecific competition for these resources is density-dependent (Verkaar, 1987). Ordinarily, insect-susceptible wild plant populations are regulated via the effects of herbivory on vegetative and reproductive growth, but the population dynamic changes when these populations are invaded by insect-resistant plants because the competitive landscape for a given individual varies with the genetic composition of its neighbors. As resistant plants have an advantage over susceptible under herbivory pressure, their prevalence would increase relative to native plants, which in turn would face an increasingly competitive environment. As an example: the disadvantage to chickweed plants (*Stellaria media*) infected by a virus increased with the proportion of healthy competitors in the population (Friess & Mallet, 1997).

The competitive relationship between insect-resistant and susceptible plants can be partitioned into two components: 1) intra-class competition between individuals of a single class; and 2) inter-class competition between resistant and susceptible plants. When insect damage reduces the size of a susceptible plant, resistant neighbors can usurp its resources and thus suppress its growth (Weis & Hochberg, 2000). However, herbivory damage not only affects the given individual, but also its neighbors and therefore regulation of the competitive relationship might be influenced by herbivory (Chase *et al.*, 2002). The interaction between competition and herbivory could be: (1) additive, when the fitness disadvantage for an insect-susceptible plant is amplified by increased competition from resistant plants (Weis & Hochberg 2000; Agrawal, 2004); or (2) antagonistic, when the disadvantage for the susceptible plant decreases because of the impacts of herbivory on neighbors (Haag *et al.*, 2004; Schadler *et al.*, 2007). There could also be no interaction, in situations where the disadvantage to the susceptible plant does not correlate with unique or combined pressure from competition and herbivory (Steets *et al.*, 2006). The outcome of the interaction between competition and herbivory will determine whether the population will evolve to be 100% insect-resistant or whether it will reach some intermediate equilibrium between resistant and susceptible plants.

Whether a population will evolve to equilibrium also depends on that population's reproductive output and the subsequent plant density of its progeny, which in turn determines the population dynamics (Meirmans *et al.*, 2008). Therefore, we address whether there is a population maximum seed production for a given percentage of resistant plants under insect pressure, since resistant plants could benefit from resources that would otherwise have been consumed by susceptible plants (Friess & Mallet, 1997; Rodriguez & Brown, 1998). Enhanced seed production could make a population better able to disperse at a certain point during its evolution from 0 to 100% resistant, making it more invasive than the original population and potentially more troublesome for ecosystem conservation and agriculture. Conversely, reduced seed production could result in only rare occurrence of insect-resistant plants.

Oilseed rape (*Brassica napus*, AACC, $2n=38$) is a model crop for transgenic studies, and *Bt*-transgenic oilseed rape conferring insect-resistance has been studied for years (Stewart *et al.*, 1997). Although Ramachandran *et al.* (2000) found that *Bt*-transgenic oilseed rape is a superior competitor compared to non-transgenic oilseed rape under pressure from the diamondback moth, there are few studies of variation in the competitive interaction when the frequency of insect-resistant plants increases. *B. juncea* (AABB, $2n=36$), one of the wild relatives of *B. napus*, is a tetraploid species that is preferentially self-pollinated but that has a considerable outcrossing rate. It is frequently found as a weed in arable fields and is a ruderal component of roadsides and waste sites in China. *B. napus* and wild *B. juncea* are highly compatible, and hybrids and subsequent backcross generations have successfully been obtained by open pollination (Frello *et al.*, 1995; Bing *et al.*, 1996; Song *et al.*, 2007; Liu *et al.*, 2010). The relatively high compatibility raises a biosafety concern; transgene introgression could occur from transgenic oilseed rape to wild *B. juncea*. This could then lead to persistence of their hybrids or backcross progeny in the wild *B. juncea* habitats and, consequently, the occurrence of transgenic insect-resistance within *B. juncea* populations following interspecific hybridization.

The objectives of our study were: (1) to detect variation in competitive interactions between insect-resistant and susceptible plants present at different proportions in mixed stands under two different experimental conditions; (2) to determine whether intraspecific competition amplifies or diminishes the selective advantage of resistance; (3) to study adaptive strategies (resource allocation) under various conditions, including herbivory and

resource pressure; (4) to investigate whether population production varies with the proportion of resistant plants; (5) to determine the variation in or stability of the fitness impact on simulated insect-resistance in susceptible plant populations of wild *B. juncea*, as the frequency of resistant plants increases. We used leaf clipping to simulate herbivory on *B. juncea* and to mimic the effect of insect resistance. Simulating herbivory (e.g. defoliation at the cotyledon stage: Sutherland *et al.*, 2006) allows us to exclude interactions arising from the interspecific hybridization cost and eliminate variability inherent in natural insect attacks. Simulated herbivory via leaf clipping and hole-punching (Schooler *et al.*, 2006) is often used as a substitute for actual herbivory in ecological studies of insect-plant interactions; it offers precise control on the amount and timing of plant damage (Baldwin, 1990) and closely mimics actual impacts of herbivory (Schooler *et al.*, 2006).

Materials and methods

The 2008 experiment Wild *Brassica juncea* ($2n=36$, A ABB) was obtained from the Dijon, INRA laboratory weed collection (ref. X04-021, from Turkey). Seeds of *B. juncea* were sown in Giffy-7 in the greenhouse (22 °C under natural light). Three weeks later, in June 2008, seedlings at the three-leaf stage were transplanted to one of four insect-free cages (3m width * 6m length * 3m height, protected by a 2mm mesh nylon net) in the INRA common garden at Dijon. Each cage contained five plots (0.5m * 0.7m), and plots were separated from each other by 0.6m. In each plot we planted 24 *B. juncea* seedlings, placed one every 0.1m, in four rows and six columns. The plots were kept weed-free by hand, and the soil around the plots was covered by a porous plastic net to prevent weed emergence.

In order to simulate the performance of transgenic insect-resistant plants in insect-susceptible wild populations, we simulated insect damage by clipping some leaves of the plants designated as susceptible (clipped plants: CP = insect-susceptible damaged plants), while other plants remained intact (non-clipped plants: NC = insect-resistant healthy plants). Every second, whole leaf to emerge on the stem of each CP plant was clipped, starting at the four-leaf stage and continuing to the opening of the first flower. To simulate the invasion process of insect-resistant plants into a wild population, five different percentages of NC were added to plots, with four replicates for each percentage: 0 (T₀), 25% (T₂₅), 50% (T₅₀), 75% (T₇₅), and 100% NC (T₁₀₀). Plant types (CP or NC) were placed randomly within each plot,

while ensuring that the same ratio of CP to NC existed for the eight plants in the center and for the 16 plants at the border. This allowed us to compare performance at the two positions (Figure 3.4). Center plants experienced higher competition while border plants experienced lower competition. Houseflies, *Musca domestica*, were continuously provided inside the cages to facilitate random pollination.

The 2009 experiment The experiment was replicated in April 2009, but this time five or six *B. juncea* seeds were directly sown in each spot within the cage. After emergence, one seedling per spot was randomly selected and the others were removed. In addition, we clipped one out of three successive leaves instead of one out of two, because greenhouse experiments indicated that this would reduce the plant damage to an acceptable intensity (see Liu *et al.*, 2009).

Measurements The number of leaves on the stem and the date when the first flower opened were recorded for each plant. When the first completely mature siliques were observed (i.e. golden brown in color) the siliques were counted on every plant and then all plants were cut at the base and left to dry at room temperature. The plants were threshed to collect the seeds. Stems, leaves, and silique material (except seeds) were dried in an oven at 80°C for 48 h, and final dry weight was measured as plant weight. The seed number was counted and the seed weight per plant was measured. The number of seeds per silique was calculated (seed number/ silique number). Biomass was calculated as plant weight plus seed weight. A resource allocation index (reproductive index) was calculated as the ratio of the plant weight to the seed weight. Viability of up to 100 seeds per plant was tested by germinating on moist paper in a growth chamber (15°C during 8h in the dark, and 25°C during 16h under artificial light). Germinated seedlings were checked once every three days for a period of three weeks, and then trays were sprayed with 0.4 % gibberellic acid and put in continuous darkness to break seed dormancy. The number of viable seeds was calculated as the seed number multiplied by the germination rate.

Statistical analysis The mean value for all CP or NC plants in the center and at the border of each plot was used for the statistic analysis of all measured traits. For seed weight, total biomass, and seed number, a per-plot analysis was carried out using the sum of the data from all CP or NC plants in the center or all at the border. Bartlett's test was used to test for homogeneity of variance, and as a result the data were log transformed to ensure a Normal

distribution of residuals for all characteristics measured. A four-way fixed-model ANOVA was used that included effects of clipping, position within plot, and plant type percentage. The two experiments were analyzed separately because of the different conditions in the first and second experiment (different experimental conditions includes clipping 1/2 versus 1/3 of the leaves, transplanting versus direct sowing, and 2008 versus 2009 year effect). Tukey's HSD test was used for multiple comparisons. A contrast test was used to analyze the effects of clipping and plot position on growth and reproductive performance. Data on flowering date, seed weight, biomass, number of viable seeds, and reproductive index were fitted to a linear regression model in terms of the plant type percentage, $Y = a + b * X$, where X was the percentage (0, 25, 50 and 75 for CP, 25, 50, 75 and 100 for NC). The linear regression slopes were compared using R software's `Diffslope` (simba) package (<http://www.r-project.org/>). Regressions were also calculated for total seed weight, biomass and number of viable seeds per plot. In order to investigate the difference between NC and CP, relative to the CP pure stand T_0 performance, and according to the percentage of NC, we used the Helmert Contrast test on the calculated $DI = (NC_{Ti} - CP_{Ti}) / CP_{T0}$. All statistic analyses were conducted in R software.

Results

Effect of simulated herbivory on individuals The 2008 and 2009 experiments were analyzed separately because the experimental conditions were so different. In 2009, plants generally performed better than in 2008. Specifically, the 2009 plants: flowered later, produced more leaves, had higher seed weight, had higher plant weight, had more biomass, had more seeds per silique, had more viable seeds, and had higher germination rate. However, 2009 plants had a lower reproductive index than those in the 2008 experiment (Figure 3.5). Neither silique number nor seed number differed between the two experiments.

Plant position (center versus border) was an important variable in both years (Table 3.5). Plants at the plot border produced heavier seeds, greater biomass, and more viable seeds (but had a lower resource allocation index) than those in the center (Figure 3.5, Table 3.5). In 2009, plant position did not affect leaf number, flowering date, or germination. There was no interaction between clipping leaves and plot position in either experiment. The effect of leaf clipping was much more pronounced in the first experiment; CP plants had lower plant weight,

silique number, seed number, seed weight, biomass, number of viable seeds (all $P < 0.001$), and seed number per silique ($P < 0.05$) than NC plants. The clipping effect was less evident in the second experiment; in this case CP plants had fewer siliques, lighter seed weights, and lower biomass than NC plants (Figure 3.5, Table 3.5). Clipping had no effect on flowering date, number of viable seeds, or the resource allocation index in either year (Table 3.5).

Effect of the percentage of simulated insect-resistant plants

In the 2008 experiment, an increased percentage of NC plants resulted in reduced seed weight, biomass, and number of viable seeds in both plant types ($P < 0.01$ for all variables), though the presence and magnitude of this effect varied with plot position. There was no effect on flowering time or the resource allocation index. There was also a significant interaction between leaf clipping, the percentage of NC plants, and plot position (Table 3.5), suggesting that NC and CP plants reacted differently to different combinations of conditions. In the plot centre, an increased percentage of NC plants resulted in decreased seed weight, biomass, and number of viable seeds of both plant types. However, at the plot border, these variables only decreased for NC plants (Figure 3.6, Table 3.6). Each of these variables was plotted against the percentage of NC plants separately for each plant type at the border, and again for those at the center of the plots (Figure 3.6). The regression slopes did not differ between NC and CP plants situated at the center of the plot. The slopes for NC plants situated at the border did not differ from those situated at the centre. In all cases, the low values of the regression coefficient R^2 indicated a poor fit and a great deal of variability among plots (Table 3.6).

In the 2009 experiment, there was no link between the percentage of NC plants and any of the measured characteristics, and no interaction among treatments. There was only one significant linear regression; flowering date of the NC plants situated at the plot center increased with the percentage of NC plants (Figure 3.6).

In the 2008 experiment, the relationship between the variables and percentage of NC plants was non-linear for CP plants at the plot border (Figure 3.6). The resource allocation index was lowest at T_{50} for all NC and CP plants at both plot positions (Figure 3.6). In the 2009 experiment, the relationship between the variables and percentage of NC plants was also non-linear for both NC and CP plants at the plot center. In this experiment, extreme values of NC plants were observed at T_{75} in the plot border and the resource allocation index of NC and

CP plants reached a maximum at T_{75} in the plot border (Figure 3.6). There was no difference in performance of NC and CP plants in pure plots in either experiment (Figure 3.6). The resource allocation index for NC and CP plants did not differ between mixed and pure stands (contrast test, $P > 0.05$) for either plot position in either experiment.

Competition between simulated insect-resistant and susceptible plants The relative difference (DI) between NC and CP varied both with the plot position and between the two experiments (Table 3.7, Figure 3.7). From the first experiment to the second and from plot center to border, there were fewer stands that had high DI values (Figure 3.7). According to the Helmert contrast test, the DI value at T_{25} was higher than that at T_{50} in the center in both experiments. The same was not true at the border (Table 3.7). DI at T_{75} was different from T_{25} and T_{50} for: 1) the plot center in the first experiment, 2) the plot border in the first experiment, and 3) the plot border in the second experiment. DI between NC and CP in pure stands was lower than in the mixed stands. The only exception was at the plot center in the second experiment, where DI was lowest at T_{50} (Table 3.7).

Comparison of summed performances per plot Plot position was the only significant factor within plots (Table 3.8). The group of plants at the plot border produced higher biomass (3.5-fold in 2008 and 4.6-fold in 2009) and more viable seeds (3.1-fold in 2008 and 4.2-fold in 2009) than the group in the plot center ($P < 0.001$) (Figure 3.8). The growth conditions were much more favorable in the 2009 experiment than in the 2008 one, especially for plants at the borders. At the plot border, there were more viable seeds ($F_{1,38} = 22.45$, $P < 0.001$) and greater biomass ($F_{1,38} = 310.47$, $P < 0.001$) in the 2009 experiment than in the 2008 experiment. In the plot center, there was more biomass ($F_{1,38} = 69.11$, $P < 0.001$) in the 2009 experiment than in the 2008 experiment, but no difference in viable seed number ($F_{1,38} = 0.01$, $P = 0.94$) (Fig 3.8). For a given position, center or border, there was no variation in the total viable seed number or biomass linked to the percentage of NC plants, except for the number of viable seeds in plot borders in the 2009 experiment at T_{75} (Figure 3.8). In particular, there were no differences in seed and biomass production between pure stands of CP or NC plants, in either plot position or experiment.

Discussion

Effects of simulated herbivory on plant growth and reproduction

Our garden experiments showed that sufficient defoliation significantly decreased plant growth and fecundity, because of the effects of defoliation on photosynthesis. Clipping one out of two successive leaves in the 2008 experiment had a negative impact on the performance of *Brassica juncea*: NC plants produced higher seed weight, biomass, and viable seeds than CP plants. Clipping one out of three successive leaves, as in the 2009 experiment, did not generate any differences between NC and CP. Although this difference between experiments could be attributed to seasonal effects (2008 vs. 2009) and planting mode (transplanting vs. direct sowing), it is more likely resulted from the leaf clipping rate. This would be consistent with the results of our preliminary greenhouse experiment (Liu *et al.*, 2009). The percentage of defoliation of non transgenic oilseed rape by diamondback moths in a field experiment was only around 20% (Ramachandran *et al.*, 2000), but this ratio applied to all foliage while we clipped only the leaves on the main stem. Clipping one out of three leaves did not affect plant growth, which can likely be explained by the observed compensatory growth of numerous leaves at axils (data not shown). Compensation has been shown to increase via plant growth, after tissue loss to herbivores (Strauss & Agrawal, 1999; Hawkes & Sullivan, 2001). Boalt & Lehtila (2007) found that foliar damage resulted in compensatory leaf growth in wild radish (*R. raphanistrum*), and foliar damage of up to 30% of the leaf area had no effect on plant height and seed yield.

Although the same percentage of damaged leaves generated from clipping leaves versus real herbivory does not impact plants equally, simulated herbivory by clipping leaves is a feasible alternative when molecular containment of transgenes is forbidden in field experiments (Letourneau & Hagen 2009) and given that the efficiency of leaf damage by insects is very unstable and depends on many environmental conditions. In the current study, NC plants represent transgenic insect-resistant plants, mimicking a transgenic crop or advanced stable backcross generation of hybrids between the transgenic crop and *B. juncea*. CP represents insect-susceptible wild relatives. Letourneau & Hagen (2009) showed that the *Bt*-based *B. rapa* did not possess a fitness advantage when the plants were subject to low herbivory pressure, but the advantage was significant when they were subject to high herbivory. Moon *et al.* (2007) demonstrated significantly higher seed output in transgenic hybrids formed between *B. napus* and *B. rapa*, than in pure populations of *B. rapa* in greenhouse conditions, while there was no effect in field experiments with low herbivory. Ramachandran *et al.* (2000) reported that non-transgenic plants infested by diamondback

moth larvae suffered higher defoliation and produced less biomass and seed yield than transgenic oilseed rape in greenhouse and field experiments. These results demonstrate that the fitness of insect-resistant plants is higher than that of insect-susceptible ones under moderate to high herbivore damage. High herbivore pressure might promote the invasion and persistence of transgenic (*Bt*-transgene) insect-resistant plants within a wild population (Vacher *et al.*, 2004). Under these conditions, it is expected that the transgene could invade the whole population.

