Behavioral, genetic, and transcriptomic analyses of a recent host range expansion of diamondback moth to pea

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von Kathrin Henniges-Janssen

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1. Abbreviations and symbols

Abbreviations

AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
BC	backcross
bp	base pairs
Cab.	cabbage
cDNA	complementary DNA
CSP	chemosensory proteins
C-strain	cabbage-adapted host strain of DBM from Australia
DBM	diamondback moth
DBM-C	cabbage-adapted host strain of DBM from Kenya
DBM-Cj	cabbage-adapted host strain of DBM from Kenya
DBM-P	pea-adapted host strain of DBM (same as P-strain)
DBM-Pc	pea-adapted host strain of DBM (same as P-strain) reared on cabbage
EIF1	elongation initiation factor 1
EST	expressed sequence tags
G88	DBM strain reared on glucosinolate-free artificial diet
GO	gene ontology
GST	glutathione s-transferase
GSS	glucosinolate sulfatase
ICIPE	International Centre of Insect Physiology and Ecology, Nairobi, Kenya
L:D	light:dark
LG	linkage group
MPICE	Max Planck Institute for Chemical Ecology, Jena, Germany
OBP	odorant binding protein
PI	proteinase inhibitor
P-strain	pea-adapted host strain of DBM (same as DBM-P)
qRT-PCR	quantitative real time PCR
REEP	receptor expression enhancing protein
RH	relative humidity
RPS	ribosomal protein
ss	single stranded
SSH	suppression subtractive hybridization
TDF	transcript derived fragment
UGT	uridine 5'-diphospho glucosyltransferase

Abbreviations and symbols used in statistics

χ^2	chi square
d.f.	degrees of freedom
n	sample size

n.s.	not significant
P	probability
SEM	standard error of the mean
SD	standard deviation
*	indicates a significant result ($P < 0.05$)
**	indicates a significant result ($P < 0.01$)
***	indicates a significant result ($P < 0.001$)

2. General introduction

Terrestrial biodiversity is dominated by the class Insecta. The number of one million described insect species is well known to naturalists, and this figure is likely to be an underestimate, because a large portion of the undescribed species is thought to be insects, with estimates as high as 30 million species (Mitter et al. 1988; Grimaldi and Engel 2002). Insect evolution is closely related to the evolution of flowering plants and indications are that most modern insect lineages coradiated with the evolution of angiosperms during the Cretaceous Period, 130 million years ago (Labandeira and Phillips 1996; Grimaldi and Engel 2002). Approximately one half of all insects can be accounted for by species that feed on tissues of green plants and today, every extant green plant is occupied by herbivores (Bernays and Chapman 1994; Schoonhoven et al. 2005). Each insect-host plant association that we find today is the result of an herbivore colonizing a (novel) plant. Most of these associations are evolutionarily ancient while a few result from recent host plant shifts. It is amongst the most fundamental aims of ecologists and evolutionary biologists to understand the means by which herbivores are able to colonize novel host plants and eventually adapt to them.

Host plant adaptation

In order to be specifically adapted to a host plant, the insect must find and lay eggs on it and feed and develop to adulthood on it. Thus, the generic trait 'host plant adaptation' can be divided into two traits: adult and larval adaptation. Larval adaptation includes preference and performance traits, such as host plant recognition, acceptance, digestion and successful development to adulthood represented by parameters such as host plant choice, development time, survival, etc. (Thompson 1988; Nylin et al. 1996). Adult adaptation traits comprise the sequence of behavioral traits involved in oviposition preference, which is the location and evaluation of a potential host plant leading to the decision to oviposit or not. Host plant adaptation is the key set of traits determining the range of plants an herbivore can thrive on.

Host plant range in herbivorous insects

Herbivorous insects run the gamut from feeding on a single plant species to feeding on a wide range of plants from different families (Jaenike 1990; Bernays and Chapman 1994). Depending on their diet range, herbivores are categorized into generalists (polyphagous species) and specialists (mono- and oligophagous species). Amongst the most striking features of herbivorous insects is that the vast majority of insect herbivores are specialists (Schoonhoven et al. 2005).

There has been a long debate on the reasons for the predominance of specialists. Intuitively the advantages of polyphagy in terms of resource use are obvious. If a host plant becomes extinct, a generalist can use an alternative host whereas the specialist runs the risk of also going extinct. However, since polyphagous insects do not accept all plants indiscriminately, they must evaluate a greater diversity of host plants than specialists and face a cognitive challenge that is predicted to constrain the efficiency and accuracy of their choices (Bernays and Funk 1999; Bernays 2001). Another argument for the evolution of specialists is seen in their enhanced physiological efficiency in dealing with noxious plant compounds, e.g. specialists are found more often on herbaceous plants, which show a broader diversity in chemical composition than woody plants, the typical host of generalists (Schoonhoven et al. 2005). Further, specialist herbivores can enjoy an enemy-free space advantage over generalists by effectively using host plant-derived chemical defenses against predators (Strauss and Zangerl 2002).

The overwhelming presence of specialist herbivores has given rise to the classical concept that host plant specialization is the derived evolutionary form of generalism and an evolutionary dead end (Mayr 1963). More recent work has shown this to be false (Nosil and Mooers 2005). Several studies on insect herbivores demonstrated that generalized lineages are often derived from specialists and host specialization appears to be a dynamic trait. Janz et al. (2001), when studying the butterfly tribe Nymphalini, found out that specialization on Urticales was followed by expansions and concentrations of host range. This result was further supported by Nosil (2002) who compared the transition rates between generalization and specialization in 15 insect groups using previously published phylogenetic studies, and found only a slightly higher overall rate from generalization to specialization than vice versa, with trends varying strongly among taxa. Thus, host plant specialization is not a dead end but a dynamic trait.

Changes in host plant range

A change in host plant range can either be a ‘host range expansion’ or a ‘host shift’. A ‘host range expansion’ refers to a situation where a new host is added to the existing host range with continued utilization of the previous host (Agosta 2006). This differs from the conditions for a ‘host shift’ whereby the original host is excluded from the diet in favor of the novel host. Usually, host shifts occur over evolutionary time ranges whereas host range expansions can happen rapidly and in some cases they are intermediate stages leading to a complete host shift (Janz et al. 2001; Murphy 2007). A general pattern underlies the majority of host shifts and range expansions: host switches in phytophagous insects occur more typically between host plants that

are phytochemically and/or taxonomically related than between distantly related ones (Winkler and Mitter 2008; Feeny 1992).

Two requirements must be met for a host shift or range expansion to occur in an herbivore. First, ecological conditions favoring colonization of the novel host plant must be present, such as extinction of the original host and/or reduced competition, predation and parasitism on the novel host (Ehrlich and Raven 1964). And second, the herbivore must be able to accept and survive on the novel host plant. Thus adaptation to a novel host plant requires either behavioral and/or metabolic pre-adaptation, standing genetic variation or genetic changes. Escape from intraspecific competition is believed to be the reason for host range expansion of the long-horned beetle *Dectes texanus* from the original host plant range Compositae to the novel host soybean (Michaud and Grant 2005; Table 1); whereas enemy free space was the major driver for the parsnip webworm *Depressaria pastinacella* acquiring cow parsnip as novel host plant (Zangerl et al. 2002; Table 1). Under the assumption that host plant chemistry plays a major role in behavioral and/or metabolic pre-adaptation to attractants, deterrents and/or toxins of the novel host plant are proposed mechanisms for facilitating the transition to a novel host (Ehrlich and Raven 1964, Feeny 1991). Murphy and Feeny (2006) have shown that plant chemistry governed the establishment of the host shift within the *Papilio machaon* L. group of swallowtail butterflies to species of the plant family Asteraceae. When herbivores are not behaviorally or metabolically pre-adapted to their novel host plant, genetic variation must have governed the host shift. A classical scenario is that a *de novo* mutation arises leading to a phenotype of improved performance on the novel host plant. The frequency of this beneficial mutant allele increases, because it enhances the reproductive success of individuals carrying it and eventually becomes fixed in the population. There is more than one exception to this scenario, as not only a single mutation might be involved or alternatively, adaptation arises from standing genetic variation, i.e. from pre-existing mutants in the population (Orr 2005).

Historically, changes in host plant range have been seen as longstanding evolutionary events, but in recent times evidence has been found that intermediate stages in dietary changes, such as range expansions before the original host plant is totally neglected, can happen in short periods and be witnessed in a human's life span (Table 1). A well established theory describing host shifts over evolutionary periods is the so-called 'co-evolutionary arms-race' between plants and their insect pests, whereby plants employ novel mechanical and/or chemical defenses to deter herbivores (Ehrlich and Raven 1964). Reciprocally, herbivorous insects invent novel adaptation mechanisms to face these plant defenses and thus are able to explore these previously unpalatable plants to include them in their diet. An example of this theory is the shift of Pieridae but-

terflies from Fabales to Brassicales (Wheat et al. 2007). This shift happened after the diversification of the glucosinolate-containing Brassicales about 80 million years ago and within a period of about 10 million years during diversification of Brassicales (Ehrlich and Raven 1964; Braby and Trueman 2006). Such a long adaptation period allowed for the evolution of a key innovation, the glucosinolate detoxification mechanism, and adaptation to host plant species outside the previous host plant range (Wheat et al. 2007; Fischer et al. 2008). Further evolutionary longstanding ancient transitions, with shifts to unrelated plant families are the above mentioned radiation of Nymphalini butterflies (Weingartner et al. 2006), the shift of small ermine moth species (Yponomeutidae) from Celestraceae to Rosaceae (Menken et al. 1992; Menken 1996), and the shift of Papilionidae butterflies. *Papilio* shifted from Aristolochiaceae to Rutaceae and Apiaceae (Berenbaum 1983; Zakharov et al. 2004) and *Parnassius* to Crassulaceae and thereafter, to Papaveraceae and Fumariceae (Fordyce 2010; Michel et al. 2008).

Still, a host switch is not necessarily the product of longstanding evolution. Certain situations such as decline of the original host plant or the availability of a novel host plant could favor rapid host shifts. The introduction of exotic plants or agricultural crops into novel environments with novel herbivores offers spectacular examples of the rapidness with which a plant can recruit herbivores or a herbivore can expand its list of host plants "without evolution occurring". Introduced plants mainly comprise agricultural crops which are grown predominantly in monoculture and thus present a stable and highly abundant food source during the growing season as compared to the rather scarce and scattered wild host plant. Moreover, such introduced plants are un-adapted to local insect species and especially domesticated crops show a reduced chemical and physical resistance. Several cases of recent host range expansions from native to introduced and wild to cultivated plants have been reported (Table 1). Examples for such scenarios almost always describe host range expansions. The majority occurred between related plant species and only in a few cases were taxonomic and/or phylogenetic unrelated plant species colonized.

Genetic basis of host plant adaptation and range expansions

Studying the genetic basis of host plant adaptation is the study of adult and larval traits. As a shift or expansion to a novel host plant requires the successful adaptation to it, changes in host plant range are likely to be mediated by changes in host plant adaptation traits. Current knowledge of the genetic architecture of host plant adaptation traits is summarized in Table 2, which comprises the mode of inheritance (dominant versus recessive), location of the genes (on autosomes versus sex-chromosomes), and the number of genes involved (many versus few genes).

The availability of fully sequenced genomes and the advance of transcriptome profiling methods have recently enabled the identification of specific or candidate genes involved in host plant adaptation.

Given a genetic basis as proven in crosses and backcrosses, the classical procedure of determining the genetic basis of a trait involved in host plant adaptation trait is to perform crosses between closely related interbreeding species (e.g. *Heliothis virescens* and *H. subflexa*) or intraspecific host races living on different host plants (e.g. pea-breeding and non pea-breeding strain of *Sitophilus oryzae*). The survival rate of hybrid offspring on each others' host plant is indicative of the mode of inheritance. Quantitative trait locus (QTL) analysis is used to infer the number of loci (genes) involved in a trait (e.g. 'survival on host A'). For this analysis F_2 or backcross progeny are phenotyped for the specific trait and genotyped using genetic markers. Markers with the same segregation pattern are clustered together and represent a linkage group, i.e. chromosome. If the trait mean of surviving offspring with one marker allele is significantly higher than for another, this indicates that there is an association between the chromosome to which the marker maps and the trait 'survival on host A'. Depending on whether one or several of such associations are detected, the trait is inherited mono- or oligo- to polygenic.

A range of different genetic architectures underlying host plant adaptation has been found with the above-described methods. In host races of *Mitoura* butterflies, the ability to successfully use cedar as a host in the larval stages was expressed as a recessive trait (Forister 2005). However, the majority of studies reports on dominant inheritance of host plant adaptation traits (see Table 2). In two populations of the brown planthopper *Nilaparvata lugens,* one feeding on cultivated rice *Oryza sativa* and the other feeding on a weed grass *Leersia hexandra*, there was evidence for dominance of rice population alleles over *Leersia* population alleles (Sezer and Butlin 1998). As for the number of genes being involved, a classical view was that most adaptations result from small changes in numerous genes (Fisher 1930), whilst recent laboratory studies (Orr and Coyne 1992; Orr 2005) and theoretical models (Orr 1998) led to the conclusion that a few major genes account for a large portion of adaptation. Hawthorne and Via (2001) detected polygenic inheritance in pea aphids, in other cases evidence for the influence of a limited number of genetic factors (oligo- to monogenic inheritance) was found (Jones 1998; Sezer and Butlin 1998; de Jong et al. 2000). For the location of host plant adaptation genes a general pattern seems to apply: genes affecting larval performance consistently map to autosomes (Tang et al. 2006). Genes controlling oviposition preference are less consistent: they are sex-linked in *Papilio* butterflies (Thompson 1988), but autosomal in moths, *Heliothis virescens* and *H. subflexa,* (Sheck and Gould 1995) and *Yponomeuta* species (Hora et al. 2005).

Each larval and adult trait conferring host plant adaptation can be determined by a number of genes. First, genes with a role in adult chemoreception and neuronal processing leading to oviposition are likely involved, as well as genes underlying larval chemoreception, digestion and nutritional metabolism, enabling larval feeding, growth and survival. Successful adaptation to a (novel) host plant by an herbivore likely requires changes in these adult and larval traits and thus, most likely in the genes underlying these traits. In model organisms for which a sequenced genome exists, specific loci, genes and mutations enabling adaptation have been identified. For example, the genetic basis of oviposition choice in the specialist fly *Drosophila sechellia* was deciphered and attributed to mutations in odorant binding protein (OBP) genes (Matsuo et al. 2007). This fly is a specialist on *Morinda citrifolia*, a plant that is toxic to the closely related *D. melanogaster*, the females of which do not oviposit on this plant. Replacing two *D. melanogaster* OBP genes with *D. sechellia* versions resulted in transformed *D. melanogaster* females whose oviposition preference resembled that of *D. sechellia*.

The recent advance of transcriptional profiling techniques, which do not require prior sequence knowledge, enables and facilitates the identification of candidate genes and gene families responsible for host plant adaptation in non-model organisms. In a cDNA-amplified fragment length polymorphism (cDNA-AFLP) approach Yang et al. (2005) identified transcripts responsive to feeding on resistant rice cultivars in the brown planthopper *Nilaparvata lugens*. Using a different technique, suppression subtractive hybridization (SSH), Brioschi et al. (2007) studied adaptation to soybean in *Spodoptera frugiperda* and revealed that more than one third of the obtained sequences were related to the digestive process.