Competitive interaction between insect-resistant and insect-susceptible plants as the percentage of resistant plants increases

Our results show that insect-resistant plants (simulated by NC plants) have a competitive advantage in a mixed population, but this advantage varies with the relative proportion of resistant plants in the plots. As the percentage of insect-resistant plants increased, the performance of both susceptible (simulated by CP plants) and resistant plants decreased under the high herbivory pressure simulated in the first experiment (Figure 3.6). The equivalent slopes indicate that fitness of the resistant plants relative to the susceptible plants increased geometrically with their increasing representation in the population. The difference between NC and CP plants in seed weight, biomass, and viable seed number increased as the combined effects of competition and defoliation increased, which suggests that these combined effects are additive (in the sense of Weis & Hochberg, 2000; Figure 3.6). As insect-resistant plants suffered no damage, they would have access to resources that their insect-susceptible wild counterparts were unable to exploit. Low resource availability exacerbates the misfortune of susceptible plants. Weis & Hochberg (2000) demonstrated that competition magnified the effect of herbivore pressure on the relative biomass advantage of resistant plants. Vacher *et al.* (2004) reported that high-density patches of highly damaged wild plants are the most vulnerable to *Bt*-transgene invasion.

Allocation of resources to reproduction was not affected by simulated herbivory, but it decreased from the plot center to the plot border due to the competitive interaction among NC and CP plants for available resources (Figure 3.6). This means that proportionally more resources were invested in producing progeny in harsh conditions, and that resource availability may limit plant plasticity. However, Rodriguez and Brown (1998) found that slug herbivory reduced reproductive investment in *Poa annua* in a greenhouse experiment. Other variable cases have been reported in the literature. In purslane (*Portulaca oleracea*)

experiment, harsh growth conditions increased investment in reproduction (Friess & Maillet, 1996), but in similar experiments with chickweed (*Stellaria media*) reproductive allocation decreased at higher density, although it was stimulated at moderate plant density (Friess & Maillet, 1997). There could be also varied responses depending on the adaptive strategy of different genotypes: competition could decrease reproductive investment because high investment in growth increases competitive ability (Pagan *et al.*, 2009). The results suggest different adaptive strategies of *B. juncea* under different growth conditions, including resource availability, competition, and herbivory, which could ultimately result in different population dynamics.

Population production and dynamics with increasing insect-resistant plants

Population production was affected by experimental conditions, including both planting procedure, percentage of clipped leaves and yearly conditions. In general, high defoliation caused a greater decline in population production than low defoliation. In a two-year experiment, mammalian herbivores altered vegetative growth, including growth rate and timing of reproduction, of *Cirsium occidentale* and ultimately this herbivory affected the population dynamics (Palmisano & Fox, 1997). Spataro and Bernstein (2004) found that the effect of competition on population dynamics was linked to the presence of parasitoids in the system. However, estimating the impact of herbivory is complex because herbivory affects neighboring plants in addition to target plants, and seed dispersal in addition to growth and reproduction (Verkaar, 1987). In the current study, populations at the plot border had higher production than those in the plot center, while the number of viable seeds of the populations in plot centers did not differ between the two experiments. Therefore, the difference in population production between the two experiments might be better explained mainly by the different growth conditions than by the different defoliation levels. In other words, like the resource allocation of plants discussed above, growth conditions may play a more important role in population production than defoliation, especially for reproductive production.

Maximum population reproduction was observed at T_{75} at the plot borders of the second experiment, although population production varied little among the various percentages of healthy plants. Reproductive production was important for population composition, since more seeds from insect-resistant plants would be released than from susceptible ones under high herbivory. Reproductive production is also important for the kinetics of population demography, because enhanced seed production could make a

population more able to spread to neighboring populations. Because the advantage of insect-resistant plants increases under harsh conditions, it is likely that resistant plants would dominate the population and that the population would evolve from 0 to 100% resistant within a short time. Letourneau and Hagen (2009) have found that *B. rapa* plants (including those protected and exposed to *Bt*-susceptible herbivores) produced more seeds in disked soil or field margins than in natural vegetation. If this population were to spread into disturbed habitats, the fate of adjacent wild populations could quickly depend on the prevalence of the invading insect-resistant plants.

Implication for the invasion of transgenic volunteers into wild populations

The current study shows that sufficient simulated herbivory decreases plant fitness, showing that healthy plants have a clear competitive advantage. This advantage varied with the percentage of resistant plants in the population, and was more obvious under low resource availability than when resources were high. In addition, the interaction between herbivory and competition had an additive effect on plant fitness. However, plants had the ability for compensatory growth following injury by herbivory. Low competition and sufficient resources support regrowth in semi-natural environments. Thus, at the final equilibrium (i.e. resistance gene fixed in a population) vegetative and reproductive production of wild populations was not significantly altered. The overall productive output of the pure resistant population was the same as the insect-attacked pure population (Figure 3.8), meaning that changing the population from susceptible to insect-resistant would not result in different demographic kinetics. Therefore, these results suggest that, although insect-resistant plants can invade and completely replace wild susceptible populations, which still is one of major concerns on commercial release of transgenic crops, there will be no change to the plant community equilibrium due to the intrinsic change in the *B. juncea* demography, unless herbivorous insects switch to another species. This could result in more resources available in the habitat for the insect-resistant species.

Acknowledgements

This work was supported by a PhD joint fellowship between France and China (CNOUS, No. 20072315) and a fund from the National Natural Science Foundation of China (NSFC grant No. 30970432).

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Table 3.5. F values of the analysis of variance for individual data of *B. juncea* plants.

Plant characteristics	Clipping	Position	Percentage of NC	Clip. × Pos.	Clip. × Per.	Pos. × Per.	Clip. × Pos. × Per.
First experiment							
Leaf number	0.03 ^{ns}	0.03 ^{ns}	0.95 ^{ns}	0.05 ^{ns}	3.45 [*]	0.57 ^{ns}	0.32 ^{ns}
Flowering date	0.89 ^{ns}	0.05 ^{ns}	1.34 ^{ns}	0.04 ^{ns}	2.47 ^{ns}	1.00 ^{ns}	1.54 ^{ns}
Plant weight	30.31 ^{***}	62.06 ^{***}	4.34 ^{**}	2.26 ^{ns}	0.02 ^{ns}	0.96 ^{ns}	3.89 [*]
Silique number	22.63 ^{***}	52.33 ^{***}	2.97 [*]	1.41 ^{ns}	0.44 ^{ns}	0.87 ^{ns}	2.47 ^{ns}
Seed number	25.14 ^{***}	35.56 ^{***}	3.20 [*]	1.19 ^{ns}	0.30 ^{ns}	1.16 ^{ns}	3.69 [*]
Seed weight	35.12 ^{***}	15.23 ^{***}	6.22 ^{***}	1.76 ^{ns}	0.11 ^{ns}	1.74 ^{ns}	4.73 [*]
Biomass	32.60 ^{***}	52.30 ^{***}	4.93 ^{**}	2.21 ^{ns}	0.02 ^{ns}	1.11 ^{ns}	4.27 [*]
Germination	0.21 ^{ns}	18.30 ^{***}	3.34 [*]	0.01 ^{ns}	2.52 ^{ns}	0.45 ^{ns}	3.99 [*]
Number of viable seeds	24.99 ^{***}	29.87 ^{***}	4.21 ^{**}	0.64 ^{ns}	0.35 ^{ns}	1.05 ^{ns}	4.39 [*]
Reproductive index	0.18 ^{ns}	60.74 ^{***}	1.92 ^{ns}	0.01 ^{ns}	0.09 ^{ns}	0.16 ^{ns}	0.55 ^{ns}
Number of seeds per silique	5.95 [*]	13.16 ^{***}	0.58 ^{ns}	0.13 ^{ns}	0.24 ^{ns}	2.70 [*]	2.29 ^{ns}
Second experiment							
Leaf number	0.58 ^{ns}	0.79 ^{ns}	0.93 ^{ns}	0.18 ^{ns}	0.50 ^{ns}	0.66 ^{ns}	0.10 ^{ns}
Flowering date	1.99 ^{ns}	1.18 ^{ns}	1.12 ^{ns}	0.12 ^{ns}	0.31 ^{ns}	0.51 ^{ns}	0.61 ^{ns}
Plant weight	3.67 ^{ns}	50.51 ^{***}	0.19 ^{ns}	0.23 ^{ns}	1.40 ^{ns}	0.13 ^{ns}	0.66 ^{ns}
Silique number	4.10 [*]	36.64 ^{***}	0.07 ^{ns}	0.23 ^{ns}	1.38 ^{ns}	0.08 ^{ns}	0.41 ^{ns}
Seed number	2.48 ^{ns}	33.11 ^{***}	0.17 ^{ns}	0.10 ^{ns}	1.41 ^{ns}	0.07 ^{ns}	0.37 ^{ns}
Seed weight	4.61 [*]	36.97 ^{***}	0.27 ^{ns}	0.23 ^{ns}	1.20 ^{ns}	0.17 ^{ns}	1.03 ^{ns}
Biomass	3.99 [*]	49.37 ^{***}	0.18 ^{ns}	0.24 ^{ns}	1.38 ^{ns}	0.12 ^{ns}	0.75 ^{ns}
Germination	0.03 ^{ns}	0.09 ^{ns}	2.72 [*]	0.10 ^{ns}	1.24 ^{ns}	3.90 ^{**}	0.84 ^{ns}
Number of viable seeds	2.20 ^{ns}	32.70 ^{***}	0.17 ^{ns}	0.08 ^{ns}	1.51 ^{ns}	0.08 ^{ns}	0.33 ^{ns}
Reproductive index	0.66 ^{ns}	5.06 [*]	1.44 ^{ns}	0.03 ^{ns}	0.03 ^{ns}	0.18 ^{ns}	0.26 ^{ns}
Number of seeds per silique	0.01 ^{ns}	4.33 [*]	1.89 ^{ns}	0.01 ^{ns}	1.02 ^{ns}	0.69 ^{ns}	1.55 ^{ns}

Levels of significance: ns, non significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Table 3.6. Parameters of significant linear regression ($Y=A+B*X$) of flowering date, seed weight, biomass and number of viable seeds in terms of the percentage of NC plants (see Fig. 3.6).

Experiments	Traits	Position	NC/ CP	A	B	R²	F	P
The first	Seed weight	Border	NC	3.72	-1.23	0.23	4.26	0.06
		Center	NC	3.96	-1.92	0.30	5.92	0.03
		Center	CP	2.12	-1.36	0.55	16.8	0.001
	Biomass	Border	NC	19.93	-6.92	0.21	3.75	0.07
		Center	NC	16.14	-8.24	0.35	7.37	0.02
		Center	CP	7.46	-4.20	0.32	6.53	0.02
	No. viable seeds	Border	NC	3238	-1401	0.41	9.51	0.008
		Center	NC	2304	-1374	0.22	3.84	0.07
		Center	CP	1288	-825	0.36	7.93	0.01
The second	Flowering date	Center	NC	44.76	3.31	0.21	3.60	0.08

Table 3.7. Results of Helmert contrast test of the relative difference between NC and CP [DI= (NC_{Ti}-CP_{Ti})/CP_{T0}] in plot center and border of the first (A for center; B for border) and the second experiment (C for center; D for border)

Contrasts	Percentage of NC				t-values			
	25%	50%	75%	100%	A ^a	B	C	D
1	-1	1	0	0	-7.17 ^{***}	1.79 ^{ns}	-12.42 ^{***}	-0.37 ^{ns}
2	-1	-1	2	0	3.21 [*]	-11.40 ^{***}	-1.16 ^{ns}	5.85 ^{***}
3	-1	-1	-1	3	-20.25 ^{***}	-7.27 ^{***}	-0.41 ^{ns}	-7.02 ^{***}

Levels of significance: ns, non significant; *, P<0.05; **, P < 0.01; ***, P < 0.001.

^a Letters are consistent with that in Fig 5

Table 3.8. F values of the analysis of variance of per-plot data of *B. juncea*.

Factors	DF	Seed weight	Biomass	No. viable seeds
The first experiment				
Position	1	132.41 ^{***}	189.66 ^{***}	138.72 ^{***}
Percentage	4	1.01 ^{ns}	0.58 ^{ns}	0.38 ^{ns}
Position × Percentage	4	0.27 ^{ns}	0.34 ^{ns}	0.11 ^{ns}
The second experiment				
Position	1	156.91 ^{***}	172.89 ^{***}	112.09 ^{***}
Percentage	4	0.81 ^{ns}	0.36 ^{ns}	0.71 ^{ns}
Position × Percentage	4	0.20 ^{ns}	0.22 ^{ns}	0.10 ^{ns}

Levels of significance: ns, non significant; ***, $P < 0.001$

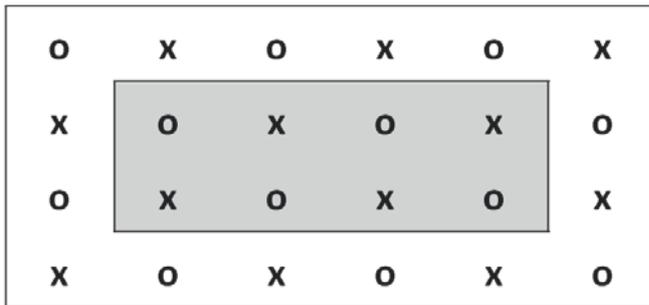
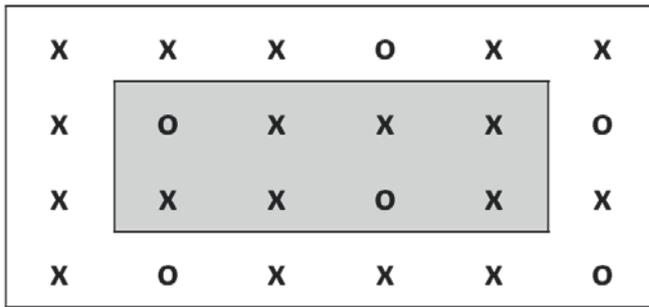


Fig. 3.4. Examples of experimental designs for different percentage of NC plants: T₂₅ (top: 'O' is NC, and 'X' is CP), T₅₀ (bottom), and T₇₅ (top: 'O' is CP, and 'X' is NC). Grey region indicates plot center, and white region is plot border.

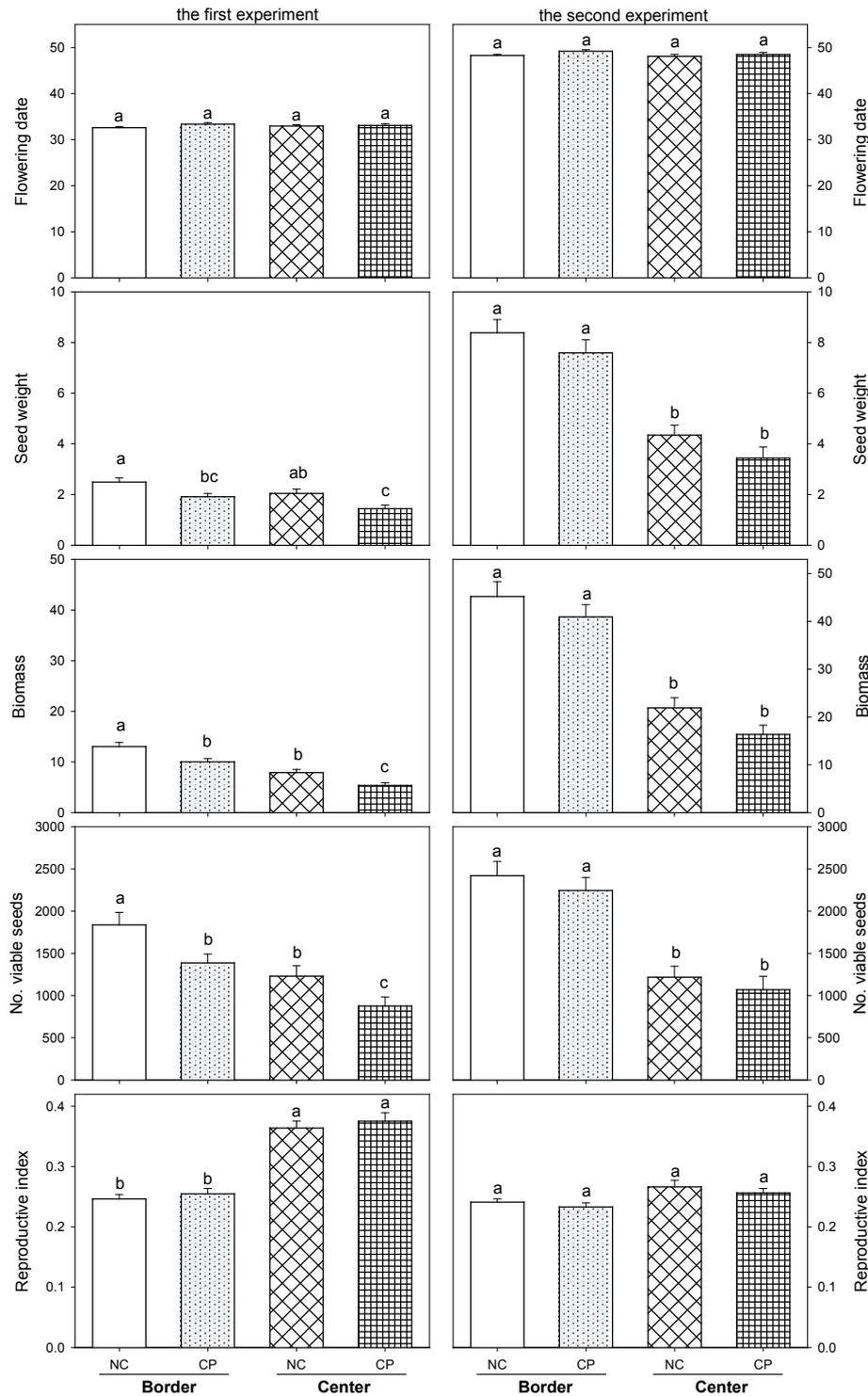


Fig. 3.5 Mean values of flowering date, seed weight, biomass and number of viable seeds of NC and CP plants in the border and center of plots in the first experiment (left column) and the second experiment (right column). Vertical bars denote SE of the mean. Different letters indicate significant differences between mean values according to Tukey's HSD ($\alpha = 0.05$) and Contrast tests.