Aim of this thesis

The aim of my research was to provide insight in the genetic basis underlying host range expansion and adaptation to a novel host plant in the diamondback moth (DBM) *Plutella xylostella* (Lepidoptera: Plutellidae). With its recent host range expansion to sugar pea and the availability of two host strains, one feeding on the original host plant crucifers and the other one feeding a novel host plant (sugar pea) of an unrelated family (Fabaceae), DBM offers the unique possibility to study the genetic basis of a recent host switch event of an important pest species. A characterization of the female phenotype of the new pea host-strain, i.e. female oviposition preference, preceded a detailed analysis of the genetic architecture of larval survival by means of backcrosses and linkage analysis and transcriptional profiling techniques.

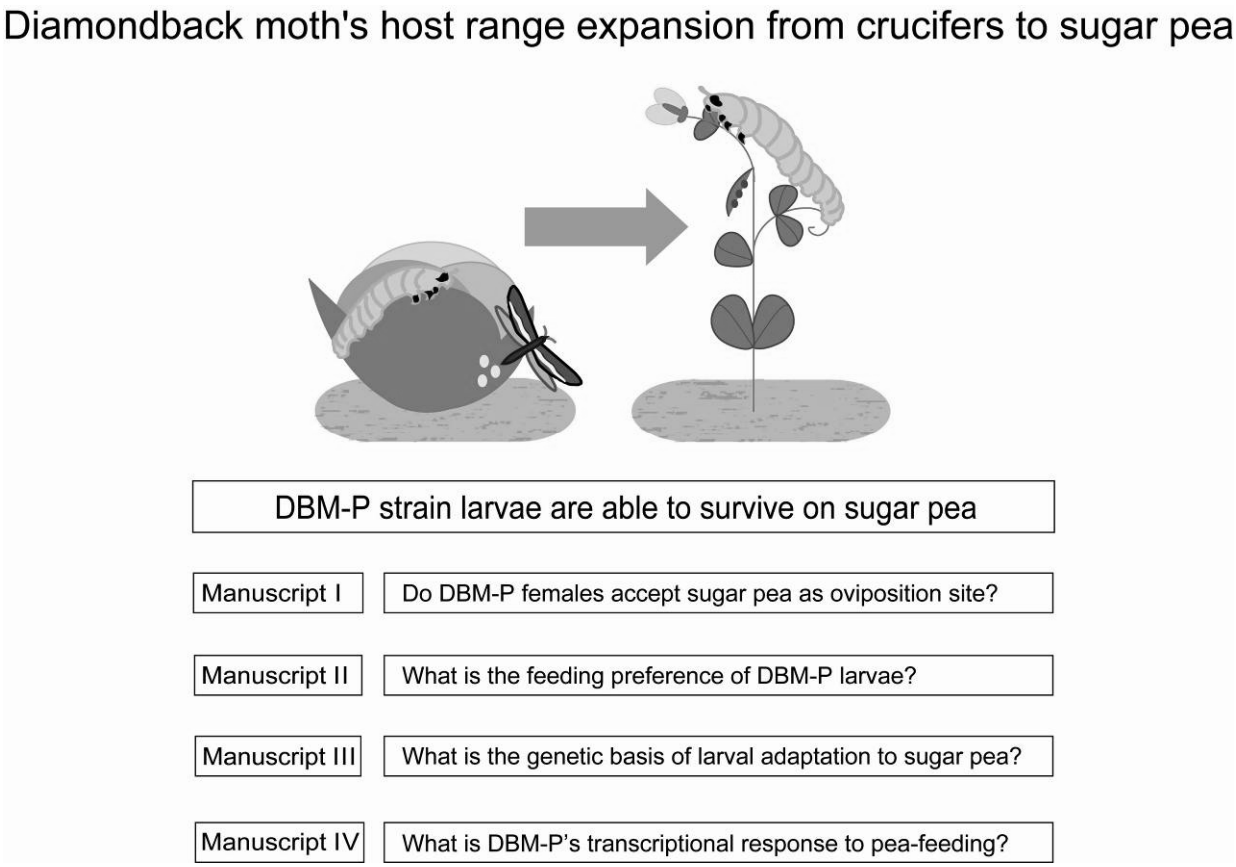


Figure 1 Study system and outline of this thesis. DBM-P is the newly evolved pea-adapted host strain of the diamondback moth.

Study system of this thesis

In this thesis, I analyzed a recent host range expansion of the Brassicaceae specialist DBM to sugar pea, *Pisum sativum* L. var. *macrocarpon*, cultivar Oregon Sugar Pod (Fabaceae), in Kenya. A detailed description of DBM and its host plants is given in Boxes 1 and 2. The host range expansion occurred when DBM densities on the original cabbage hosts were extremely high, and a neighboring pea field became infested (Löhr 2001). Because of its well-known status as a crucifer specialist, the identity of the pest as DBM on this unrelated host plant was doubted until confirmed by an entomologist. In 2000, this local population even expanded to an adjacent field of mangetout peas (*Pisum sativum* L. var. *macrocarpon*, cultivar Snow Green). As the population persisted as an uncontrollable pest on the pea crop in the following two years, the farmer stopped growing peas, so that this population either became extinct in the field or rejoined the populations feeding on the neighboring cabbage (B. Löhr, personal communication). Larvae were collected from the pea crop in 2000 and 2002, and have been reared on Oregon Sugar Pod peas in the laboratory since then (Löhr 2001). While other populations of diamondback moth that we (Janssen et al. 2008) and others (Zhang et al. 2007) have tested cannot survive on pea plants, this population can complete development on a pea host alone and is

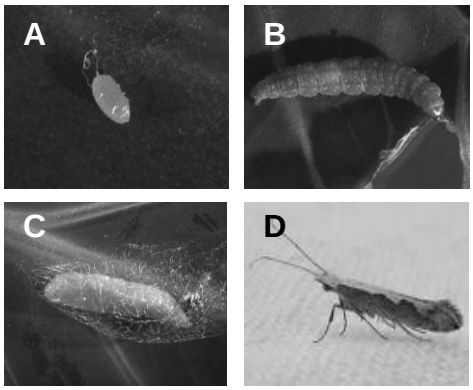
now referred to as DBM-P (Löhr and Gathu 2002), the pea-adapted strain of the diamondback moth. Since this strain can still feed and develop on cabbage, host range expansion rather than switch seems to have occurred, although it may also be in a transitional phase. Thus, this DBM population represents a unique and very recent switch or expansion from the original plant family (Brassicaceae) to a new and dissimilar host plant family (Fabaceae) in the field. A short description of the DBM strains used for experiments in this thesis can be found in Table 3.

BOX 1 Study organism: Diamondback moth

The experimental work in this thesis has been done on the diamondback moth (DBM), *Plutella xylostella* (Lepidoptera: Plutellidae), a specialist pest feeding exclusively on plants of the family Brassicaceae. It is considered one of the most destructive cosmopolitan insect pests of brassicaceous crops with annual management costs of 1 billion US$, not considering the costs of crop losses (Talekar and Shelton 1993). Larvae attack a wide range of economically important crops, and only in the absence of these favoured cultivated crops do they feed on alternate cruciferous weed hosts. DBM was believed to have originated in the Mediterranean region (Harcourt 1957), the origin of cultivated crucifers, although its origin in South Africa (Kfir 1998) and China (Liu et al. 2000), has been discussed recently. Today, DBM is present wherever crucifers are grown and considered the most widely distributed lepidopteran pest (Shelton 2001).

DBM is specifically adapted to brassicaceous plants and can circumvent the crucifer-specific glucosinolate-myrosinase defence system that is toxic to most other herbivores by producing a specific enzyme, the glucosinolate s-sulfatase (GSS) which desulfates the glucosinolates, thereby rendering them invisible to the myrosinases which normally activate glucosinolates to their toxic breakdown products. This enzyme allows DBM to feed on crucifers with impunity. Moreover, glucosinolates have been found to act as feeding and oviposition stimulants for larval and adult DBM, respectively (Nayar and Thorsteinson 1963).

Despite the common belief that DBM is restricted to cruciferous plants, the species has been found to accept host plants other than crucifers for feeding and oviposition under laboratory conditions and has occasionally been found on plants outside its original host range in the field. Gupta and Thorsteinson (1960a) showed that out of 62 species of plants from 37 different families (excluding crucifers) offered as leaf disks to DBM larvae, nine species were eaten, six of them from the Fabaceae. If confined to prevent escape, DBM larvae fed on whole plants of three legumes in the laboratory, and 5% overall developed to pupae on *P. sativum* (Gupta and Thorsteinson 1960a). A Kenyan cabbage strain was selected for survival on sugar peas within four generations (Löhr and Gathu 2002). In a no-choice oviposition assay Gupta and Thorsteinson (1960b) also found low acceptance of *P. sativum* by DBM females, with more eggs being laid on the pot or vial than on the plant, but considered the plant to contain only weak inhibitors of oviposition if any.



Developmental stages of diamondback moth: egg (A), larva (B), pupa (C) and adult (D). Sources A-C Kathrin Henniges-Janssen; D http://lepidoptera.butterflyhouse.com.au/plut/xylost9.jpg.

Several records of DBM on non-cruciferous hosts in the field (e.g. Fabales) can be found in the HOSTS database of the Natural History Museum London (Robinson et al. 2010), but also in literature: In Ghana, *P. xylostella* was apparently recorded on okra in 1971 (Anonymous in Löhr and Gathu 2002), in northern Russia it was reported on chickpea and on a chenopodiaceous vegetable, *Salsola kali* (Reichart 1919 in Löhr and Gathu 2002) and in India the non-cruciferous crop *Amaranthus viridis* L. has been reported to be a host of DBM (Chelliah and Srinivasan 1986).

The DBM is multivoltine with four to 20 generations per year in temperate and tropical regions, respectively (Vickers et al. 2004). A single female can lay up to 200 eggs. Eggs hatch after four to eight days and first instar larvae usually mine the spongy mesophyll tissues (Harcourt 1957). Second, third and fourth instar larvae are surface feeders and consume every aboveground part of the plant. Once mature, larvae spin a cocoon that is attached to the leaves or stems of the plant. The duration of the pupal period varies from four to 15 days depending on the temperature. After adults hatch, mating occurs on that same day and the oviposition period lasts about four days (Talekar and Shelton 1993). Adults feed on water drops or dew and are short lived (Talekar and Shelton 1993).

DBM's remarkable adaptability, e.g. to thrive in many different climates, is also demonstrated by its ability to having developed resistance to almost every synthetic insecticide applied in the field (Sun et al. 1986). DBM became the first crop pest in the world to develop resistance to DDT in Java and Indonesia and to the bacterial insecticide *Bacillus thuringiensis* (Kirsch and Schmutterer 1988).

BOX 2 Host plant range of diamondback moth

Original host plant range: Brassicaceae

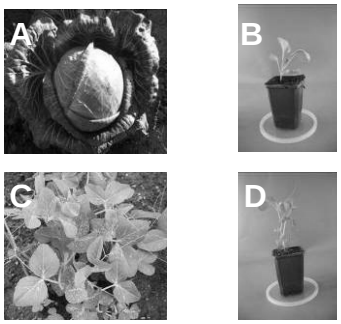
Brassicaceae, also known as Cruciferae, crucifers or mustard oil family, comprise the natural host range of DBM. Brassicaceae originate from the Mediterranean basin and the Near East, and became distributed worldwide after their domestication (Nieuwhof 1969). Different portions of the plant were emphasized during domestication, e.g. broccoli and cauliflower were selected for large edible inflorescences and brussels sprouts and cabbage for leafy buds. With exception of certain cabbages and some types of kale, brassicaceous vegetables have a strong middle stem with alternate leaves with lobed or wavy to highly dissected margins. Leaves are thick and succulent, with or without a waxy bloom.

Phytochemically, Brassicaceae are characterized by mustard oil glucosides, so-called glucosinolates, which are herbivore-deterrents. The glucosinolate-myrosinase defence system is a two-component system. In intact plant tissue, myrosinases and glucosinolates are compartmentalized in different cell compartments. Upon tissue damage (e.g. herbivory), the formerly compartmentalized myrosinase comes into contact with the non-toxic glucosinolates and hydrolyzes them into toxic break-down products (e.g. isothiocyanates). DBM's ability to thrive on brassicaceous plants is associated with the ability to escape this plant defence with the enzyme glucosinolate s-sulfatase (GSS; see Box 1). The vegetable parts of the brassica crops have high water content, and are low in lipids and carbohydrates.

Cruciferous vegetables are important components of many traditional human diets and are grown on small subsistence farms as well as large scale farms. In Kenya, brassica vegetables are grown in the mid and high altitude zones throughout the year and provide an important income for small holder farmers (Oduor et al. 1996).

Novel host plant: Sugar pea

In Kenya 1999, DBM was reported to feed on the fabaceous crop sugar pea (*Pisum sativum* var. Oregon Sugar Pod). Sugar peas originated in Central or South East Asia but now are grown worldwide. They are an annual legume with slender stems. The leaves end in one or more tendrils and have one to three pairs of leaflets. The pod is about 8 cm long and straight or curved containing up to 10 globular smooth or wrinkled seeds. Sugar peas are grown for their edible pods. In contrast to the succulent, waxy and compact leaves of DBM's brassica hosts, pea leaves are soft and thin.



Original and novel host plants of DBM. Fully-grown (A) and 6-week-old (B) cabbage (*Brassica oleracea*) plant. Fully-grown (C) and 6-week-old sugar pea (*Pisum sativum*) plant. Source: Kathrin Henniges-Janssen.

Phytochemically, sugar pea seeds are well characterized but information on the phytochemistry of sugar pea leaves, the part of the plant consumed by DBM larvae, and their compounds is rare. Herbivore defence compounds commonly found in leaves of other Fabaceae species are proteinase inhibitors, especially lectins.

In Kenya, sugar peas have become an important export crop in recent years and sugar pea production gained a growing importance due to an increasing demand for sugar peas in Europe. They are grown year round in cooler high altitude tropical areas.

Manuscripts and main results of this thesis

A shift or expansion to a new niche is almost always initiated by a change in behavior. It is evident that a change in larval feeding behavior must have occurred, as DBM-P larvae are able to maintain themselves on pea in the field as well as in the lab (Löhr 2001; Löhr and Gathu 2002; Henniges-Janssen et al. 2011). The adult phenotype, i.e. female oviposition preference, had not yet been analyzed, which was therefore my first aim, described in Manuscript I. In Manuscript I I address the following questions: How readily do DBM-P females accept peas for oviposition? Do they still utilize the ancestral plant or do they prefer pea? To address these questions, I performed two types of tests with females of DBM-P as well as with another Kenyan cabbage strain (DBM-Cj) for comparison. To assess oviposition acceptance, no-choice experiments were conducted, in which females were confined with either a cabbage or a pea plant. To assess oviposition preference, females were offered both plant species at the same time. Surprisingly, DBM-P females laid most eggs on cabbage and very few on peas. However, they laid significantly more eggs on the cabbage plant when pea plants were present. These findings suggest that DBM-P manifested the initial stages of an evolutionary host range expansion, which is incomplete due to lack of oviposition fidelity on pea plants. Whereas larval performance has already been studied by Löhr and Gathu (2002), knowledge on the preference behavior in the newly evolved DBM-P strain is rare. Manuscript II describes the preference behavior of DBM-P larvae in comparison to DBM-Cj and cabbage reared DBM-P larvae. Larvae were offered pea and cabbage leaf discs and larval position and percentage of consumed leaf area was recorded after certain time intervals. This revealed that DBM-P larvae preferred their novel host plant pea whereas DBM-Cj fed exclusively on cabbage. The cabbage-reared DBM-P larvae also preferred cabbage, with a few larvae being observed on pea and consuming pea leaf discs.