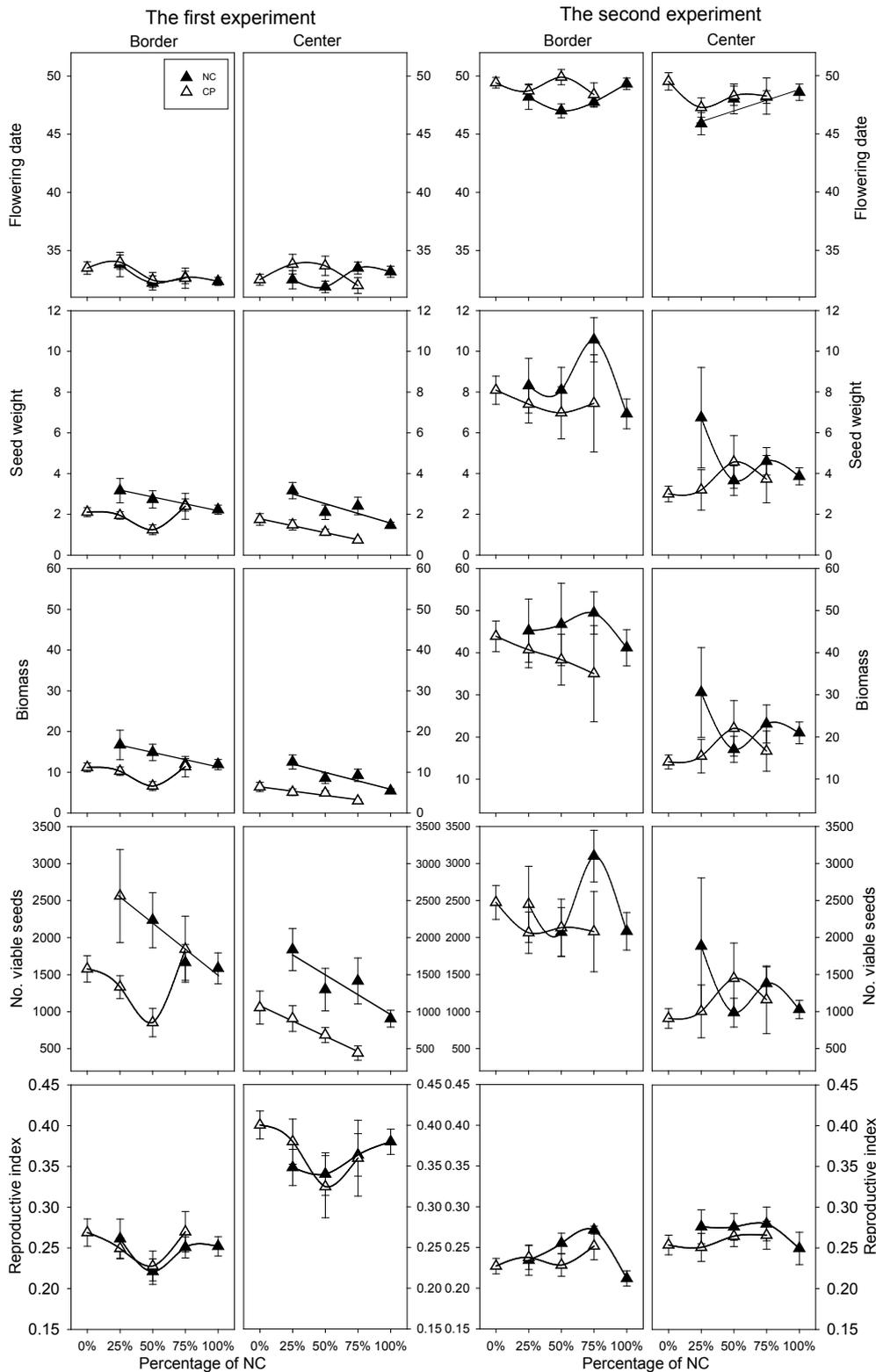


Fig. 3.6. Mean values and linear regressions of flowering date, seed weight, biomass, number of viable seeds and allocation of resources to reproduction (Index=seed weight/ plant weight) in terms of the different percentage of NC plants for NC (full symbols) and CP plants (empty symbols) in the plot border (left column) and center (right column) in the first experiment (left group) and the second experiment (right group). Vertical bars denote SE of the mean.

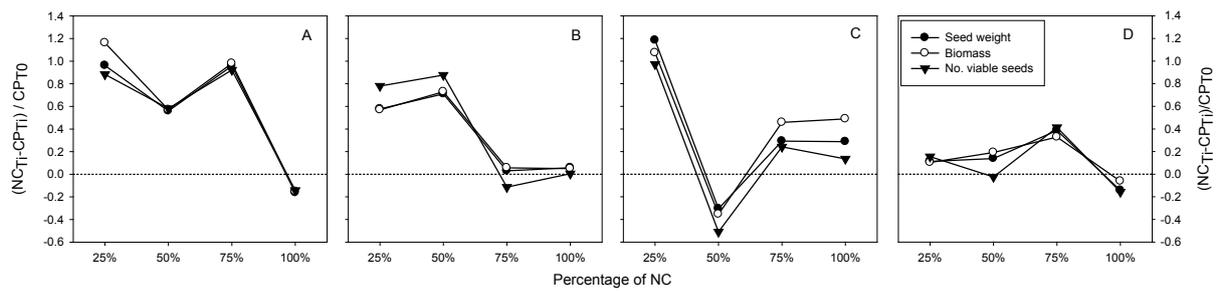


Fig. 3.7. Relative difference between CP and NC plants ($DI = (NC_{Ti} - CP_{Ti}) / CP_{T0}$) according to NC percentage for three plant characteristics in four conditions with decreasing competition intensity from left (A) to right (D): plot center (A) and border (B) of the first experiment, and plot center (C) and border (D) of the second experiment. (Value at T_{100} is calculated as $DI = (NC_{T100} - CP_{T0}) / CP_{T0}$).

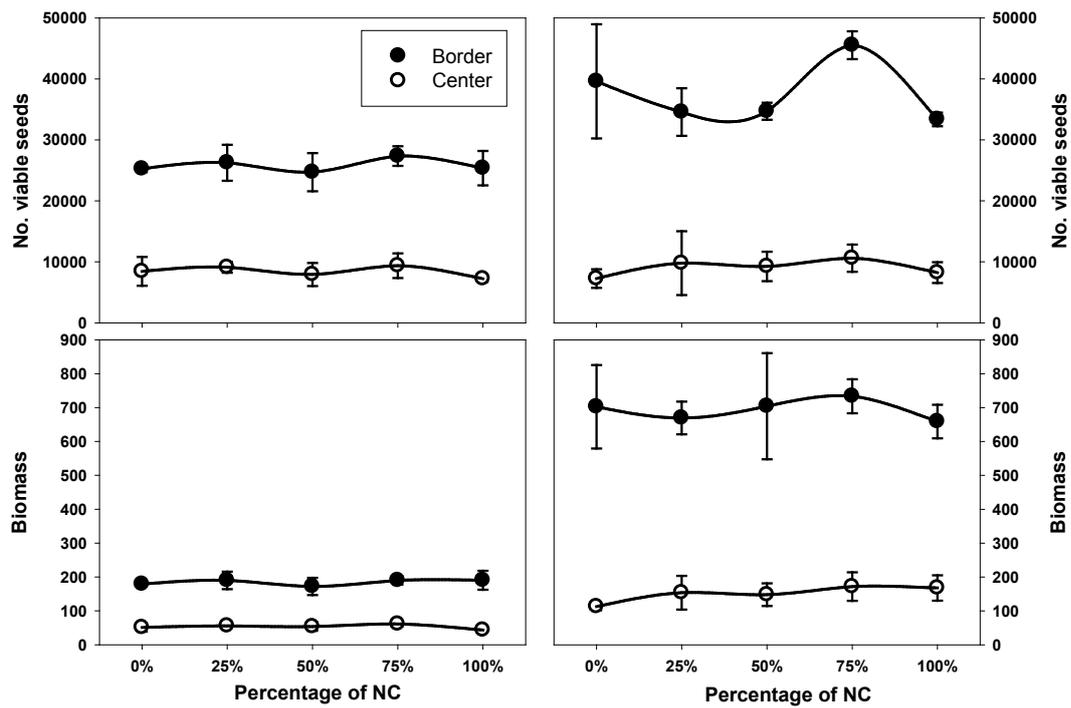


Fig. 3.8. Total number of viable seeds and biomass per plot against NC percentage in plot border and center in the first experiment (left column) and the second experiment (right column). Vertical bars denote mean SE.

3.4 Article 5: Effet de la proportion relative de *B. juncea* résistants aux insectes (gène Bt) sur la fitness des populations

Une génération B C₂ a été obtenue en rétrocroisant de la moutarde avec les hybrides transgéniques ou non décrits dans l'article 1. Les BC₂ transgéniques (trBC2) et non-transgéniques (ntrBC2) ont été semés à différentes proportions dans des cages où des chenilles ont été introduites tardivement, quand les premières fleurs s'ouvraient. Hauteur, biomasse, nombre et poids de semences ont été mesurés.

La présence des insectes a réduit la croissance des plantes mais n'a pas affecté la reproduction. Nous n'avons pas observé de différences significatives entre les BC₂ transgéniques et non transgéniques quelque soit la présence des insectes. Cependant, les deux types de plantes ont vu leurs performances augmenter avec la proportion de résistants transgéniques en présence des insectes seulement. La production totale par cage a augmenté dans les mêmes conditions.

Ces résultats montrent que le gène Bt n'a pas d'effet négatif observable sur la fitness des résistants en l'absence d'insectes et qu'il ne devrait pas modifier la dynamique des populations de moutardes. Cependant, comme dans l'expérience en serre, mais au contraire de la simulation au champ, la pression de sélection par les insectes conduit à une augmentation globale de la productivité de la population qui pourrait la rendre plus invasive et disséminer encore plus facilement le transgène hors des milieux cultivés. Les différences enregistrées dans nos différentes expériences pourraient cependant être dues à différents niveaux de ressources, de densité de plantes ou de dommages causés par les insectes, au cas où les expériences n'étant finalement plus proche qu'une autre des conditions réelles du champ.



Photo 3.3. Cotton bollworm (*Helicoverpa armigera* H.) were put on plants in cages

ARTICLE 5

EFFECTS OF THE RELATIVE PROPORTION OF *Bt*-TRANSGENIC PLANTS ON POPULATION PRODUCTION AFTER THE TRANSGENE INTROGRESSION

Yongbo LIU, Henri DARMENCY, C. Neal STEWART Jr, KePing MA, Wei WEI

Abstract

- The dynamics of populations composed of insect-resistant plants received few attentions, although it determines the invasive potential of both the resistant plants and the populations containing resistant plants.
- Nearly isogenic backcrossed generations formed between wild *Brassica juncea* and *Bt*-transgenic oilseed rape (*B. napus*) were cultivated in a common garden, with pure and mixed populations of insect-resistant and/or susceptible plants under insect pressure and control.
- Insects significantly decreased vegetative growth but did not affect reproduction in term of both individual plants and populations. With increasing percentage of insect-resistant plants, in the presence of insects, both resistant and susceptible plants had slightly increased productivity. The population production increased with increasing percentage of transgenic plants in populations under insect pressure. It was not the case in the absence of insect when a fitness cost occurred for the transgenic plants at transgenic percentage of 50%.
- These results imply that the occurrence of insect attacks is the major determinant of the dynamics of mixed resistant and susceptible populations, a feature whose frequency is quite different in cultivated and wild habitat, which could hamper the spread of the *Bt*-transgenes among wild populations.

Key words: transgenic oilseed rape, *Bt*, population production, fitness

Introduction

Spontaneous introgression from crops to their relatives should be evaluated as a component of the risk assessment for the release of genetically modified (GM) crops (Darmency 1994; Ellstrand et al. 1999). Several studies on advanced-generation hybrids find that some interspecific hybrid genotypes persist for several generations under certain conditions, which suggests that long-term introgression could occur under natural environments (Linder et al. 1998; Lexer et al. 2003; Al Mouemar and Darmency 2004; Warwick et al. 2008).

Studies on the direct fitness effects of transgenes on introgressed progeny aid in assessing their phenotype and their effect on the dynamics and demography of the introgressed populations. The rate of transgene spread is mainly governed by fitness effects (Rieseberg and Burke 2001). However, most experimental studies only involved F_1 hybrids that may display either heterosis, a transient condition that may overestimate the probability of persistence of crop genes within wild populations (Arnold and Hodges 1995; Lexer et al. 2003), or outbreeding depression and near sterility, a transient condition that may underestimate the probability of persistence of crop genes within wild populations (Burke and Arnold 2001). Such interacting effects are reduced in advanced generations of backcrossed progeny that are better suited material to estimate the benefits and costs of an introgressed transgene in a wild population.

As transgene introgression will inevitably lead to populations composed of both transgenic and non-transgenic progeny (Vacher et al. 2004), the fecundity and resulting evolutionary impact of crop-wild introgressed offspring may depend on their competition ability (Campbell and Snow 2007). Transgenic plants generally are expected to be a competitive advantage when transgenes involve fitness-related traits, like the *Bacillus thuringiensis* (Bt), insect-resistance transgene. Wild plants receiving the Bt gene are likely to display insect-resistance and less herbivore damage as Bt crops do, which in turn confers greater plant fitness (survival, growth and fecundity potentiality) compared with insect-susceptible plants (Stewart et al. 1996; Ramachandran et al. 2000; Snow et al. 2003; Vacher et al. 2004; Moon et al. 2007). As the fitness advantage of insect-resistance allows higher competition, the resistant plants are expected to suppress the growth of susceptible ones in the populations in which the transgene moves in. However, the dynamics of mixed populations including resistant and susceptible plants might be a complex process, depending on

interacting factors such as genetic background, intensity of herbivory, resource level and relative abundance of resistant plants (Verkaar 1987).

Studies on the dynamics of mixed populations involving resistant and susceptible plants and their possible change from a pure susceptible population to a pure resistant population are scant (Ramachandran et al. 2000). In addition, it is not clear if results from a given interspecific cross can be validated in the case of other wild species with different growth habit. *Brassica juncea* ($2n=36$, AABB), the wild brown mustard, is closely related to *B. napus* ($2n=38$, AAC), the oilseed rape, and both are polyploids. They easily intercross and produce viable progeny (Frello et al. 1995; Song et al. 2010; Liu et al. 2010a). In this study, we aimed at detecting if the fitness advantage conferred by the Bt transgene to wild populations of *B. juncea* varies according to its frequency within the population. Backcrossed Bt and non-Bt hybrid progeny were employed to detect the vegetative and reproductive output of mixed population with various proportions of nearly isogenic resistant and susceptible plants, and under no insect and insect pressure.

Material and methods

Plants

Seeds of wild brown mustard (*Brassica juncea* var. *gracilis*, $2n = 36$, AABB) originated from a local field collection provided by Nanjing Agricultural University (NAU). Transgenic oilseed rape (*B. napus* cv. Westar, $2n = 38$, AAC) was obtained after transformation by pSAM12 plasmid containing genetically linked GFP and Bt Cry1Ac cassettes (GT) regulated by independent CaMV 35S promoters (Halfill et al. 2001). The third generation of the progenies of the transgenic event 1 was used in this study. The non-transgenic oilseed rape parent (Westar), a spring type, was also employed. Transgenic and non-transgenic F₁ hybrids were formed from wild mustard as female fertilized by the transgenic and the Westar parent, respectively. Backcrossed generations BC₂ were obtained through pollinating mustard as the female by pollen of hybrids.

Field experiment

Medium seeds ($1.2\text{mm} < \text{seed diameter} < 1.6\text{ mm}$) of BC₂ were germinated in a greenhouse in Beijing on 19 April 2010. Seedlings at four-leave stage were checked with GFP fluorescence

screening as transgenic BC2 (trBC2) and non-transgenic BC2 (ntrBC2), and then transplanted in cages protected by a mesh nylon-net (0.5m * 0.5m width * 1.5 m height) in a common garden. The trBC2 and ntrBC2 were cultivated in pure stands or in mixtures containing 0, 25, 50, 75 and 100% of trBC2 (proportions P₀, P₂₅, P₅₀, P₇₅ and P₁₀₀, respectively). Eight plants were planted in a circle with a 0.3m diameter in each cage. The five proportions were randomly distributed with two replicates in a block, and there were four blocks. On May 28, when the first flower just open, there were 25% of plants had flowering bud (81 out of 320 plants), 30 cotton bollworm (*Helicoverpa armigera* H.) were put per plant in one cage of every proportion and block. Such an advanced leaf-stage was chosen because of the possibility of early-season damage-induced resistance (Agrawal 1998; 1999). Every plant was labeled. Plants were harvested when all siliques were matured in brown color, and plant height, biomass, seed weight and seed number was measured. Thousand-seed weight (seed weight*1000/ seed number) and reproduction allocation (seed weight/ biomass) was estimated.

Statistics

The mean value of all transgenic or non-transgenic plants in a cage was used as one value to statistical analysis. All data were log-transformed to ensure a Normal distribution of residuals. Five-way mixed ANOVA ($Y \sim I * T * P + F + B$) was employed to detect the fixed effects of insect (I), plant type (T) and proportion of trBC2 (P), and random effects of flowering (F, flowering or not at the date insects were deposited on plants) and blocks (B) on plant growth and reproduction. Linear regression function ($Y = a + b * X$) was used to study the measured variable in terms of the proportion of trBC2. The population production was evaluated as the sum of the contribution of all plants in a cage, including transgenic and non-transgenic plants. Linear regression function ($Y = a + b * X$) was also used to study the variation of the population production (biomass and seeds) against the proportion of transgenic plants.

Results

Growth and reproduction of trBC2 and ntrBC2 plants

The presence of insects (I) significantly decreased plant height, biomass and thousand-seed weight, but increased reproductive allocation, and did not affect seed number and weight (Table 3.9). TrBC2 and ntrBC2 did not have significantly different performances. There were

significant differences among the different proportions (P) of trBC2 for plant height, biomass, seed weight, seed number and reproductive allocation, but that was not the case for thousand-seed weight. The plant stage (F) when the insects were deposited had effects on biomass, seed weight and number, and thousand-seed weight, but not reproductive allocation: the values were lower for the early flowering plants. There were significant interactions between the presence of insects and plant type (I*T) for biomass, seed number and weight, and between the presence of insect and the proportion of transgenic plants (I*P) for biomass, seed number and weight and reproductive allocation. There was no interaction among the three controlled factors (Table 3.9).

Under no insect pressure, there was no relationship between the six variables and the proportion of trBC2 plants, whatever ntrBC2 or trBC2. A maximum value was observed for both types of plant at P50 (Fig. 3.9). Under insect pressure, both trBC2 and ntrBC2 showed a trend to increase their performances with increasing proportions of trBC2, but the regression was significant for seed weight ($P=0.05$) and for reproductive allocation in ntrBC2 ($P<0.001$) only. Increased seed number in both ntrBC2 ($P=0.07$) and trBC2 ($P=0.09$) could also be considered (Fig. 3.9).

Productivity of cages according to proportions of transgenic BC2

Insects significantly decreased the vegetative production (biomass) but did not affect reproductive production (seed number and weight) (Table 3.10). There were significant differences among the different proportions of trBC2 for reproduction but not for growth. There was no interaction between effects of insect and proportion of trBC2. In the absence of insect pressure, the variations of total vegetative and reproductive productions per cage were non-linear in terms of the proportion of trBC2, with a maximum at P50 (Fig. 3.10). Under insect pressure, vegetative and reproductive productions increased with increasing proportion of trBC2 in cages ($Y=3.24+0.86*X$, $R^2=0.31$, $P=0.011$ for biomass; $Y=8.51+1.26*X$, $R^2=0.32$, $P=0.010$ for seed number) (Fig. 3.10).

Discussion

Effects of insects on plant growth and reproduction

Insects significantly decreased plant growth but did not affect plant reproduction. In general, herbivory had a negative effect on plant fitness (Escarré et al. 1999; Rogers and Siemann

2002), but certain studies showed no effects because of compensatory growth (Hawkes and Sullivan 2001; Boalt and Lehtila 2007). In an experiment in which we simulated herbivory by clipping leaves of *B. juncea* we also observed compensatory growth at low herbivory level (Liu et al., 2009; 2010b). The effect of herbivory on plant growth and reproduction depends on lots of factors such as plant species (Rogers and Siemann 2002), resource level (Hawkes and Sullivan 2001; Rogers and Siemann 2002), induced insect-resistance and damage time (Agrawal 1998; 1999).

In the current study, trBC2 and ntrBC2 were exposed to insects only at plant late-growth stage so that it resulted in poor effects. The timing of the insect attack is important. Agrawal (1999) found that insect-resistance was induced by caterpillar herbivory at the fourth true leaf of wild radish, and this resistance persisted in newly formed leaves of damaged plants, reducing the mass of caterpillars feeding on induced plants compared to un-induced controls. Thus, the herbivory at early-growth stage of plants could induce the resistance to herbivory for insect-susceptible plants, which could underestimate the differences between resistant and susceptible plants. In this study, the late flowering plants showed higher fitness than the early flowering ones at the time of insect deposit. Most studies involving Bt insect-resistant focused on the effects of insects from the early-growth to mature, and they found insect-resistant transgenic plants showed higher fitness only under moderate or higher insect pressure (e.g. Ramachandran et al. 2000; Letourneau and Hagen 2009).

The impact of the insects on the seed weight and number decreased with the increased proportion of transgenic plants. In contrast, when plants were not subjected to insects, the growth and reproduction of both trBC2 and ntrBC2 showed apparently erratic variations, with a maximum in populations composed by one half of resistant plants. This genotype proportion in a limited resource environment could correspond to the spatial plant distribution that maximizes an eventual fitness difference between the transgenic and non-transgenic plants. In other words, in years without insect attack, the spread of transgenic plants would be stopped by a low reproduction value relative to the native susceptible plants if the transgenic proportion reaches 50%. Once over this threshold, the transgenes would not confer any reproduction cost, which open the way for its fixation in the populations. Under insect pressure, the growth and reproduction in both trBC2 and ntrBC2 showed linearly increasing trends with the increased proportion of transgenic plants. This could be interpreted by a protection effect applied to all the plants in a cage, perhaps because the insects leaved from

the damaged transgenic plants possibly inducing defense mechanisms and moved towards healthy and attractive transgenic plants. Alternatively, it could be a further evidence of the lower innate fitness of the transgenic plants, leaving more and more resources to their neighbor.

Population production with varied proportion of transgenic plants

Population production, biomass and seed output, increased with the percentage of transgenic BC2 in populations under insect pressure, perhaps because of the generalized protection effect as proposed above. It was not the case when the populations were not subjected to herbivory, again with a maximum of seed production at a 50% proportion of transgenic plants. The increased population production in the presence of insects is important to predict the increase of the colonizing ability of the *B. juncea* populations hosting more and more insect-resistant plants. Few studies focused on the dynamics of resistant plants in susceptible populations, although some studies showed that herbivory strongly affected population dynamics of plants (e.g. Palmisano and Fox 1997). Ramachandran et al. (2000) showed that insects decreased total biomass but did not affect seed yield in mixed and pure stands of Bt-transgenic and/or non-transgenic oilseed rape. They found that no significant differences occurred in total biomass and seed production when there was no insect, and that biomass and seed yield was significantly different among mixed plantings for insect-infested plots with a maximum at the proportion of 50% in two field experiments, but that it was not different in another field experiment in the presence of insects. In our experiments simulating insect-resistant plants through clipping leaves, the increase of healthy plants in a population of damaged plants did not increase the population production but resulted in a maximum seed output when healthy plants dominated the population (75% of healthy plants) (Liu et al. 2010b). Enhanced seed production could make a population more able to spread to neighboring habitats, even displacing other species suffering of insect damages. Thus, the occurrence of insect attack is the major determinant of the fate and dispersal of the transgene: while pests are over-frequent in arable lands covered by the same crop over hectares, the occurrence of such insect infestations is not so permanent in wild habitats, which could seriously hamper the spread of the Bt transgene in and among wild populations, as observed in the case of virus-resistance genes in squash (Fuchs et al. 2004a ; 2004b). Laughlin et al. (2009) showed that virus-resistance transgenes and conventional crop genes can increase fecundity of wild plants under virus pressure, and viruses play a role in the population dynamics of wild squash (*Cucurbita pepo*).

Acknowledgements

We thank Zhixi Tang, Xinjing and Hanbin Wu for their assistances in experiment manipulating. This work is supported by a project of the Natural Science Foundation of China (grant no. 30970432) and a PhD joint fellowship between China and France (CNOUS, No. 20072315).

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Table 3.9. F-values of four-way mixed ANOVA results for fitness of transgenic BC₂ in pure and mixed stands under insect and non-insect pressure (*, P<0.05; **, P<0.01; ***, P<0.001)

	DF	Plant height	Biomass	Seed number	Seed weight	TSW ^a	RA ^b
Insect (I)	1	10.71**	14.78***	1.43	0.74	5.38*	38.32***
Plant type (T)	1	0.07	1.84	0.04	0.12	0.87	1.13
Proportion (P)	4	7.64***	3.05*	8.51***	9.98***	1.03	11.79***
Flowering	1	2.88	7.62**	6.82**	4.75*	20.98***	0.14
I*T	1	0.74	9.17**	4.68*	5.00*	0.03	0.26
I*P	4	1.95	2.58*	3.08*	5.52***	0.37	6.42***
T*P	2	1.37	0.03	0.52	0.57	2.34	0.99
T*I*P	2	2.57	2.18	0.02	0.03	0.44	1.76

^a TSW, thousand-seed weight; ^b RA, reproductive allocation

Table 3.10. F-values of three-way ANOVA results for per-cage data of all trBC2 and ntrBC2 plants in pure and mixture stands under insect pressure and control (*, P<0.05; **, P<0.01)

	DF	Biomass	Seed number	Seed weight
Insect (I)	1	7.45**	0.02	0.07
Proportion (P)	4	2.62	3.34*	3.64*
I*P	4	1.42	1.64	1.17

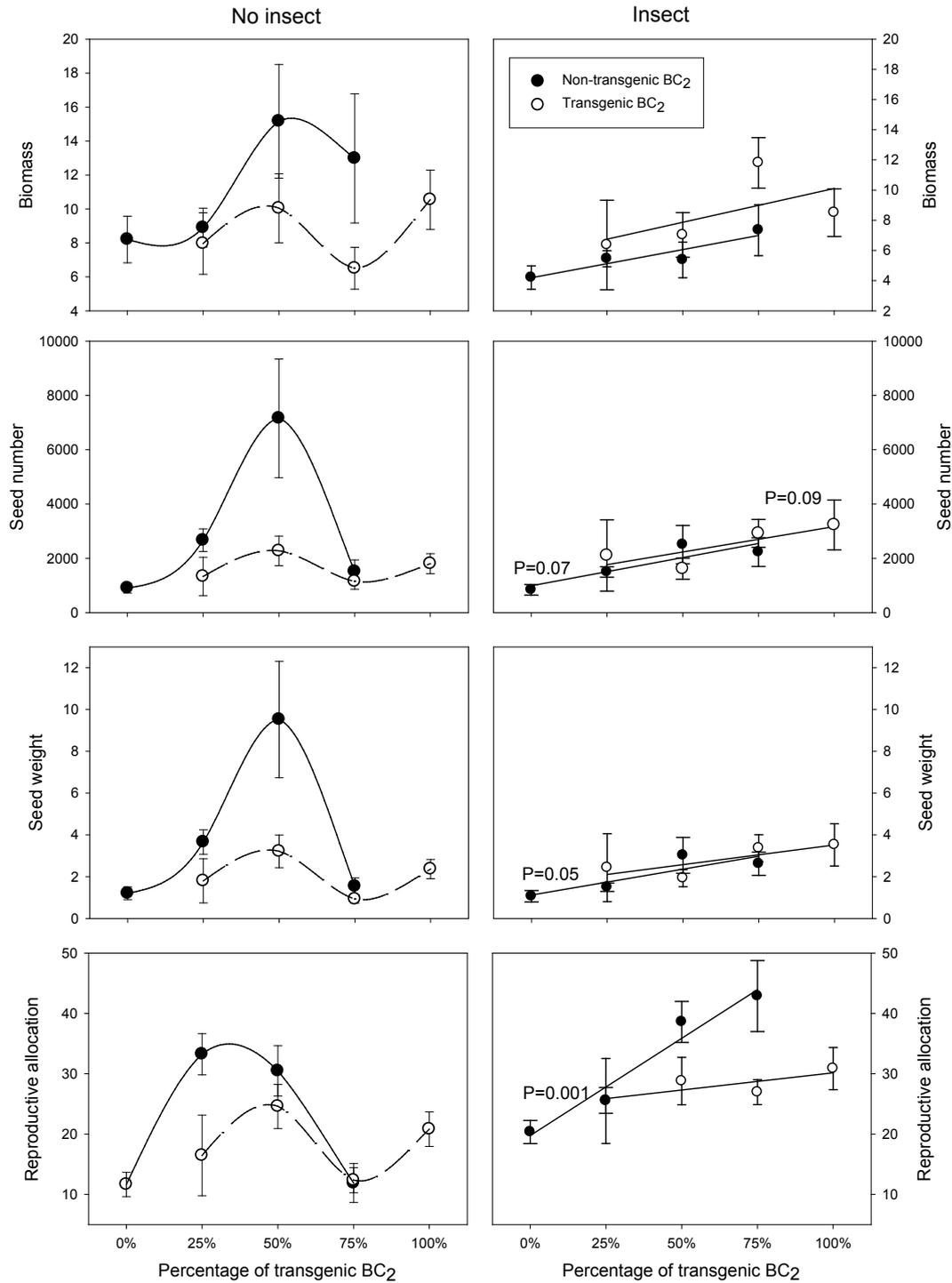


Fig. 3.9. Biomass, seed weight and number and reproductive allocation of transgenic trBC₂ and non-transgenic ntBC₂ according to the proportion of trBC₂ under non-insect pressure (left column) and presence of insects (right column). Smooth lines were added in the case there was no insect, while the P values of linear regressions are given at left side for ntrBC₂ and at right side for trBC₂ in the case of insect pressure.

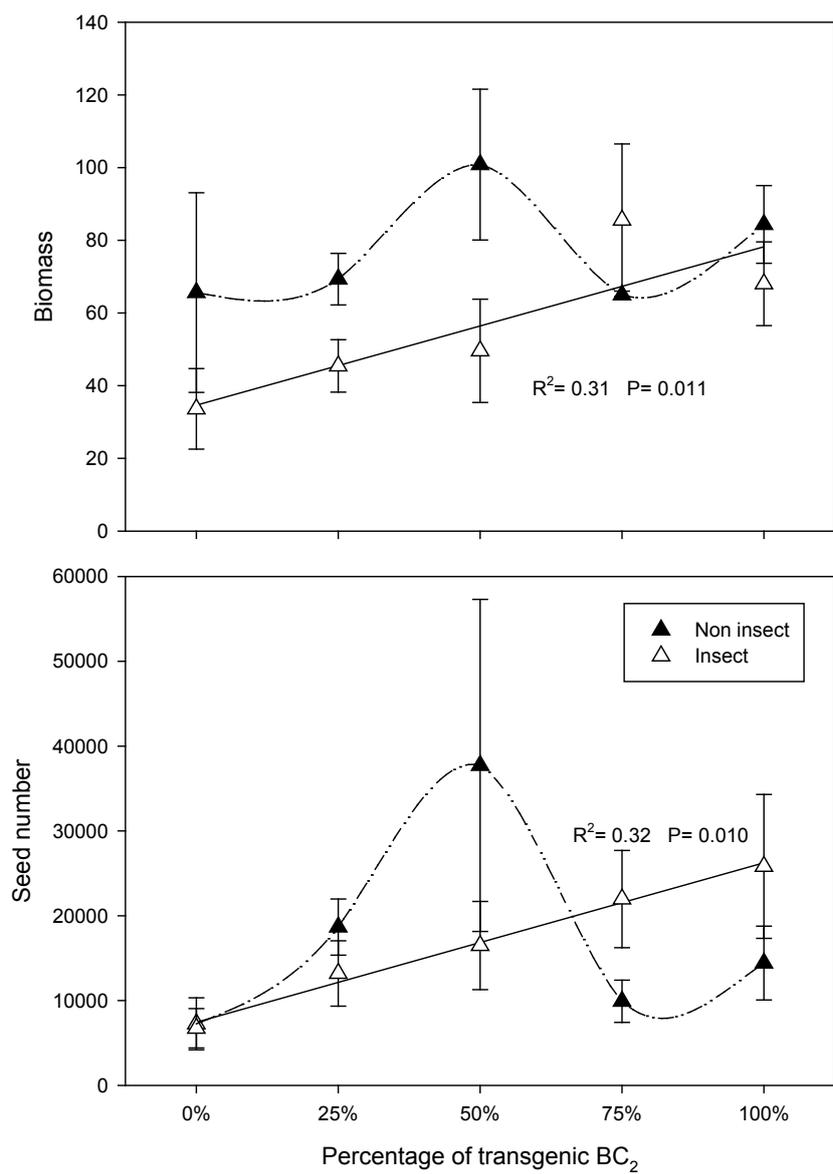


Fig. 3.10. Population production of both ntrBC₂ and trBC₂ per cage (mean value and standard error of four blocks) in pure or mixture cages under insect or non-insect pressure

Chapter 4

Recherche des consequences d'introggression ancienne

CHAPTER 4

RECHERCHE DES CONSÉQUENCES D'INTROGRESSION ANCIENNE

4.1 Introduction

L'étude des conséquences d'introgessions anciennes et stabilisées reste le but ultime des études liées aux hybridations interspécifiques car le flux de gène entre les cultures et leurs apparentés sauvages peut modifier les traits d'histoire de vie et la trajectoire évolutive des populations sauvages (Lee and Snow 1998; Snow et al. 2001; Lenormand 2002; Campbell et al. 2009a). Ceci peut conduire à des populations montrant des particularités biologiques, voire des dynamiques modifiées. Cependant, il est difficile de détecter de telles conséquences expérimentalement à cause du manque de matériel transgénique sauvage approprié et les contraintes temporelles.

En fait, le flux de gène et l'introgession sont des phénomènes naturels qui ont façonné en partie de nombreuses plantes cultivées que nous connaissons aujourd'hui, mais qui ont également touché en retour leurs apparentés sauvages. La recherche de telles introgession et de leurs conséquences sur la biologie des populations sauvages, même en l'absence de transgène, peut apporter un éclairage inestimable sur les potentialités offertes aux plantes sauvages par ce moyen et le rôle que pourraient avoir les transgènes dans le futur.

Dans ce chapitre, nous abandonnons les moutardes pour examiner le cas d'une autre *Brassicée*, la ravenelle, *Raphanus raphanistrum*. À l'aide de marqueurs moléculaires, des chercheurs de l'INRA de Rennes ont détecté la possibilité d'introgession de gènes des groupes de linkage C1 et C9 du colza dans quelques populations de ravenelles originaires de Normandie, une région où le colza a été cultivé depuis au moins 200 ans (Chèvre, non publié). La durée des échanges possibles entre les deux espèces étant une condition favorable à l'introgession, nous avons cherché à savoir si ces populations montraient des différences remarquables par rapport à d'autres provenant de régions où le colza a été cultivé plus récemment (**Article 6**).

4.2 Article 6 : Le rôle d'une longue coexistence entre colza et ravenelle sur la morphologie et les caractéristiques de populations de *Raphanus raphanistrum*

A part les populations de Normandie citées ci-dessus, nous avons utilisé des populations de ravenelle échantillonnées en Bretagne, une région voisine, et en Bourgogne et au Danemark qui en sont géographiquement éloignées. Les caractéristiques des fleurs et des siliques ont été mesurées, et les plantes ont été cultivées ensemble dans un champ de colza ou à faible densité en cage afin de comparer leur capacité de croissance et de reproduction.

Les caractéristiques morphologiques des siliques et des fleurs permettent de discriminer les populations des quatre régions. Les populations de Normandie semblent les plus polymorphes mais se situent dans la moyenne de la variabilité totale observée. Aucun hybride avec le colza n'a été trouvé pour aucune des populations. Des différences très significatives de croissance et de reproduction ont été trouvées entre les plantes des différentes régions quand elles poussaient dans le colza, mais beaucoup moins et avec un autre classement relatif lorsqu'elles poussaient en cages à plus faible densité et mieux pollinisées par les insectes que nous apportions. Il est remarquable que les populations locales étaient les moins productives.