Unraveling the genetic basis of larval adaptation to pea was the foremost goal of the present thesis. Understanding the genetic architecture underlying host shifts and range expansions is an attractive research field for evolutionary biologists, because of the relevance of such events in host race formation, ecological speciation, sympatric speciation and insect diversity (Ehrlich and Raven 1964; Bush 1969; Bush 1975; Funk et al. 2002). In addition, there is considerable interest from an applied agricultural perspective. A complete understanding of the mechanisms by which an herbivore evolves as a new pest species can help in minimizing these events and has implications for pest control and growing procedures. Manuscript III describes my approach to determine the mode of inheritance. First, larval survivorship on the novel host plant pea and a typical crucifer host (kale) was measured in reciprocal F_1, F_2 and backcrosses between the DBM-P strain and a strain reared only on crucifers (C-strain) to reveal a) whether the

trait ‘larval adaptation to pea’ is inherited dominantly or recessively, and b) whether this trait is sex-linked or not. Subsequently, backcross individuals were genotyped for linkage analysis using AFLP markers to determine whether the trait is inherited mono-, oligo- or polygenically. As a lepidopteran insect, DBM is well-suited for linkage analysis, because crossing-over during meiosis is absent in females and maternally inherited linkage groups are actually chromosomes (Heckel 1993). Backcrosses to DBM-P produced higher survivorship on pea than C-strain backcrosses, suggesting recessive inheritance. The linkage analysis using three different P-strain backcross families revealed two, four and five linkage groups contributing to survival on pea respectively, indicating oligogenic inheritance. Thus the newly evolved ability to survive on pea has a complex genetic basis, and the DBM-P strain is still genetically heterogeneous and not yet fixed for all the alleles enabling it to survive on pea.

Manuscript IV presents my approach to identify candidate genes underlying host range expansions and adaptations. Whereas a number of studies identified genes and genetic mechanisms underlying host plant adaptation in model-organisms, insight remains scarce on the mechanisms in important pest insects and the transcriptional response of specialist feeding in general. I applied two transcriptome profiling techniques, cDNA-AFLP and subtractive suppression hybridization, appropriate for non-model organisms such as DBM, to characterize the transcriptional response of specialist larvae feeding on a novel host plant and to identify functional groups of genes enabling the shift to sugar pea. This approach revealed a globally altered transcriptome profile of pea-feeding DBM larvae involving a large number of genes, affiliated with a variety of different functional classes (e.g. metabolism, detoxification, response to stimulus, response to stress), which can be hypothesized to mediate DBM’s host range expansion to peas.

In conclusion, DBM-P seems to have initiated a host-range expansion, which is governed by a number of recessive genes that are autosomally inherited and not yet fixed. The most likely candidate gene(s) involved in this expansion are those with a role in host plant perception, digestion and detoxification. It was very surprising that this sudden host range expansion involves a number of genes, and is thus a complex trait. One would expect that sudden change involves one or very few major genetic changes. It was also surprising to find that different backcross families varied in at least some loci involved in being able to survive on pea. Obviously, the genetic basis of this trait is not yet fixed in the DBM-P strain. The main future task is to identify the actual genes underlying host plant expansion in the pea-adapted strain of DBM. After identification it will become possible to trace back possible genetic variation(s) in these genes among DBM populations, which is necessary to unravel the mechanism(s) of how a sudden change in host plant use may arise.

Table 1 Examples of recent host range expansions in chronological order; n/a = not applicable.

Species	Original host	Novel host	When	Where	Conditions	Reference
A) Shift to phylogenetically related host						
Yucca moth (*Prodoxus quinquepunctellus*) (Lepidoptera: Prodoxidae) (S)	Yucca palm *(Yucca filamentosa)*	Yucca palm *(Yucca aloifolia)*	1500s	Southeastern Atlantic coast, USA	Introduction of *Y. aloifolia* from Mexico to Southeastern Atlantic coast around 1500.	Groman and Pellmyr 2000
Edith's checkerspot (*Euphydryas editha*) (Lepidoptera: Nymphalidae) (G)	*Collinsia parviflora* (Scrophulariaceae)	Plantain (*Plantago lanceolata)* (Plantaginaceae)	1880s	Sierra Nevada, USA	Introduction of *P. lanceolata* into North America from Europe ~150-200 years ago.	Singer 1971; Singer et al. 2008
Melissa blue (*Lycaeides melisssa*) (Lepidoptera: Lycaenidae) (S)	Wild Fabaceae	Cultivated alfalfa (*Medicago sativa*) (Fabaceae)	1800s	North America	Introduction of *M. sativa* into North America ~200 years ago. Some *L. melissa* populations exclude native hosts that are still locally available.	Forister et al. 2009
Parsnip webworm (*Depressaria pastinacella*) (Lepidoptera: Oecophoridae) (S)	Apiaceae	Cow parsnip (*Heracleum lanatum*) (Apiaceae)	1800s	North America	Introduction of *D. pastinacella* from Europe to North America ~150 years ago.	Berenbaum 1983; Zangerl et al. 2002
Gall midge (*Rhopalomyia yomogicola*) (Diptera: Cecidomyiidae) (S)	Native *Artemisia* species	Introduced *Artemisia* species	1850s	Japan	n.a.	Nohara et al. 2007
Apple maggot fly (*Rhagoletis pomonella*) (Diptera: Tephritidae) (S)	Hawthorn (*Crataegus* spp.)	Apple (*Malus pumila*)	1860s	North America	Introduction of apple into North America from Europe almost 400 years ago, establishing a mamximum possible age for the host shift.	Bush 1969
Yellow sulphur (*Colias philodice eriphyle*) (Lepidoptera: Pieridae) (S)	Fabaceae	Cultivated alfalfa (Medicago sativa) (Fabaceae)	1890s	Colorado, USA	Introduction of cultivated alfalfa to North America ~200 years ago.	Tabashnik 1983
Ladybird (*Epilachna yasutomii)* (Coleoptera: Coccinilidae) (S)	Wild Solanaceae	Cultivated potato (Solanaceae)	1930s	Central Honshu, Japan	Increased potato cultivation in the 1930's in mountainous area of Honshu Japan.	Shiral and Morimoto 1999
Soapberry bug (*Jadera haematoloma*) (Heteroptera: Rhopalidae) (S)	Balloon vine (*Cardiospermum corindum*) Sapindaceae	Flat-podded goldenrain tree (*Koelreuteria elegans*)	1950s	Florida, USA	Increasing plantation of *K. elegans* since 1950's for landscaping purposes. Rapid host range expansion within 20-50 years.	Carrol and Boyd 1992; Carroll et al. 2001
Seed beetle (*Stator limbatus*) (Coleoptera: Bruchinae) (S)	Fabaceae	Texas ebony (*Chloroleucon ebano*) (Fabaceae)	1970s	Phoenix, Arizona, USA	Introduction of Texas ebony post -1972. *S. limbatus* has never been observed on *C. ebano* before.	Fox 2006
Tephritid fly (*Tephritis conura*) (Diptera: Tephritidae) (S)	Melancholy thistle (*Cirsium heterophyllum*)	Marsh thistle (*Cirsium palustre*)	1980s	Northern Britain	n.a.	Diegisser et al. 2009
Winter pine processionary moth (*Thaumetopoea pityocampa)* (Lepidoptera: Notodontidae) (S)	Austrian black pine (*Pinus nigra)*	Scots pine (*Pinus sylvestris*), Mountain pine (*Pinus mugo*)	1990s	Italian Alps, Italy	Latitudinal and altitudinal range expansion of *T. pityocampa* from Southern Europe to Northern Italy (geographical range of novel host plants) over recent decades.	Stastny et al. 2006
Seed beetle (*Acanthoscelides macrophthlamus*) (Coleoptera: Bruchinae) (S)	*Leucaena* species (Fabaceae: Mimosoideae)	*Falcataria moluccana* (Fabaceae: Mimosoideae)	2000s	Taiwan	Introduction of *F. moluccana* from Moluccas and New Guinea. *F. molucca* is first and only host besides *Leucaena* species.	Tuda et al. 2009
Laboratory conditions						
Seed beetle (*Callosobruchus maculatus)* (Coleoptera: Bruchinae) (S)	Mung bean (*Vigna radiata*) (Fabaceae)	Lentil (*Lens culinaris*) (Fabaceae)	n/a	n/a	Survival rate on lentil over 85% within <20 generations.	Messina et al. 2009
B) Shift to phylogenetically unrelated host						
Baltimore checkerspot (*Euphydryas phaeton)* (Lepidoptera: Nymphalidae) (S)	Turtlehead (*Chelone glabra)* (Scrophulariaceae)	Plantain (*Plantago lanceolata)* (Plantaginaceae)	1850s	North America	Introduction of *P. lanceolata* into eastern North America from Europe ~150-200 years ago.	Bowers et al. 1992
Long-horned beetle (*Dectes texanus*) (Coleoptera: Cerambycidae) (G)	Compositae	Soybean (*Glycine max*) (Fabaceae)	1940s	North America	Intensive commercial growing of soybean started in 1940s. *D. texanus* /soybean association evolved over the past 50-60 years.	Michaud and Grant 2005
Ladybird (*Epilachna vigintioctopunctata*) (Coleoptera: Coccinilidae) (S)	Solanaceae	*Centrosema pubescens* (Leguminosae)	1990s	Indonesia and Malaysia	Introduction of *C. pubescens* from South/Central America into Southeast Asia as plantation plant and green manure.	Shirai and Katakura 2000
Pale green triangle (*Graphium eurypylus)* (Lepidoptera: Papilionidae) (S)	Annonaceae	*Michelia champaca* (Magnoliaceae)	2005-2006	Brisbane, Australia	Observed in Brisbane botanical garden.	Larsen et al. 2008
Laboratory conditions						
Twospotted spider mite (*Tetranychus urticae*) (Acari: Tetranychidae) (G)	Lima bean (*Phaseolus vulagris*)	Cucumber (Cucurbitaceae)	n/a	n/a	Laboratory experiment over 21 months (~50 mite generations).	Gould 1979