Ces résultats montrent la variabilité des populations de ravenelle. Les différences observées ne correspondent pas systématiquement à des distances géographiques. Elles pourraient aussi être expliquées par des introgressions, ce dont nous discutons les indices dans l'article. Dans tous les cas, les populations de Normandie ne se démarquent pas des autres d'une manière particulière telle qu'une éventuelle introgression ait pu modifier leur évolution depuis 200 ans. Il se pourrait cependant que seules les plantes possédant des gènes introgressés puissent montrer des différences, ce qui est en cours d'étude.



Photo 4.1. Wild radish in a herbicide-resistant oilseed rape field



Photo 4.2. Wild radish sampled from different regions were cultivated together in cages

ARTICLE 6

The role of long-term coexistence with oilseed rape on the morphological and life-history trait variability of *Raphanus raphanistrum* populations

Yongbo LIU, Wei WEI, KePing MA, AM CHEVRE, Henri DARMENCY

Summary

- Plant divergence and adaptation is challenged by the occurrence of gene flow and introgression from related species. A particular case consists in gene flow from genetically modified (GM) crops to their wild relatives.
- Wild radish (*Raphanus raphanistrum*) populations were sampled from geographically distant regions, of which one had a long history of oilseed rape cultivation. Plants were cultivated together, and their morphological, growth and reproductive traits were measured.
- The morphological traits discriminated the wild radish populations from the different regions. The populations were different for growth and reproduction amongst them, but the differences changed when the plants were grown in an oilseed rape field or in intra-specific competition condition.
- The geographic distance cannot account for all the differences among regions. There is no direct evidence of introgression with oilseed rape, but this hypothesis could fit the data. The longer coexistence time one wild radish populations with oilseed rape in one region had no dramatic effect on the studied traits.

Key words: introgression, life history, morphology divergence, natural selection, wild radish

Introduction

Plant divergence is generated by isolation by distance (genetic drift) or by changes in mating systems and adaptation driven via natural selection (Hey 2006; Ross-Ibarra et al. 2009; Zheng and Ge 2010). In contrast, gene flow plays a key role in maintaining cohesion among geographically separated populations and makes species tend to evolve as units (Morjan and Rieseberg 2004; Abbott and Comes 2007; Hey 2006; Nosil 2008). Introgression of wild plants by genes from crops could result in poor divergence when gene flow is strong enough, with no correlation between genetic differences and geographic distances (Kercher and Conner 1996; Sahli et al. 2008).

Although it is a natural phenomenon associated to crop development, gene flow and introgression from crops to their wild relatives receive now increasing attention in the framework of the commercial release of genetically modified (GM) crops. Because GM crops confer new traits, such as resistance to insects, herbicides and diseases, they could result in undesired consequences of eventual transgene introgression through the dispersal of interspecific hybrids and their progeny. Introgression after hybridization between GM crops and their wild relatives may result in increasing: (1) genetic variation that promotes adaptive evolution (Rattenbury 1962; Postma and Van Noordwijk 2005), (2) weediness of wild species (Darmency 1994; Pilson and Rendeville 2004), (3) competitiveness or colonizing abilities through hybrid vigor or new traits (Ellstrand et al. 1999; Ramachandran et al. 2000), (4) invasiveness via enhanced fitness (Vacher et al. 2004), and (5) ability to match new environment (Campbell et al. 2006; Whitney et al. 2006), which eventually could result in the displacement of natural community equilibria, including the elimination of desirable species through competition (Tiedje et al. 1989).

Several studies have found long-term introgression from crops to their wild relatives, such as in rice (Suh et al. 1997), radish (Snow et al. 2001; Hedge et al. 2006), oilseed rape (Hansen et al. 2001) and sunflower (Whitton et al. 1997; Linder et al. 1998). The crop-wild introgression may alter morphological and life history traits as well as the evolutionary trajectory of wild populations (Lee and Snow 1998; Snow et al. 2001; Lenormand 2002; Campbell et al. 2009a), which could lead to populations exhibiting peculiar traits and population dynamics. In addition, local natural selection exerted on new genetic backgrounds could make introgressed wild populations to converge to or diverge from their parental taxa,

depending on whether phenotypes are beneficial or deleterious, or even to develop new life attributes and strategy. For example, in North America, pollinator-mediated selection has been found to be acting on floral traits such as stamen dimorphism, anther exertion, and flower size of wild radish (Conner et al. 1996; 2003).

Wild radish (*Raphanus raphanistrum*, RrRr, 2n=18) is a member of the Brassicaceae, a family of world-wide distribution. It is a self-incompatible, annual to biennial broadleaved species, growing on deep and rather low-pH soils. Wild radish has successfully colonized a variety of locations, leading to its naturalization on all continents except Antarctica (Holm et al. 1997), and it is often a major agricultural weed (Sahli et al. 2008). Studies on the geographic distribution of the genetic variability and morphological traits have shown that divergences among wild radish populations did not correspond to a geographic pattern due to distance (Tokunga and Ohnishi 1992; Kercher and Conner 1996; Sahli et al. 2008). Kercher and Conner (1996) have tentatively explained this by: (1) the self-incompatibility of wild radish that promoted gene flow, (2) the long-distance movement of radish seeds, and (3) the introgression from the cultivated radish (*R. sativus*).

There are some evidences that the long-term introgression from cultivated radish to wild radish has caused concerns on morphology and reproduction divergences of introgressed descendants (Kercher and Conner 1996; Snow et al. 2001; Campbell et al. 2006; Hedge et al. 2006). Campbell et al. (2006) showed that hybrid radish populations after crop gene introgression had greater fecundity and survival than wild populations, which could eventually lead to the replacement of their parents if the introgression succeeded. Using morphological and allozyme evidences, Hedge et al. (2006) found that hybrids-derived descendants supplanted the two local *Raphanus* species in California, suggesting the aggressive colonizing behavior of the hybrids, and that California wild radish separated from both of its parents as an evolutionary entity. The flowering phenology and leaf length of these plants were intermediate between those of the wild and cultivated radishes (Hedge et al. 2006), indicating introgression-caused morphology divergence.

In this study, we wonder if hybridization of wild radish with oilseed rape, *Brassica napus*, could result in long term introgression and morphological divergence. Spontaneous hybridization between these two species was demonstrated to occur at low frequency (Darmency et al. 1998; Chèvre et al. 2000) although its success could depend on the genotype

of the population of wild radish (Guéritaine and Darmency 2001; Guéritaine et al. 2003; Ammitzbold and Jorgensen 2006). Advanced backcross generations (BC6) resemble closely typical wild radish (Guéritaine et al. 2002) and the resilience of a transgene encoding for a herbicide-resistance was shown to be stable over eleven generations under selection pressure, although not introgressed (i.e. located on a supernumerary chromosome, Al Mouemar and Darmency, 2006). For these purposes, wild radish populations were collected from the Normandy region (France) where oilseed rape was cultivated for more than 200 years. Introgression, if any, is likely to occur in locations where co-occurrence of the two species spanned for the longer time. The putative introgressed populations (Normandy), populations from an adjacent region (Brittany) and others from two distant regions (Burgundy and Denmark) where oilseed rape cultivation is recent were cultivated together to study the morphological, growth and reproductive differences among them. The objective was to detect the roles of introgression opportunities and geographic distance in the divergence of morphologic traits, and whether the putative introgressed populations have a particular behavior in the field.

Materials and methods

Plant material

Wild radish populations were sampled in fields in three regions in France, two in Brittany (near Rennes: BT), two in Burgundy (near Dijon: BG1 and BG2), three in Normandy (near Caen: NM1, NM2, NM3), and one in Denmark (DM, provided by RB Jorgensen) (Fig.4.1). The cultivation of oilseed rape near Caen, in Normandy, is established by records on Communal Archives since 1802, and then it has been continuously grown in the region (D. Poulain, unpublished data), while oilseed rape is a recent crop in the other regions, at best for 40 years (X. Pinochet, unpublished CETIOM data). Thirty plants of each population were grown in Dijon in 2008 in separated insect-proof cages (3m length× 3m width× 2m height) continuously provided with house flies, *Musca domestica*, in order to facilitate random pollination. The seeds harvested in each cage were used for the following experiments.

Silique and flower morphology

The fruit of wild radish is a silique with a slender beak, constricted between seeds; it is easy to break into distinct segments (articles) when mature, and each article containing one seed. Silique characteristics in the seven cages in 2008 were measured, including diameters of largest and smallest articles, diameter of largest and smallest constrictions, number and total length of articles per silique, silique length (including the beak) and silique weight (Fig. 4.2). Beak length (silique length- total length of articles per silique) and article weight (silique weight/ number of articles per silique) were estimated. Mean values of diameters of the largest and smallest articles and constrictions were calculated as article diameter and constriction diameter, respectively.

A sample of seeds dehulled from articles of each population was germinated in greenhouse and grown in pots placed at random at 15-25°C under natural light in 2009. The flower characteristics of three to six flowers per plant and 29-42 plants per region were measured, including length and width of two adjacent petals per flower. Petal color was noted in white, pale, yellow and intense yellow, and petal patterns were classified as round or long. Mean values of length and width of two adjacent petals were estimated as petal length and petal width, respectively. Petal shape (petal width/ petal length) and flower area regarded as a disk ($\pi \times \text{petal length}^2$) were estimated (Fig. 4.3).

Field experiment

The same seeds as above were germinated in greenhouse in January and again in February 2009 in order to insure getting plants flowering at the same time as a sulfonylurea-resistant oilseed rape line grown in a field at the INRA experimental station. They were grown on a bare ground in 20 L pots with water tank, one plant per pot. 41 NM pots and 40 BT+BG+DM pots with plants at the onset of flowering were transferred in the field when the sulfonylurea-resistant oilseed rape plants started flowering. Already opened flowers of wild radish were cut before putting pots in the field. Pots were put 10 meters apart each other, at random along ten rows in a 1 ha field sown at oilseed rape density of 40 pl m⁻². Each pot position was located by GPS.

Wild radish was harvested when the first siliques turned brown. The vegetative parts were separated from the pods and dried in an oven for 48h at 80°C and weighted. Number of siliques and seeds per plant and weight of seeds per plant were measured. Per seed weight (seed weight/ seed number) and reproduction allocation (seed weight/ plant weight) were

estimated. All the seeds of every harvested plant were germinated in greenhouse in conditions as above. Seedlings were counted and sprayed with 2.5 g a.i. ha⁻¹ chlorsulfuron in 300 L of water (Glean, Du Pont de Nemours, France) at two to four leaves stage. Survived seedlings were checked two weeks after spraying. Since the herbicide-resistance is endowed by a dominant allele, every resistant seedling would be hybrid (Darmency et al. 1998)

Cage experiment

The experimental design consisted of 6 cages, each containing 30 positions (18 positions for the putative introgressed NM plants and the rest 12 positions for two populations chosen from the three other regions). Positions were located at random and sown with 5 seeds per position. The emerged seedlings were counted to calculate the emergence rate, and then randomly thinned to one plant per place. Houseflies, *Musca domestica*, were continuously provided inside the cages to facilitate random pollination. The day when the first flower opened was noted, and the number of days to flower was calculated. Wild radish was harvested when the first siliques turned brown. The vegetative parts were separated from the pods and dried in an oven for 48h at 80°C and weighed. The silique weight was measured, and articles were counted and weighed. Per article weight (article weight/ article number) and reproduction allocation (article weight/ plant weight) were estimated.

Statistic analysis

The Fisher test was employed for the percentage of petal color and flower pattern categories. A Student-Newman-Keuls (SNK) test was used to compare the mean values of the flower and silique characteristics among the four regions, and the fitness components of wild radish in the field and the cages. All data, except of the petal color and flower pattern, were square-root transformed to ensure a Normal distribution of residuals. A Discriminant Function Analysis (DFA) was applied to the six independent morphological characteristics of the siliques to identify the characteristics that best discriminate the four regions. A stepwise DFA was used to eliminate variables with very little or no contributions to discriminant functions. Discriminant functions were tested for their significance using Wilk's lambda. The discriminant scores of the first two functions were used to carry out ANOVA and Contrast test to check the difference among regions. In order to distinguish the differences among the four regions, a Principal Components Analysis (PCA) was used for the variables that were measured directly on each individual: plant weight, number of siliques and seeds per plant,

weight of seeds per plant, and number of germinated seeds for the field experiment; emergence rate, plant weight, biomass, number and weight of articles per plant for the cage experiment. The first principal component was extracted for one-way ANOVA, TukeyHSD test and Contrast test. The analysis was conducted by SPSS (SPSS version 13.0) and R (<http://www.r-project.org/>).

Results

Silique characteristics

All eight silique characteristics showed significant differences among regions ($P < 0.001$, Fig. 4.4) but very little among populations within region, so that the results are represented for populations bulked within regions. BT produced the largest article and highest constriction diameter and had the highest silique weight and per article weight, BG and DM had the lowest values, and NM was intermediate. BG produced the highest number of articles per silique, BT and DM had the lowest values, and NM was intermediate. NM had the highest length of articles per silique compared to the other regions. Silique length in NM and DM was the highest, BG the shortest, and BT was the intermediate. DM had the largest beak length, BG the smallest, and BT and NM were intermediate.

The discriminant analysis showed a unique set of silique characteristics that accounted for 90% of the variance (Fig. 4.5). The high score on the discriminant function 1 (DF_1) resulted from article diameter (correlation coefficient $r = 0.79$) and constriction diameter ($r = 0.71$). The number of articles ($r = 0.76$) accounted for DF_2 that absorbed 7% of the variance, only. The DF_1 effectively separated the four regions in three groups: BG and DM together, NM intermediate, and BT apart ($P < 0.001$, Tukey HSD and contrasts test).

Flower characteristics

The DM populations was homogeneous at 100% and showed an intense yellow color, BG was almost homogeneous at 94% for the yellow, while BT and NM showed more polymorphism (Fig. 4.6). Petal pattern was mostly round for BG and DM, while it was shared between round and long in BT and NM. NM showed the lowest petal length, width and area than the other regions.

Field experiment

Number of siliques and seeds per plant, weight of seeds per plant, reproduction allocation and number of germinated progeny were higher in wild radish from the DM region than that from the other three regions, BT, BG and NM, where these traits were not significantly different each other (Fig. 4.7). Plants from BT had the highest weight, NM the lowest, and BG and DM were intermediate. BT and DM had the greatest per seed weight, and BG produced the smallest seeds. The germination of the BT and DM seeds was the highest, and that from BG was the lowest (Fig. 4.7).

The first principal component of PCA explained 74 % of the variance and consisted of contribution by plant weight ($r=0.26$), number of siliques per plant ($r=0.94$), number and weight of seeds per plant ($r=0.96$ and $r=0.96$), and number of germinated seeds ($r=0.96$). The performance of DM plants along this axis 1 was significantly higher than that of the other three regions where there was no difference (Fig. 4.8).

Wild radish in BT, BG, DM and NM produced on average 16, 66, 114 and 42 seeds per plant, respectively. All the 1134 and 1526 seedlings germinated from the seeds of NM and BT+BG+DM, respectively, were killed by the herbicide, indicating that no interspecific hybrid was produced. BT showed 55% yellow and intense yellow petal, BG 95% yellow, DM 80% intense yellow and NM 65% yellow. There were no differences in seed production among plants with different petal colors within the regions ($P>0.05$), with except for NM. For NM, plants with different petal color produced different seed number ($F_{2,34}=3.58$, $P=0.039$), and plants with yellow petal produced more seeds than plants with white petal (TukeyHSD's test, $P=0.041$): 12 seeds per plant for yellow vs. 4 for white petals, on average.

Cage experiment

The emergence rate of BT seedlings was the same as that of DM, which was higher than that of BG and NM. Wild radish from BT flowered latest compared with the other regions, and DM flowered the earliest. The reproduction allocation of wild radish was not significantly different among regions (Fig. 4.9). Plant weight, biomass, number and weight of articles per plant, and per article weight were lowest for the BG region than that from the other three regions, BT, DM and NM. Wild radish from BT produced higher biomass, weight of articles per plant, and per article weight than DM and NM did; the three regions produced similar plant weight and number of articles per plant (Fig. 4.9).

The first principal component of PCA explained 76 % of the variance and consisted of the contribution by five life-history traits: emergence rate ($r=0.44$), plant weight ($r=0.96$), biomass ($r=0.99$), number and weight of articles per plant ($r=0.89$ and $r=0.95$). The resulting value on the first axis was significantly lower for the BG region than for BT, the other two regions being intermediate (Fig. 4.10; and contrast test). TukeyHSD test showed that NM was significantly higher than BG.

Discussion

Variation of flower and silique characteristics

Silique and flower characteristics in wild radish sampled from four regions were significantly different. Silique characteristics discriminated the BT wild radish from others: BG and DM together, and NM intermediate (Fig. 4.5). A large variation of morphological traits was already reported for wild radish in Europe, which lead nomenclaturists to distinguish several subspecies (Tutin et al. 1993). They mainly took into consideration the silique diameter: diameter less than 5 mm would correspond to subsp. *raphanistrum* (3-4 mm) and *microcarpus* (1.5-2 mm), while diameter more than 5 mm to subsp. *maritimus*, *rostratus* and *landra*. Wild radish from BT region had the largest silique, with a mean value of 5.51 mm (ranging from 3.41 to 7.83 mm), and could tentatively correspond to the subsp. *maritimus* as the BT region borders the sea. However, NM and DM that border the sea too, had silique diameter ranging from 2.1 to 6.13 mm for NM and 1.92 to 3.62 mm for DM. It is therefore difficult to arrange the plants from the four regions to every subspecies based on silique diameter. Similarly, all plants in the four regions belong to subsp. *landra* based on petal length (10-15 mm), but they will belong to subsp. *maritimus* when based on beak length (6-20 mm) (Tutin et al. 1993; Fig. 4.4). In contrast, BG plants with silique diameter ranging from 1.7 to 3.37 mm could correspond to typical subsp. *raphanistrum*. However, nobody knows the biological validity of the subspecies classification. All the plant types inter-crossed in the cage experiment, so that there is no reproductive isolation. The apparent differences just could be the consequence of regional fixation of alleles encoding for silique shape and flower color, without any taxonomical value and ecological meaning.

In California, based on two distinct traits, predominantly silique diameter of less than 5.1 mm and yellow flowers (85-93%), Panetsos and Baker (1967) distinguished pure wild *R.*

raphanistrum from hybrids and *R. sativus* (Hedge et al. 2006). We could apply these criteria to our results rather than trying to draw a clear-cut assignment to given subspecies. The frequency of yellow and intense yellow petals was nearly 100% in BG and DM, and all silique diameters measured less than 5.1 mm. The existence of regional homogeneity of flower color was pointed out in some instances: just bright yellow in Scotland, just white around York, but mixed colors near Rothamsted (Cousens, personal communication); intense yellow in Poitou and white in other Brittany populations and North Western France, but mixed color in the Rhone valley (authors' personal observations). In contrast, BT and NM had mixed color, and their siliques were most often wider than 5.1 mm in diameter. It could be indirect evidence of gene flow from cultivated radish (for the white color) or oilseed rape (for the intense yellow color) to wild radish as observed elsewhere (Kercher and Conner 1996; Snow et al. 2010). Besides silique diameter and flower color, other silique and flower characteristics were also scored in this study. In every case as well as globally (Fig. 4.5), these traits showed more polymorphism or larger range of variation in BT and NM than in BG and DM.

Vegetative growth and reproduction

Seed set per radish plant was very low in the oilseed rape field, 53 seeds per plant on average, and this may result from the small population size (81 plants) and the low density (0.01 pl m^{-2}) because wild radish is self-incompatible and need both a variety of self-incompatibility alleles in the stand and active pollinators. Small population size and low density limit opportunities for individuals to mate effectively and influences maternal reproduction (e.g. Elam et al. 2007). In addition, foraging insects were probably poorly attracted by rare radish flowers embedded in huge amount of oilseed rape flowers. These conditions should have been favorable for interspecific crosses due to the abundant oilseed rape pollen available in contrast to the poor amount of wild radish pollen, except in the case there was a specific pollinator to wild radish. However, there was no herbicide-resistant progeny, which indicated that interspecific hybridization, if any, can be comprised between 0 and 0.2% (95% confidence limits of the data), and not different between NM and BT+BG+DN. The frequency of such interspecific hybridization in the field was found to be extremely low (Chèvre et al. 2000; Thalmann et al. 2001; Warwick et al. 2003), although once observed at 0.5% (Darmency et al. 1998). Genotype and population polymorphism for the interspecific barriers to hybridization could explain such widely different estimates (Guéritaine and Darmency 2001; Guéritaine et

al. 2003; Ammitzboll and Jorgensen 2006). Ammitzboll and Jorgensen (2006) suggested that the presence of cultivated radish genes introgressed in wild radish populations might result in higher ability to hybridize with *B. napus*. Gene flow between wild and cultivated radish is known to occur rather easily (Klinger et al. 1992; Eber et al. 1994; Snow et al. 2001), and the hybridization rate between oilseed rape and cultivated radish seems to be again easier (Ammitzboll and Jorgensen 2006). If NM were really ancient crop-introgressed populations, our results cannot support the hypothesis of such an improved interspecific outcrossing ability.

Another feature in this experiment is that plants from BT and NM showed lower seeds output than that from BG and DM. Again, it might relate to the introgression from crops if the putative introgression was proved. The successful sex mate might be lower in introgressed plants under low pollen density because of the greater heterozygosity in introgressed lineages (Snow et al. 2010). For example, Campbell and Snow (2007) found that hybrid progeny produced lower fitness than wild radish under no competition conditions. It is noteworthy that the populations producing the most seed number were DM that was the farthest one from the location where the experiment was carried out, in Burgundy, from which the BG populations belonged. This suggests that DM had the most flowers or the highest outcrossing rate compared to plants from the other three regions. It has also higher reproduction allocation than BG although they have similar silique morphology. The adaptation to northerly and colder region could have caused DM to be the best competitor in our experiment, indicating that population divergence can occur among separated regions and concern growth and reproduction capacities.

When grown in cages in condition of intra specific competition, but at a low density of 3.3 pl m^{-2} , plants of DM had again higher performances than the local BG plants, but BT and NM had the highest values for most traits (Fig. 4.8). Due to lower plant density than in the oilseed rape, seed set was on average 4000 seeds per plant. The constant and abundant presence of a non-specific pollinator, the domestic fly, which certainly saturated the flowers with pollen belonging to all the present plants, could explain the absence of difference of reproductive allocation among regions while there was marked differences in the field experiment. The difference of ranking between the cage and the field experiments could be due to differential adaptation to the habitat, or again to possible introgressed crop genes that make the BT and NM populations more productive under low competition, but less under high competition. For example, in Michigan, Campbell and Snow (2007) found that

competition diminished the difference between the hybrid progeny and wild plants, although hybrids produced lower fitness than wild radish under no competition conditions. Alternately, one could imagine that DM plants also displayed some introgressed crop genes, e.g. the intense yellow flower color, and these genes could help them to overcome the competition from the crop easier than non-introgressed plants. With artificial populations of wild and hybrid radishes in arable fields in USA, Campbell et al. (2006) found that introgressed hybrids showed greater survival and fitness after four generations of introgression than wild plants in a new habitat. Introgression via hybridization may lead to adaptive evolution (Ellstrand and Schierenbeck 2000), because introgression may generate novel adaptations enhancing the fitness of weedy hybrid lineages in certain environments (Rieseberg et al. 1999; Lexer et al. 2003).

Conclusion

Our results showed that the morphological traits discriminated the wild radish populations. Synthetic characteristics of the multivariate analyses showed BT and NM populations different from BG and DM, although NM was often intermediate. The geographic distance was the shortest between BT and NM, which could explain that they showed less difference between them than with the inland BG or farthest DM populations. However, the distance cannot account for the grouping of BG and DM. When analyzing specific characteristics separately, some arguments were equally in favor of the interaction between introgression and natural selection or distance-mediated divergence on morphology traits, as observed in other cases (Lenormand 2002; Postma and van Noordwijk 2005). However, the long exposition of the NM populations to pollen of oilseed rape, for at least 200 years, had no apparent or discriminant effect on the morphology, growth and reproduction of the wild radish plants in that region. Apart, perhaps, the higher polymorphism, there is no evidence of a dramatic evolution of the populations of that region with regard to the variability found in the three other regions. Molecular evidence of ancient introgression is necessary to confirm that some population would have been introgressed, and this is difficult because both *B. napus* and *R. raphanistrum* share a large part of their genetic background within the *Brassica* tribe. In case introgression were detected, future study should focus on the benefit and cost associated to these genes and their role for field adaptation compared to the potential role of the transgenes

currently engineered in GM crops. Perhaps some effect could be detected on the fitness of the plant that bear an introgressed gene while there is no any consequence at the whole population level, a question that is currently tested by genotyping every studied plant.

Acknowledgements

We thank Alain Fleury for his assistance in field experiment. This work is supported by a PhD joint fellowship between China and France (CNOUS, No. 20072315).

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Fig. 4.1. Four regions where wild radish sampled: BT, Bretagne; BG, Bourgogne; DM, Denmark; NM, Normandy



Fig. 4.2. Siliques photos from four regions: BT, Bretagne; BG, Bourgogne; DM, Denmark; NM, Normandy

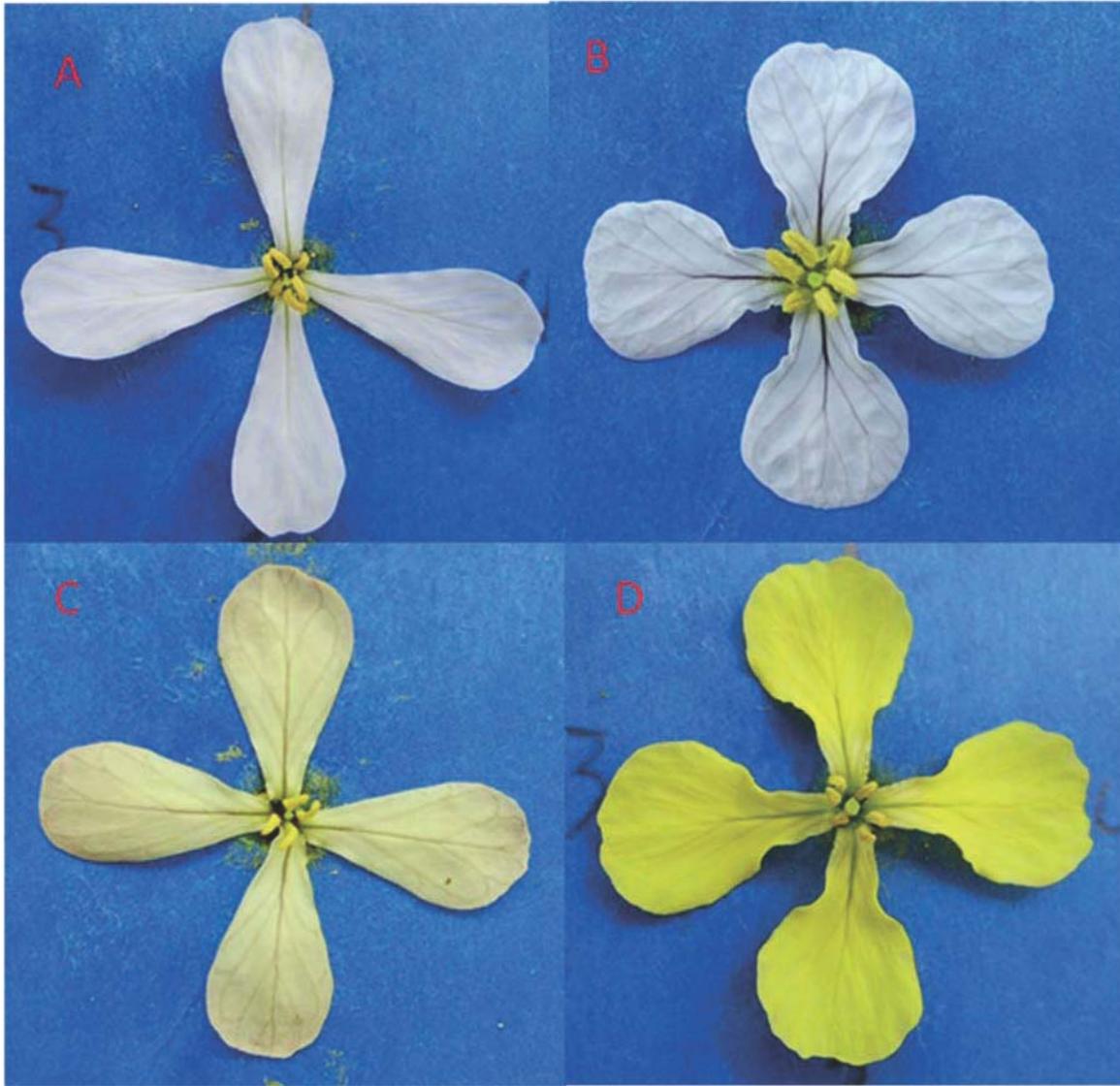


Fig. 4.3. Flower photos showing petal colors and patterns: A, white and long; B, pale and round; C, yellow and long; D, intense yellow and round.

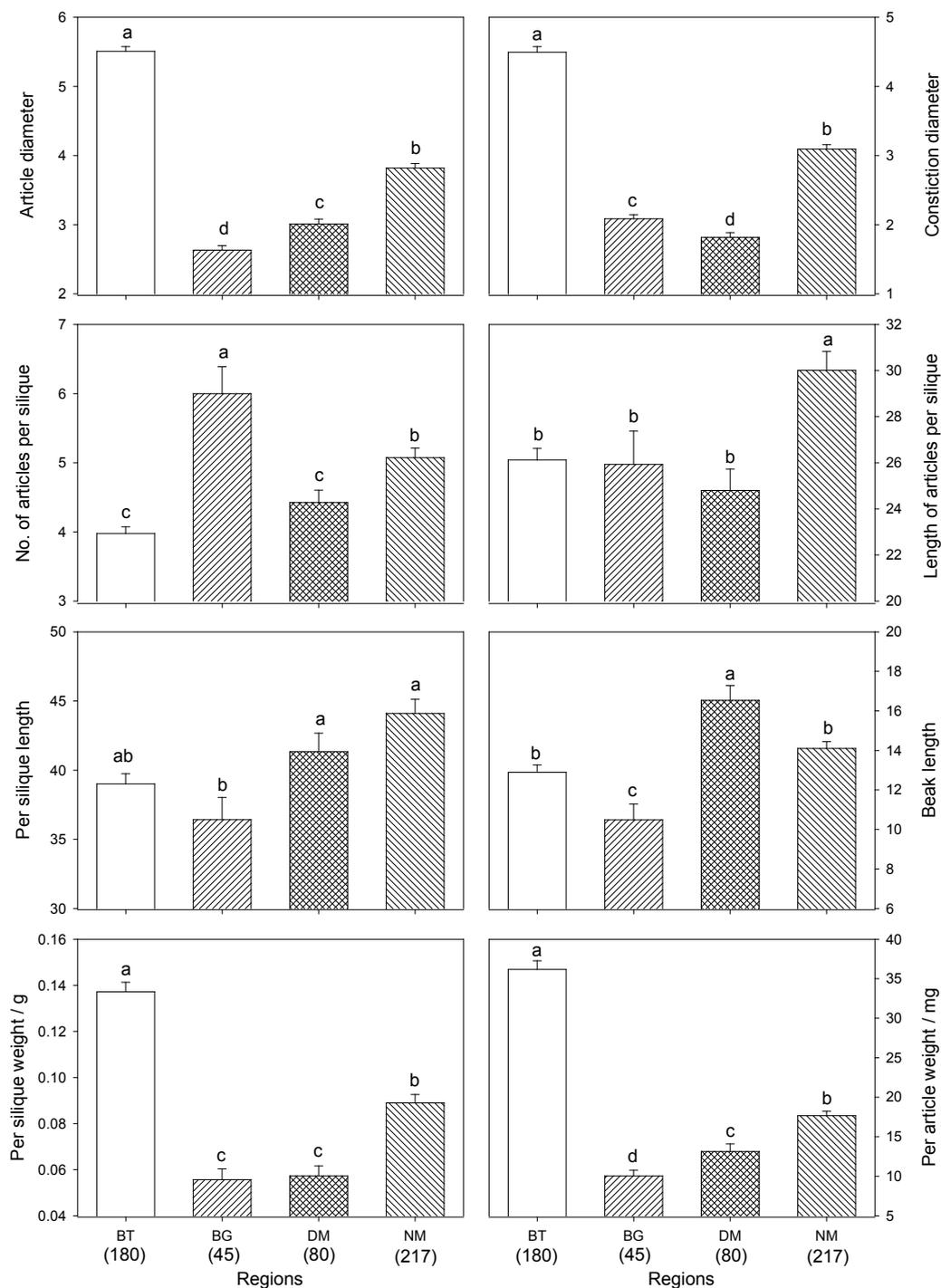


Fig. 4.4. Mean and standard error (vertical bars) of article and constriction diameter, article number and length per silique, silique length and weight, beak length and mean article weight of wild radish from four regions; different letters indicate significant difference at $P < 0.05$ from SNK test. Numbers in parenthesis at bottom were the number of siliques measured.

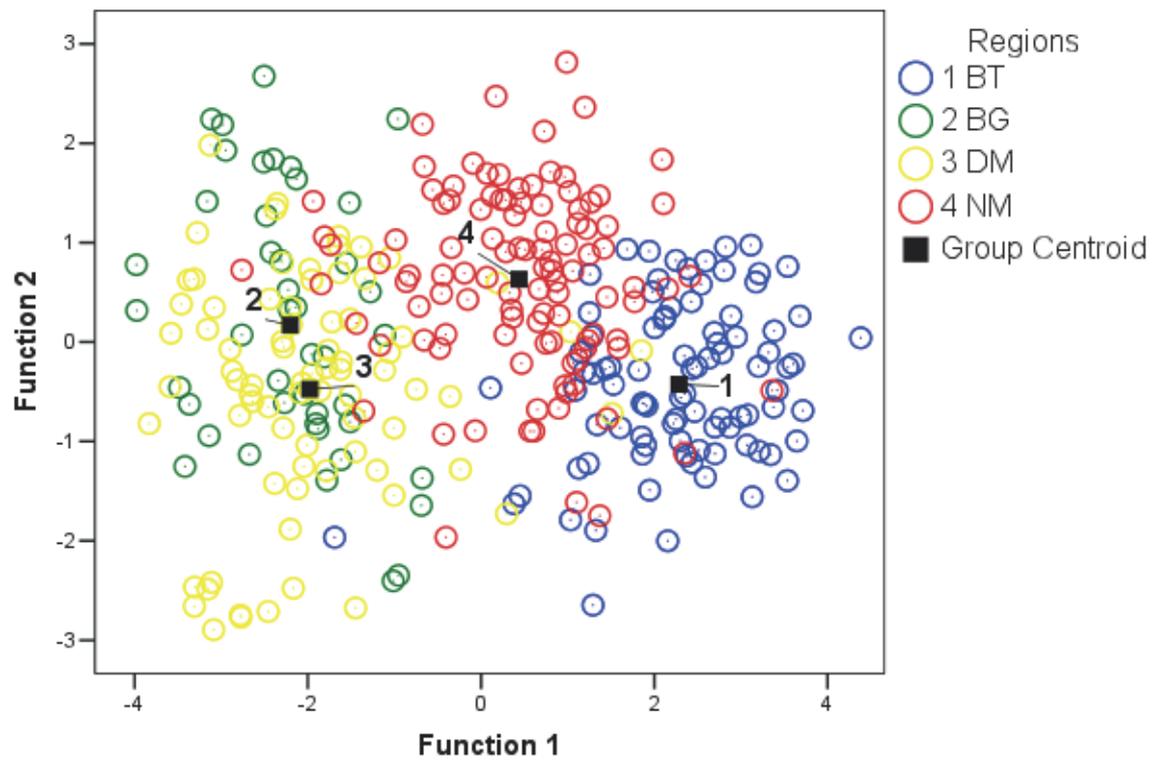


Fig. 4.5. Plot of the first two axes of discriminant analysis of morphological measurements of wild radish from four regions: B T, Bretagne; B G, Bourgogne; D M, Denmark; N M, Normandy