Table 2 Examples for the genetic basis of host plant adaptation; n/a = not applicable.

Species	Trait	Genetic basis			Reference
		Location	Number of genes	Mode of inheritance	
A) Intraspecific pairs					
Rice weevil (*Sitophilus oryzae*) (Pea-breeding strain/Non-pea-breeding strain)	Larval ability to feed on pea	Autosome	Monogenic	Recessive	Grenier et al. 1997
Brown planthopper (*Nilaparvata lugens*) (Rice strain/Weed grass strain)	Larval feeding on rice	n/a	Oligogenic	Dominant	Sezer and Butlin 1998
Seed beetle (*Callosobrochus maculatus*) (*Vicia faba* selected strain/Non-selecetd strain)	Larval survival on *Vicia faba*	n/a	n/a	Recessive	Huignard et al. 1996
Fruit fly (*Drosophila tripunctata*) (Mushroom population/Tomato population)	Oviposition preference for mushroom	Autosome	Oligogenic	n/a	Jaenike 1987
Seed beetle (*Callosobrochus maculatus*) (Asian strain/African strain)	Oviposition preference	Autosome (assumed)	Oligogenic	Dominant toward Asian strain	Messina and Slade 1997; Fox et al. 2004
Tephritid fly (*Eurosta solidaginis*) (*Solidago altissima* race/*S. gigantea* race)	Adult host preference for *S. gigantea*	Autosome	Oligogenic	Dominant	Craig et al. 2001
Flea beetle (*Phyllotreta nemorum*) (*Barbarea vulgaris* resistant strain/*B. vulgaris* non-resistant strain)	Ability to use *Barbarea vulgaris* as a host	Autosome, Sex-chromosome †	Monogenic, oligogenic †	Dominant	de Jong and Nielsen 1999; de Jong et al. 2000
B) Interspecific pairs of closely related species					
Small ermine moths (*Yponomeuta padella*/*Y. malinellus*)	Larval gustation	Autosome	n/a	n/a	van Drongelen and van Loon 1980
Flea beetles (*Altica viridicyanea*/*A. fragariae*)	Larval feeding preference for own host plant	Autosome	Oligogenic	Dominant	Xue et al. 2009
Noctuid moths (*Helicoverpa armigera*/*H. assulta*)	Larval feeding preference for *H. armigera* host cotton	Autosome	Oligogenic	Dominant	Tang et al. 2006
Small ermine moths *Yponomeuta cagnagellus*/*Y. malinellus*)	Oviposition preference for *Y. cagnagellus* host plant *Euonymus europaeus*	Autosome	n/a	Semi-dominant	Hora et al. 2005
Mitoura butterflies (*Mitoura nelsoni*/*M. muiri*)	Oviposition preference for *M. nelsoni* host plant cedar	n/a	n/a	Dominant	Forister 2005
	Larval performance on *M. nelsoni* host plant cedar	n/a	n/a	Recessive	Forister 2005
Fruit flies (*Drosophila sechellia*/*D. simulans*)	Adult resistance to plant toxins of *D. sechellia* host *Morinda citriifolia*	n/a	Oligogenic	Dominant	Jones 1998
	Larval resistance to plant toxins of *D. sechellia* host *M. citriifolia*	Autosomes	Oligogenic	Intermediate	
	Oviposition preference for *D. sechellia* host *M. citriifolia*	Autosome	Oligogenic	Recessive	
Tobacco budworms (*Heliothis virescens*/*H. subflexa*)	Oviposition preference	Autosome	n/a	n/a	Sheck and Gould 1995 a
	Larval growth	Autosome	n/a	n/a	Sheck and Gould 1995b
Swallowtails (*Papilio glaucus*/*P. canadensis*)	Oviposition preference	Sex-chromosome (X)	n/a	n/a	Sperling 1994
	Larval feeding on birch, aspen, tuliptree	Autosome	Polygenic	n/a	Hagen et al. 1991
Swallowtails (*Papilio machaon*/*P. zelicaon*)	Oviposition preference	Sex-chromosome (X)	Oligogenic	n/a	Thompson 1988
	Larval feeding	Autosome	Oligogenic	n/a	Thompson et al. 1990

†Locally varying genetic basis of this trait.

Table 3 Description of DBM strains used in experiments presented in this thesis.