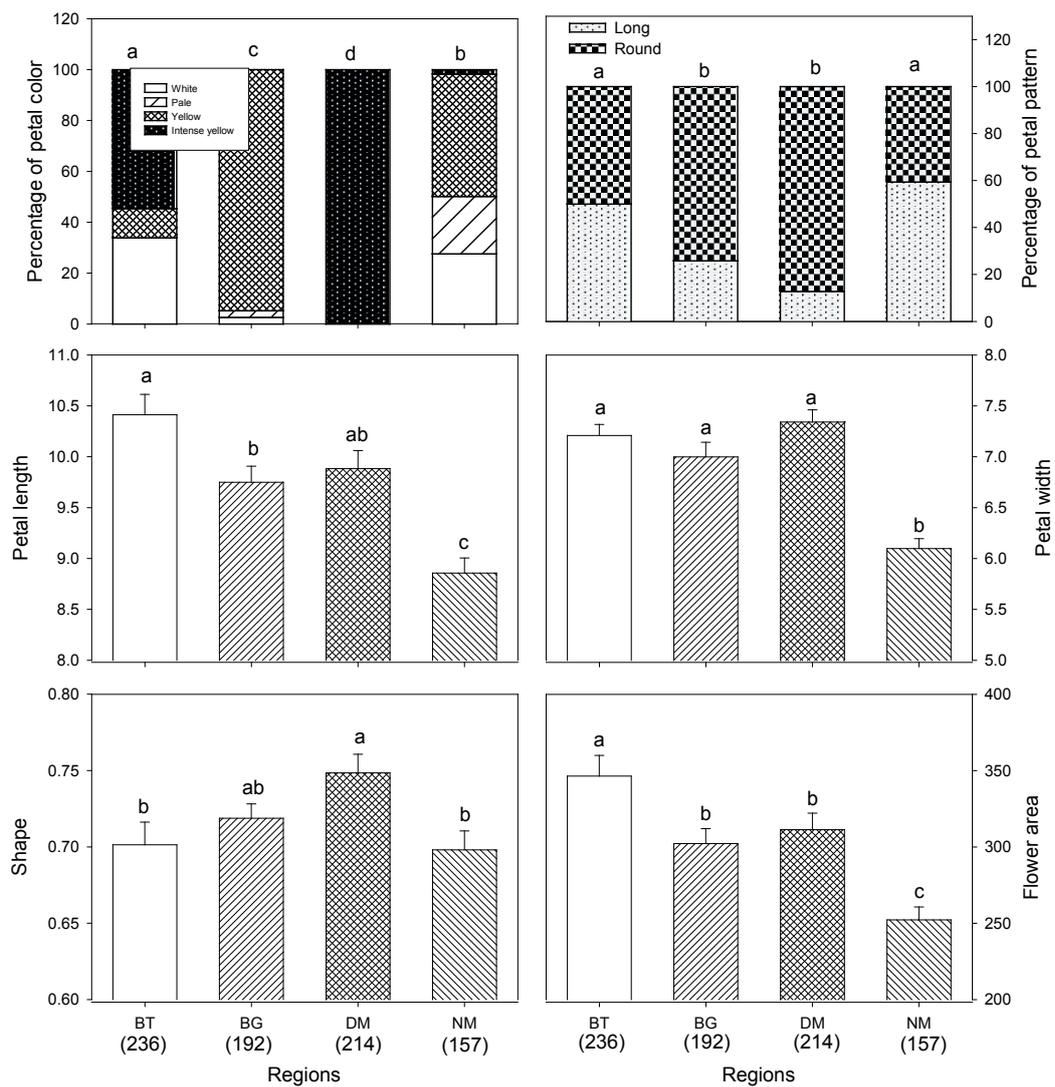


Fig. 4.6. Mean and standard error (vertical bars) of percentage of petal color and flower pattern categories (long and round), petal length and width, shape (width/length) and circle area ($\pi \times \text{length}^2$) of wild radish flowers from four regions; different letters indicate significant difference at $P < 0.05$ from Fisher test for two percentages and others from SNK test. Numbers in parenthesis at bottom were the number of flowers measured.

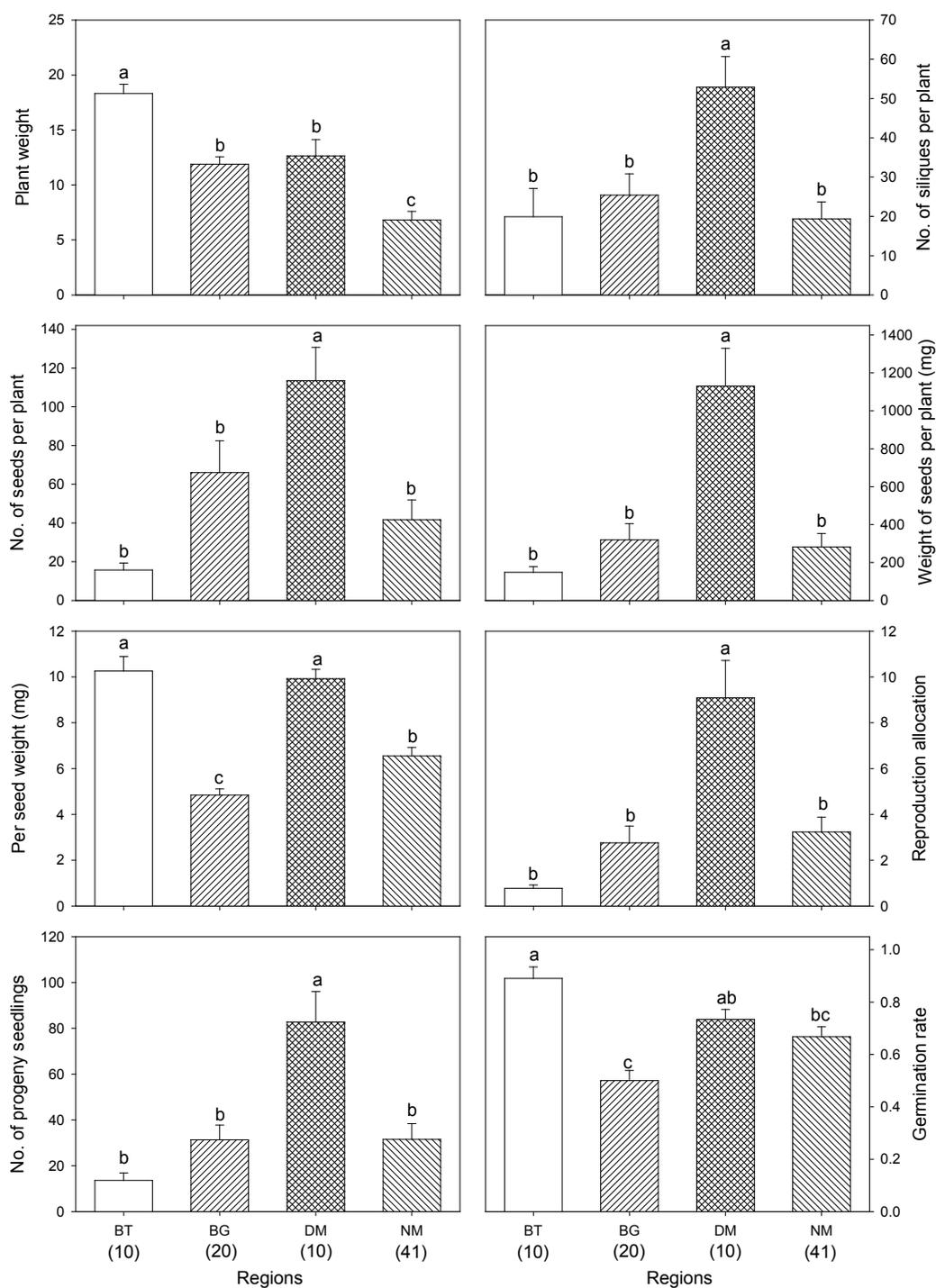


Fig. 4.7 Mean and standard error (vertical bars) of eight plant characteristics of wild radish from four regions planted in oilseed rape field; different letters indicate significant difference at $P < 0.05$ from SNK test. Numbers in parenthesis at bottom were the number of plants harvested.

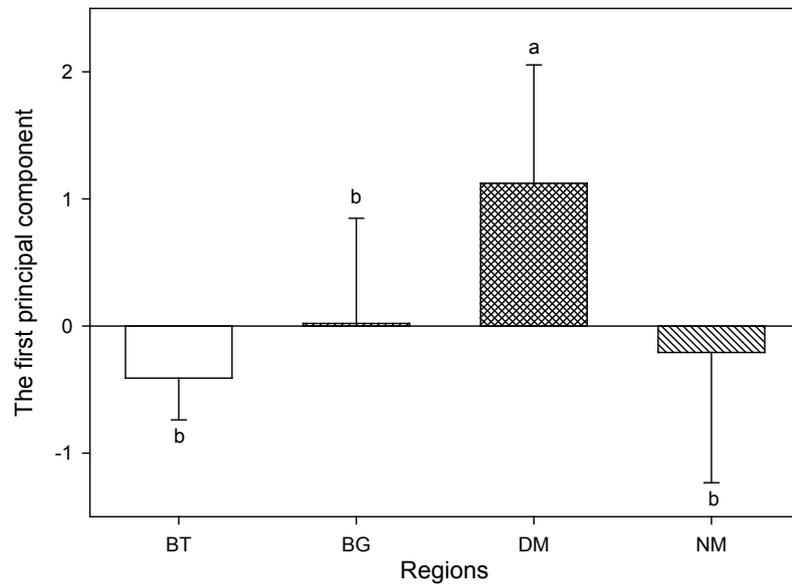


Fig. 4.8 Mean and standard error (vertical bars) of the first principal component of PCA for wild radish from four regions planted in oilseed rape field; different letters indicate significant difference at $P < 0.05$ from TukeyHSD test.

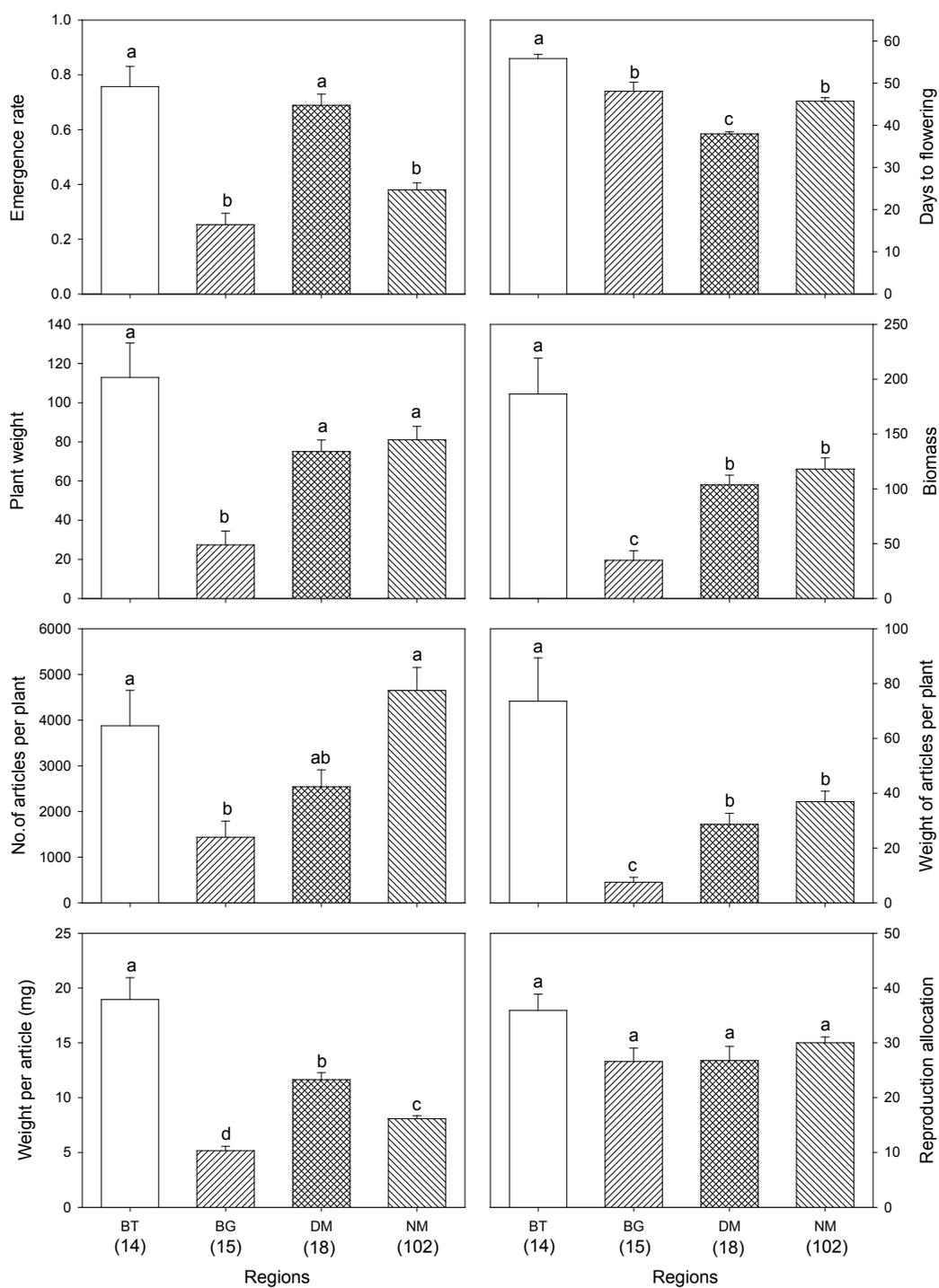


Fig. 4.9. Mean and standard error (vertical bars) of eight plant characteristics of wild radish from four regions planted in six cages; different letters indicate significant difference at $P < 0.05$ from SNK test. Numbers in parenthesis at bottom were the number of plants harvested.

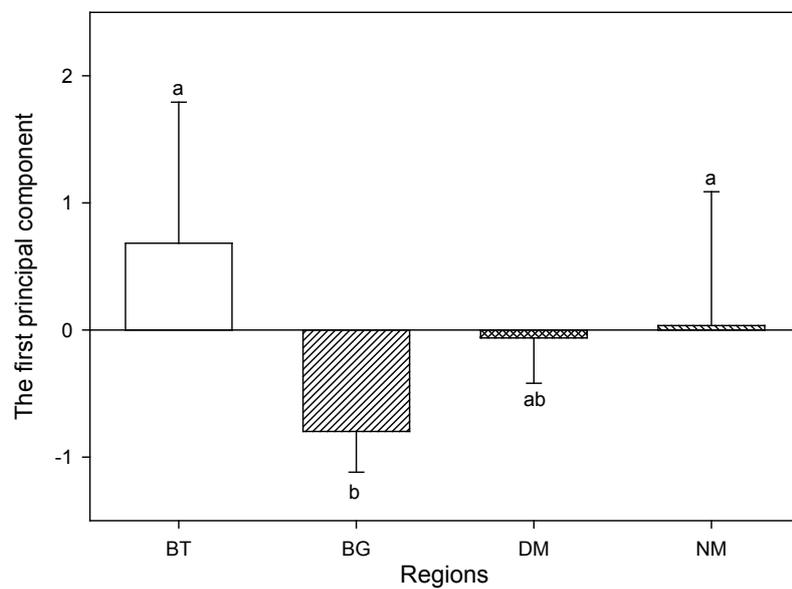


Fig. 4.10. Mean and standard error (vertical bars) of the first principal component of PCA for wild radish from four regions planted in cages; different letters indicate significant difference at $P < 0.05$ from TukeyHSD test.

CONCLUSION

CONCLUSION

Dans ma thèse sur les conséquences écologiques et évolutives des flux de gènes et de l'introgession entre du colza transgénique (*Brassica napus*) et ses apparentés sauvages, en particulier la moutarde brune (*B. j uncea*) et marginalement la ravenelle (*Raphanus raphanistrum*), j'ai mis en évidence des effets sur des caractéristiques morphologiques, des relations de compétition, et des composantes de la fitness au niveau de s i ndividus et de s populations. Ces trois ensembles d'effets sont discutés ci-dessous en intégrant les apports de chaque chapitre plutôt que de reprendre le déroulement chronologique des di fférentes étapes depuis l'hybridation jusqu'à l'introgession tel que présenté da ns l e do cument e t dont l es conclusions ont déjà été abordées dans chaque propositions d'articles.

1. Caractéristiques morphologiques

En règle générale, les hybrides montrent des caractéristiques morphologiques intermédiaires entre celles de leurs parents (Lefol et al. 1996; Choudhary et al. 2000), quoi que l'effet du cytoplasme de la plante cultivée ou de la plante sauvage introgressée soit aussi observé dans certains cas (e.g. Lefol et al. 1996; Zhang et al. 2002; Guéritaine et al. 2002; Chang et al. 2007). Dans la suite des générations, les caractéristiques morphologiques divergent selon les types de croisements possibles dans les populations, entraînant une diversité qui peut être soit sélectionnée ul térieurement soit c onservée s elon qu' elle dépende ou non du t ransgène, de s conditions de l'habitat et du nombre de générations passées.

Parmi les caractéristiques des hybrides, la première est qu'ils apparaissent sous forme de p etites s emences, p robablement d u fait d 'interactions en tre l es d ifférents g énomes d es parents à l'état hétérozygote dans l'embryon qui limitent son développement (Eber et al. 1994; Chadoeuf e t a l. 1998; W ei a nd D armency 200 8). O n pe ut s 'attendre à c e que l es pe tites semences, renfermant moins de réserves, soient désavantagées dans leur implantation dans le milieu (Aparicio *et al.* 2002; Westoby *et al.* 2002). E ffectivement, nous avons montré dans notre é tude q ue la p etite ta ille d es s emences r éduit s ignificativement l a c roissance e t l a reproduction, mais ces effets ne s ont pas s ystématiquement pr oduits e t d épendent du fond génétique, de l a d ensité d e p lantes e t de l 'intensité d e l a co mpétition dans le milie u. La signification d es d ifférences d épends au ssi d es stratégies s uivies p ar l es es pèces p our s e développer, et on pe ut observer qu'il existe autant d'espèces sauvages ou adventices à petite ou à g rosse s emence, d ans l a g amme d e v aleurs obs ervée da ns nos t ravaux, qui ont un

véritable succès adaptatif dans les milieux qu'elles colonisent. La relation trouvée chez le colza transgénique, avec moins de biomasse et de reproduction pour les petites semences, correspond à une logique de productivité d'une plante domestiquée. La réduction de taille de la semence n'a pas eu les mêmes effets chez la moutarde et les hybrides, sauf une floraison retardée, et le nombre de semences viables produites a été le même quelque soit la taille des semences hybrides. Si le statut d'hybride interspécifique implique souvent une fitness inférieure à celle du parent sauvage, la taille des semences n'ajoute pas une barrière supplémentaire au flux de gènes. Au contraire, les petites semences sont facilement triées par les moissonneuses et retombent au sol lors de la récolte et peuvent ainsi alimenter le stock de semences enfouies dans le sol ou être dispersées à la surface, aboutissant ainsi plus facilement dans des milieux incultes (Chapitre 2.2).