Strain	Origin	Reared on	Description
DBM-P or **P-strain**	Kenya	Sugar pea	Originally collected from the infested pea field in Naivasha in 2000, and repeatedly replenished with additional field-collected material from the same site for the next two years. It has been maintained as a laboratory culture since then at ICIPE in Kenya and was sent to Max Planck Institute for Chemical Ecology (MPICE, Jena, Germany in May 2005, where this strain has been reared for more than 50 generations since then.
DBM-Cj	Kenya	Cabbage	Derived from a field population from the semi-arid areas about 40 km southeast of Nairobi and was sent to the MPICE in May 2005, where it has been reared for more than 50 generations since then.
C-strain	Australia	Kale	Originally obtained from Waite Campus, Adelaide, South Australia and derived from a field collection in South Australia and was maintained as a laboratory culture for many generations in the laboratory of Dr. Nancy Endersby, Victorian Department of Natural Resources, from whom it was obtained. At MPICE it has been reared since 2005 for more than 50 generations.
G88	USA	Diet	Collected in 1988 from cabbage at the New York State Agricultural Experimental Station, Robbins Farm, Geneva, New York, USA. It has been reared on a wheat germ-casein artificial diet ever since. In 1996 it was kindly provided by A. M. Shelton (Cornell University, Geneva, New York, USA) to MPICE whereit has been reared since then.

3. Manuscript I

Oviposition of diamondback moth in the presence and absence of a novel host plant

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This manuscript addresses the oviposition acceptance and preference of females of the newly evolved pea-adapted host strain of DBM (DBM-P) on original and novel host plant. The results suggest that DBM-P manifested the initial stages of an evolutionary host range expansion, which is incomplete due to lack of oviposition fidelity on pea plants.

KHJ conceived the study and conducted the experiment. KHJ, ATG, AR and DGH discussed results and performed data analysis. GS helped with statistical analysis. The manuscript was written by KHJ and revised by ATG and DGH.

Abstract

The diamondback moth (DBM, *Plutella xylostella* L. (Lepidoptera: Plutellidae)) consumes a wide variety of brassicaceous host plants and is a common pest of crucifer crops worldwide. A highly unusual infestation of a sugar pea crop was recorded in Kenya in 1999, which persisted for two consecutive years. A strain (DBM-P) from this population was established in the laboratory and is the only one of several strains tested that can complete larval development on sugar peas. The oviposition acceptance and preference of the DBM-P strain was assessed in the presence of cabbage plants, sugar pea plants or both, in comparison to another strain (DBM-Cj) that was collected from cabbage and is unable to grow on pea plants. As expected, DBM-Cj females preferred to oviposit on cabbage plants. Surprisingly, DBM-P females also laid most eggs on cabbage and very few on peas. However, they laid significantly more eggs on the cabbage plant when pea plants were present. Our findings suggest that DBM-P manifested the initial stages of an evolutionary host range expansion, which is incomplete due to lack of oviposition fidelity on pea plants.

3.1 Introduction

We are surrounded by a plethora of herbivorous insects feeding on many different kinds of host plants. This high ecological diversity results from a dynamic process in which insect populations may change their ecological niches throughout evolution (Funk et al. 2002; Janz and Nylin 2008). When an insect species adds a new host plant to its diet, host range expansion has occurred. When it has lost the ability to feed on the original host plant as well, this is considered a host shift (Tabashnik 1983; Bernays and Chapman 1994). Research on host range expansions and shifts in herbivorous insects has been focused mostly on the crucial role that adaptation to the host plant plays in the early stages of speciation as a starting point for diversification (Bush 1969; Via 1999; Schwarz et al. 2005; Janz et al. 2006; Mercader and Scriber 2007) or understanding the coevolutionary processes between herbivorous insects and their plant hosts (Ehrlich and Raven 1964; Wheat et al. 2007; Stenberg et al. 2008). Only recently, the evolutionary dynamics of the process have received increasing attention (Janz et al. 2001 and 2006). Does the acquisition of novel food sources proceed via sudden shifts or rather via gradual expansions of the host range? How does the subsequent loss of the ability to feed on the original food source occur? In most species these changes have taken place in the evolutionary past, so that the sequence of events leading to the current state of ecological differentiation cannot be studied easily.

Successful range expansion to a new host plant requires both physiological and behavioral adaptations (Wasserman and Futuyma 1981): (i) larvae must be able to recognize, digest and fully develop on the newly acquired host plant; and (ii) adult females have to be able to find and accept the new plant as an oviposition site (Rausher 1982; Thomas et al. 1987; Bowers et al. 1992). In many cases, the newly acquired host plants are chemically similar to the original host (Ehrlich and Raven 1964; Becerra and Venable 1999; Murphy and Feeny 2006). For example, populations of the apple maggot fly, *Rhagoletis pomonella* (Diptera: Tephritidae), shifted from native hawthorn trees to introduced apple trees during the mid-19th century (Bush 1969; Feder et al. 1994; Forbes et al. 2005) so that there are now two host races. Both host trees belong to the Rosaceae. Another example is the host plant range expansion by the legume feeding butterfly *Colias eriphyle* (Lepidoptera: Pieridae) to the forage crop alfalfa (*Medicago sativa*) about 100 years ago (Tabashnik 1983), with the new host plant also belonging to the Fabaceae. It is rare to find the acceptance of a novel host plant species that belongs to a chemically and evolutionarily unrelated group of plants, which has not been previously utilized as a host within the evolutionary lineage of the herbivore (Strong 1979; Bush 1994).

The highly specialized crucifer-feeding diamondback moth (DBM) *Plutella xylostella* L. (Lepidoptera: Plutellidae) provides a unique opportunity to study a contemporary event of adaptation to a chemically and evolutionarily unrelated novel host and the underlying behavioral changes in the herbivore. The natural host range of DBM encompasses wild crucifers (Brassicaceae), and it is a significant worldwide pest of cultivated crucifers, a plant family characterized by the glucosinolate-myrosinase defense system against herbivore attack. DBM has the ability to deactivate this defense system using a highly active glucosinolate-sulfatase and, thus, is specifically adapted to brassicaceous plants (Ratzka et al. 2002). Thus, it was very surprising to find DBM feeding on sugar snap peas, *Pisum sativum* L. var. *macrocarpon*, cultivar Oregon Sugar Pod (Fabaceae) in the field in the area south of Lake Naivasha in the Rift Valley, Kenya, in 1999 (Löhr 2001). That year, DBM densities on the original cabbage hosts were extremely high, and a neighboring pea field became infested. Because of its well-known status as a crucifer specialist, the identity of the pest as DBM was doubted until confirmed by an entomologist. In 2000, this local population even expanded to an adjacent field of mangetout peas (*Pisum sativum* L. var. *macrocarpon*, cultivar Snow Green). Because the population persisted as an uncontrollable pest on the pea crop in the following two years, the farmer stopped growing peas, so that this population either became extinct in the field or rejoined the

populations feeding on the neighboring cabbage (B. Löhr, personal communication). Larvae were collected from the pea crop in 2000 and 2002, and have been reared on Oregon Sugar Pod peas in the laboratory since then (Löhr 2001). While the other populations of diamondback moth that we (Janssen et al. 2008) and others (Zhang et al. 2007) have tested cannot survive on pea plants, this population can complete development on a pea host alone and is now referred to as DBM-P (Löhr and Gathu 2002), the pea-adapted strain of the diamondback moth. Thus, this DBM population represents a unique and very recent switch or expansion from the original plant family (Brassicaceae) to a new and dissimilar host plant family (Fabaceae) in the field.