Nous n'avons pas étudié le devenir de ces plantes dans des milieux naturels. Leur reproduction s'est montrée plus facile et plus abondante par rétrocroisement avec le colza qu'avec la moutarde ou en autofécondation, et c'est donc dans les champs ou à leur proximité que des descendants ressemblant au colza seront produits. Les rétrocroisements avec le colza produisent effectivement des plantes avec des traits plutôt du type colza, par exemple avec des fleurs de type colza qui leur permettent en core plus facilement de se reproduire, via les insectes pollinisateurs affiliés au colza, avec les variétés cultivées ainsi qu'avec leurs repousses. Leurs semences sont plus grosses et germent mieux que pour les plantes produites avec du pollen de moutarde, c'est-à-dire qu'elles sont moins dormantes et donc montreront une dynamique de survie et de levée plus tôt proches de celles du colza. Dans le cas du marqueur de résistance à un herbicide que nous avons utilisé, il n'y a pas eu de biais de ségrégation, mais ce n'est pas automatique et dépend du génome A ou C sur lequel est inséré le transgène et des recombinaisons avec les génomes A ou B de la moutarde (Frello et al. 1996). Ce type de transgène, mais aussi des résistances à des insectes ou des capacités physiologiques meilleures, leur permet de perdurer dans les champs, voire de s'y multiplier de manière exponentielle. Des flux de pollen des descendants résistants vers des plantes sensibles ont été observés, indiquant que l'implantation de ces plantes dans les champs et leurs bordures se double d'un relai pour la dissémination des transgènes. Du fait de la variabilité génétique héritée de la moutarde sauvage et générée par l'hybridation interspécifique et les recombinaisons, ces plantes ressemblant au colza disposent d'un potentiel adaptatif plus fort que les simples repousses variétales. Elles représentent probablement un danger plus

important pour l'agriculteur et les risques d'infestation de milieux incultes, d'autant plus que peu de caractéristiques morphologiques permettront de les identifier lors de plusieurs années de surveillance post-commercialisation des variétés transgéniques (monitoring). Il n'y a donc pas seulement que l'introggression de s gènes cultivés dans les espèces sauvages qu'il faut surveiller (Chapitre 2.3).

Si on se projette à un pas de temps plus lointain, que peut-il rester de phénomènes d'hybridation ancienne suivie d'introggression, bien sûr sans transgène conférant un avantage distinct ? Nous avons abordé cette question en faisant l'hypothèse que les chances des flux de gènes augmentaient avec la durée de coexistence des deux types de plantes, dans ce cas le colza et la ravenelle. Les populations supposées introgressées se sont montrées polymorphes et intermédiaires entre des populations plus typées pour leurs caractéristiques de silique et de fleur. Cependant, elles étaient plus proches des populations les plus voisines, et l'effet de la distance géographique restait la principale variable corrélée aux différences enregistrées. La mise à disposition de marqueurs moléculaires spécifique du colza pour confirmer le niveau d'introggression de populations de ravenelle, ainsi que la connaissance de gènes impliqués dans des modifications de traits sélectionnés (par exemple la couleur des fleurs pour l'attraction des pollinisateurs, la forme des siliques pour la dissémination la survie et la germination des semences) sont nécessaires pour confirmer les tendances observées (Chapitre 4).

2. Interaction compétitive et fitness individuelle

Dans le cas d'une introgression réussie, le transgène est géré de la même manière que les autres gènes dans le génome de la plante sauvage, et il peut atteindre un équilibre ou bien se fixer ou disparaître selon les pressions de sélection du milieu. Il faut donc préciser dans quelle mesure et sous quelles conditions un transgène donné peut donner un avantage sélectif relativement aux plantes indigènes, ou au contraire être délétère, ou en core neutre en l'absence de la pression de sélection. Différents cas ont été rapportés chez le colza (Stewart et al. 1997; Ramachandran et al. 2000; Mason et al. 2003; Di et al. 2009; Letourneau and Hagen 2009). Nos expériences ont été menées, soit en comparant des descendants isogéniques de croisement entre la moutarde sauvage et le colza, soit en simulant un gène de résistance aux insectes chez la moutarde sauvage, ce qui nous rendait indépendant des effets de linkage et des perturbations du génome liées au croisement interspécifique.

Dans le cas des premières générations d'hybrides avec un colza résistant à un herbicide, la résistance a été associée à des plantes plus grosses produisant plus de semences (Chapitre 2.3). Au contraire, avec le colza résistant à un insecte, les rétrocroisements donnent des plantes plus petites et se reproduisant moins que les parents mutarde et colza transgénique, mais les hybrides résistants avaient une biomasse et une production de semences plus élevées (Chapitre 3). Enfin, dans le cas de s-ravenelles, les populations supposées introgressées avaient des plantes plus grosses et plus productives (Chapitre 4).

Avec les simulations, nous n'avons pas observé de pénalité à être résistant en l'absence d'insecte. En revanche, les résistants se sont montrés supérieurs sous pression d'herbivorie, et cet avantage s'est accru avec un niveau plus élevé de compétition intra-population, de ressources réduites, et aussi avec des proportions plus élevées de plantes résistantes dans la population (la compétition intra-classe résistante a accentué la compétition inter-classe sensible/résistante, Chapitre 3).

3. Productivité des populations

Avec l'arrivée d'un transgène de résistance dans une population sensible, la compétition entre les individus présente un niveau de complexité supplémentaire. La relation entre les plantes sensibles et résistants ouvre la possibilité d'un gain de productivité de la population, ce qui peut lui conférer une dynamique de colonisatrice qu'elle n'avait pas avant, entraînant à la fois sensibles et résistants dans une dispersion au-delà de la situation d'équilibre connue avant. Nous avons vu que selon la pression d'herbivorie, il pouvait y avoir soit une proportion limitée de résistants défavorable à leur propre multiplication, soit une évolution complète vers la fixation. Cependant, on sait que la pression de sélection par les insectes n'est pas systématique au champ et dans les milieux avoisinants, ce qui ne permet pas de conclure sur la rapidité de colonisation d'une population sauvage par un tel transgène. De plus, nous avons observé à plusieurs reprises des phénomènes de compensation des dommages liés à l'herbivorie (Chapitre 3). Et enfin, les conditions de compétition font émerger des différences de classement des populations comme dans l'étude des ravenelles au champ et en cage (Chapitre 4). Il faudrait pouvoir prendre en compte toutes ces interactions dans un modèle démographique avant de se risquer à des prédictions dépourvues d'estimation des chances de réalisation.

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Annex 1

GENERAL INTRODUCTION (English version)

The new traits would affect the growth and reproduction of interspecific hybrid progenies (Ellstrand et al. 1999; Snow et al. 2003; Halfhill et al. 2005; Campbell et al. 2006). When resistant plants invade susceptible wild populations, or when the transgene conferring resistance is transmitted to wild populations, the competition existing between resistant and susceptible plants would impact both the two plant types, resistant and susceptible plants. Because of the resistance advantage of the resistant plants, they might suppress the growth of the susceptible plants for competing resources. At the same time, the competitive interaction between resistant and susceptible plants is expected to vary as the relative proportion of both plant types vary in the population because of the change of neighbors. In addition, it is likely that this competitive interaction depends on the surrounding growth conditions, such as resource availability, herbivory, herbicide and virus diseases pressures (Ramachandran et al. 2000; Vacher et al. 2004; Campbell and Snow 2007).

Over the long term, the persistence of transgenes or transgenic plants in a wild population should result in shift in the population dynamics, and affect the direction and consequences of population variation. While transgenic plants dominate the wild population, the susceptible wild plants may either coexist with resistant plants or face an endanger situation, which could depend on the competition for resources and harsh conditions like high herbivory (Stewart et al. 1997; Ramachandran et al. 2000; Letourneau and Hagen 2009). Another important outcome could be the evolution of plant morphological (such as flower color or size, fruit shape or seed size) and life-history traits (such as flowering time, growth and reproduction, seed germination), because the transgene could be either directly related with these traits or affect them indirectly via the long-term evolution process. However, the study of long-term evolution processes is very difficult because of the lack of appropriate (wild) transgenic materials and the limitation of time. An alternate method is using models to carry out simulations, but models also need precise data to run. The discovery of a wild population introgressed by conventional crops could be invaluable as an alternative for detecting the possible long-term effects of gene flow and introgression between transgenic crops and wild relatives.

Therefore, after a review of the state of the art about the impact of transgene and selected genes in the introgression, my thesis focuses on the following questions:

- 1) Does small seed size of transgenic progeny hamper the gene flow and introgression between crops and wild relatives? (Chapter 2.2)
- 2) Does transgenic progeny, hybrids and backcrosses, persist in and outside of cultivated fields after gene flow? (Chapter 2.3)
- 3) What resulted from the advantage of insect-resistant in individual plant and population production: under simulated herbivory (Chapter 3.2 and 3.3) and real herbivory pressure (Chapter 3.4)?
- 4) Does ancient introgression between crops and their wild relatives could be detected and change morphological and life traits of populations? (Chapter 4)

Annex 2

CONCLUSION (English version)

In my thesis, ecological and evolutionary consequences of gene flow and introgression from transgenic oilseed rape (*Brassica napus*) and its wild relatives (*B. juncea* and *Raphanus raphanistrum*) were detected, considering the effects of morphological traits, interactive competition, fitness components in individual plants and population. The three effects that are discussed above in every chapter will be concluded integrally as follows.

1. Morphological traits

Generally, the hybrids show intermediate morphologic characteristics compared to their parents (Lefol et al. 1996; Choudhary et al. 2000), because the cytoplasm of crops or wilds introgress into hybrids would affect the morphology of the progeny (e.g. Lefol et al. 1996; Zhang et al. 2002; Guéritaine et al. 2002; Chang et al. 2007). The morphological traits the successive generations might be altered and even further diverge from others by the long-term introgression, which depends on the transgenes, growth conditions and how many generations passed.

Small seeds were produced by hybridization between crops and wild relatives because the effects of interaction between different genome and heterozygote in embryo limit their development (Eber et al. 1994; Chadoeuf et al. 1998; Wei and Darmency 2008), and small seeds are disadvantaged in their early establishment to plants in nature (Aparicio et al. 2002; Westoby et al. 2002). Indeed, in our study, seed size significantly affected plant growth and reproduction. However, its influence on plant fitness varied among genetic backgrounds and was affected by plant density and competing neighbors. The significant differences depend also on survive strategy for developing, and the same species could produce small or large seeds. Small-seeded transgenic oilseed rape plants produced less seeds, lower biomass and delayed flowering than large-seeded plants. For mustard and ntrF1, small-seeded plants delayed flowering but had similar biomass and seed set compared to large-seeded ones. Small-seeded trF1 plants had the same fitness as the large-seeded trF1. These results imply that further gene flow could not be reduced by the production of small-sized seeds in transgenic hybrids in field. Moreover, small seeds are

more easily sieved out by harvesters and fall onto the soil, and further buried in the seed bank of soil. In addition, the small seed dispersed more easily through wind and animals, such that the survival of small-sized seeds of weeds could be higher in conventional tillage systems and arable habitats. These seeds could be volunteers in the subsequent years, and they might represent the main risk of transgene escape in the field. Hence, the small size in hybrids might not be a counterbalancing force exposing them to higher competition from the neighborhood but be possible resources for further gene flow (Chapter 2.2).

We did not study the successive generations of these plants with different seed size in fields. Backcross progeny with pollens of oilseed rape (*Brassica napus*) is more easily formed and produces more seeds than that with pollens of mustard (*B. juncea*). Hence, the progeny that has traits of oilseed rape will be produced in or outside of arable fields. Most of plants backcrossed with oilseed rape pollen had crop-like traits, for example larger flower that permit pollinators more easily to access and produced more seeds. Their seeds were larger and germinated better than the seeds produced with *B. juncea* pollen, suggesting they show a similar survive dynamic and emergence with oilseed rape. In our experiment with herbicide resistance, their chromosome number followed a Normal distribution, and the herbicide-resistance gene was consistent with Mendelian ratio. However, this is not automatic, and it depends on the insertion loci of transgene on A or C genome and on the backcrossing with A or B genome in mustard (Frello et al. 1996). This transgene could also be resistant to insects or others that permit them continue survive in fields. Pollen flow to susceptible plants within the mixed stand was occurred. These results suggest that the resistant BC₁ produced with *B. napus* pollen could frequently occur and easily establish as a false feral crop population within cultivated fields and along roadsides. The establishment of crop-like progeny is much more rapid and hosts a large genetic variability compared to crops, which could be the source of further rapid adaptation and evolution (Chapter 2.3).

Does the hybridization effects on morphology will exist after long term introgression of crop gene in wild relatives, even without transgene conferring new traits? We first propose the hypothesis that gene flow increases with the increasing coexistence between two species, oilseed rape and wild radish (*Raphanus raphanistrum*). The putative ancient introgressed populations showed polymorphism and intermediate characteristics in siliqua

and flower compared to other populations. It is necessary to find appropriate molecular markers of oilseed rape species for confirming the introgression in populations and genes that imply the modification of selected traits, for example flower color for attracting pollinators and silique form for dispersal and survival (Chapter 4.2).

2. Competition interaction and individual fitness

As it is inevitable that populations composed transgenic and non-transgenic individuals after the occurrence of introgressions (Ramachandran et al. 2000; Vacher et al. 2004; Moon et al. 2007), their relative competition capacity will affect the individual growth and reproduction and further affect population dynamics.

Insect-resistant plants had competitive advantage when competing with insect-susceptible plants under insects. This relative advantage was increased with the percentage of healthy plants in susceptible populations. However, as the increasing of percentage of resistant plants in populations, the intra-class competition among resistant plants affects their inter-class competition between resistant and susceptible plants. In addition, this competition advantage of resistant plants was magnified under harsh conditions, like limiting resources, high plant density. The effects of competition and herbivory on plant fitness were additive. Competition interaction among plants in populations was changed because of the presence of introgressed resistant plants. (Chapter 3.3)

In case of the introgression succeed, the transgene will be treated as other genes in wild relatives, and it could arrive at equilibrium situation or fixed or disappeared that depends on the selection pressure. Hence, which traits and in which condition a transgene confers fitness advantage should be detected. A transgene transferred from transgenic crops to wild relatives might be neutral, but it is likely to increase or decrease the fitness of receiving hybrid/ backcross plants (Stewart et al. 1997; Ramachandran et al. 2000; Mason et al. 2003; Di et al., 2009; Letourneau and Hagen 2009). This depends on the transgenic character, its selection and costs, the context of transgene introgressed in the genome, and the population composition.

Transgenic F1 produced higher biomass, lower seed number and weight and reproductive allocation than mustard and transgenic oilseed rape (Chapter 2.2). Herbicide-resistant BC1 was associated with greater plant and produced more seeds than their susceptible counterparts (Chapter 2.3). Insect-resistant plants produced higher plant fitness: higher biomass and seed weight and more seeds (Chapter 3.3). However, Bt-transgenic BC2 showed similar fitness compared to susceptible BC2 when they were planted together in the presence of insects because of the protection of transgenic plants on non-transgenic plants (“halo effect”) (Chapter 3.4). The ancient introgressed wild radish plants did not show significant higher fitness but intermediate values compared with others (Chapter 4).

Moreover, the fitness effects of transgenes were affected by other factors, such as morphological traits (seed size, flower color etc.), competition, herbivory, and resources availability. Large-seeded plants in transgenic oilseed rape showed higher plant fitness than small-seeded ones (Chapter 2). Ancient introgressed wild radish plants with white petal produced more seeds than plants with white petal (Chapter 4). Increased plant density significantly decreased plant biomass and seed output (Chapter 2). Competition magnified the fitness advantage of the insect-resistant plants, but as their frequency increases, neighbor competition limits their growth. High resources availability and low herbivory induced compensatory growth in susceptible plants, and accordingly decreased the fitness differences between susceptible and resistant plants (Chapter 3.3). The mixed cultivation with Bt-transgenic plants and non-transgenic plants increased production of both transgenic and non-transgenic plants (Chapter 3.4).

3. Population production

Population equilibrium might be broken in case of the invasion of transgenic plants or the transferring of transgenes in wild populations because of the competition interaction between transgenic and non-transgenic plants. In long-term, population might diverge from others after successively introgression because of the transgenes conferring new traits.

The total vegetative and reproductive production of mixed populations of healthy and damaged plants was the same as that of pure populations of either plant type, but

population reproduction had a maximum when healthy plants occupied 75% of total individuals. The outcome was the same no matter how the two plant types were distributed within the plots, although both population production and individual fitness were significantly higher in plot borders than in centers (Chapter 3.3). Insects decreased population vegetative production but did not affect reproduction, and population production, including vegetation and reproduction, increased with percentage of insect-resistant transgenic BC₂ in susceptible non-transgenic BC₂ populations (Chapter 3.4). Although these results showed the insect-resistant plants had advantage of competition and fitness in the presence of insects, insects are not a regular selection pressure in natural fields, and thus we could not conclude that Bt-transgene conferring insect-resistance could colonize rapidly susceptible wild populations. Moreover, the compensatory growth of susceptible plants and halo effect observed decreased the differences between resistant and susceptible plants. Finally, competition caused differences among populations in the wild radish experiments (Chapter 4).

In summary, my thesis present new evidences for the possible effects of gene flow and introgression between transgenic crops and their wild relatives on morphology and population dynamics in wild relatives by detecting the relative competition and fitness of individual and population, and exploring the factors enhanced the introgression from crops to wild relatives. These evidences showed that it is not easy to alter morphological traits and population composition through the insertion of transgene in wild relatives, and the consequences of gene flow between transgenic crops and wild relatives should be evaluated.