To determine whether DBM-P was just in the initial phases of a host range expansion, or has gone further towards a complete host shift, the process of adaptation in larvae and adults must be examined. Evidence for possible pre-adaptation to peas as a novel host was obtained by Gupta and Thorsteinson (1960a), who showed that out of 62 species of plants from 37 different families (excluding crucifers) offered as leaf disks to DBM larvae only nine species were eaten, six of them from the Fabaceae. If confined to prevent escape, DBM larvae fed on whole plants of three legumes in the laboratory, and 5% overall developed to pupae on *P. sativum*. How well and by what means have DBM-P larvae adapted to the new host compared with the original crucifers? In feeding assays Löhr and Gathu (2002) compared the DBM-P strain to a strain from neighboring cabbage fields (DBM-C). They showed that DBM-C survived very poorly on peas (88% survival on kale and 2% on peas), but DBM-P did equally well on both host plants (85% survival on kale and 83% on peas). Despite this similar overall survival rate on pea plants, developmental time of DBM-P larvae was still significantly longer on the new host plant peas than on the original kale host plant; and pupal weight was significantly lower for DBM-P reared on pea than on kale. When Löhr and Gathu (2002) started a new laboratory culture from the few DBM-C survivors from pea, and reared these on pea plants, larval survivorship increased gradually from 2% in the first generation to 50% in the fourth generation, suggesting that selection acted to increase the frequency of pea-adapted alleles over time. We have subsequently shown that the ability of DBM-P larvae to complete development on pea has a polygenic genetic basis, which does not diminish the ability to complete development on kale (Henniges-Janssen et al. in press). This indicates that DBM-P larvae have expanded their host range to include pea, by means of genetic changes in response to selection, but have not undergone a host shift by losing the ability to perform on the original host.

Adaptation of DBM-P adults, in contrast to larvae, has received less attention. The question of whether adult adaptation (i.e. oviposition preference) is a trait independent of larval adaptation has already been addressed (Thompson 1988), and for several butterfly species it has been found that these traits were indeed controlled by different genes (Wiklund 1975; Thompson 1988; Forister 2005). Adult adaptation, thus, could evolve independently from larval adaptation. Gupta and Thorsteinson (1960b) found low acceptance of *P. sativum* by DBM females in no-choice experiments, with more eggs being laid on the pot or vial than the plant, but considered the plant to contain only weak inhibitors of oviposition if any. The central question we addressed in this study is how readily do DBM-P females accept peas for oviposition; do they still utilize the ancestral plant or do they prefer pea? To address these questions, we performed two types of tests with females of DBM-P as well as with another Kenyan cabbage strain (DBM-Cj) for comparison. To assess oviposition acceptance, no-choice experiments were conducted, in which females were confined with either a cabbage or a pea plant. To assess oviposition preference, females were offered both plant species at the same time. We predicted that DBM-P females would either oviposit similarly well on cabbage and pea plants, or prefer their new host pea for oviposition. We found instead that DBM-P oviposition was increased overall in the presence of pea plants but not specifically targeted to pea plants, suggesting a very early stage in adaptation to the new host.

3.2 Materials and methods

Insects

Two strains of *P. xylostella* were used in the oviposition experiments: the cabbage feeding strain (DBM-Cj) and the pea adapted strain (DBM-P). Both strains originate from Kenya and were kindly provided by Bernhard Löhr from the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya. DBM-P was originally collected from the infested pea field in Naivasha in 2000, and was repeatedly replenished with additional field-collected material from the same site for the next two years (Löhr and Gathu 2002). It has been maintained as a laboratory culture since then at ICIPE in Kenya. DBM-Cj derived from a field population from the semi-arid areas about 40 km southeast of Nairobi. Both strains were sent to the Max Planck Institute for Chemical Ecology (Jena, Germany) in May 2005, where they have been reared for more than 50 generations since then. Population sizes of the strains maintained in Kenya are unknown to us, but averaged about 400 adults per generation in Jena. Insect cultures of both strains were reared from egg to adult stage on intact plants (for rearing procedure

of plants see below) in mesh cages (60×60×60 cm) at 21°C, 50% RH and 16:8 L:D photoperiod, with DBM-Cj reared on cabbage and DBM-P reared on pea. For mating and oviposition, adult moths were collected with an aspirator (BioQuip Products, Rancho Dominguez, CA, USA) and transferred from cages to mating boxes (15×15×5 cm). Each box contained at least 30 individuals, and per generation 12 to 15 boxes were set up. The bottom of the boxes was covered with tissue paper on which leaves of the respective host plants (cabbage or pea) were placed as oviposition sites. Adult moths were fed with 5% honey solution. After eggs were deposited on leaves and the tissue paper, these were transferred to cages and fresh leaves were added to the plastic box.

Plants

Seeds of pea, *Pisum sativum* L. var. *macrocarpon*, cultivar Oregon Sugar Pod, were obtained from Agri-Saaten GmbH (Bad Essen, Germany). Cabbage seeds, *Brassica oleracea* var. *capitata*, cultivar Gloria, were obtained from B and T World Seeds (Aigue-Vives, France). Plants used for rearing of insects were grown in trays (58×32×11.5 cm) in a peat-based substrate (Klasmann Kultursubstrat TS1, Geeste-Groβ Hesepe, Germany) under greenhouse conditions at 21–23°C, 50–60% RH and 14:10 L:D photoperiod. Each tray contained approximately 60 plants. These trays were put into rearing cages. Plants for oviposition experiments developed under the same conditions, except that only seedlings were grown in flat trays. After two weeks, seedlings were separated and grown individually in single pots (7×7×8 cm). For oviposition experiments, five-week-old single potted pea and cabbage plants of similar size were used. At this stage, both plant species had a leaf area of similar size of approximately 200 cm^2.

Oviposition experiments

Oviposition experiments were designed to evaluate the oviposition acceptance (no-choice) and preference (choice) of DBM adult females of the newly evolved pea host strain in comparison to the crucifer host strain. Five virgin female and male moths were collected for each trial from the colony by removing intact pupae (with cocoon) from the rearing cages and isolating them in plastic vials to prevent uncontrolled mating and egg laying. Isolated pupae were stored in a growth chamber at 21°C, 50% RH and 16:8 L:D photoperiod. After emergence, moths were provided with 5% honey solution. Thirty-six to 48 hours after emergence, adult moths were sexed and released into mesh cages (60×60×60 cm) in groups of five males and five females. Placement of moths in the cages was always done at around 5 pm. Once

placed in the cages, moths were allowed to freely mate and oviposit on the offered host plant(s). Cotton balls saturated with 5% honey solution were placed in cages to serve as feeding sites. Plants were watered regularly. Moths were left in cages for three consecutive days (72 h). After three days, plants were removed from the cage and the numbers of eggs laid on each host plant were counted; eggs laid at other sites in the cage were ignored. Eggs laid elsewhere in the cage were omitted from the analysis because of the high chance of missing some of the minute eggs. In addition, individuals from eggs off the plant would have a very low chance of survival in the field, and thus would not substantially promote a host range expansion in nature. In the acceptance (no-choice) test, either a cabbage or a pea plant was placed in the cage. In the preference (choice) test, one plant of each species, cabbage and pea, were positioned in the cage such that the distance between both plants was about 40 cm with no physical contact. This resulted in three experimental set-ups: (i) a pea plant, or (ii) a cabbage plant offered separately, and (iii) a pea and a cabbage plant offered together in one cage. Both acceptance and preference test were repeated six times for each of the strains, i.e. DBM-P and DBM-Cj. All experiments were conducted in a controlled climate chamber at 21°C, 50% RH and 16:8 L:D photoperiod.

To assess variation in developmental time between the two strains, we analyzed the generation times in days of both strains over the past four years in the laboratory, i.e. from September 2006 until January 2010.

Data analysis

A two-way analysis of variance (ANOVA) was conducted to determine differences in the total number of eggs laid on the plants in the different tests. First, combining results from acceptance and preference tests, we tested for overall effects of strain, treatment (choice, no-choice pea plant and no-choice cabbage plant) and the interaction between strain and treatment. Since we found significant differences between the strains, we subsequently assessed differences in the total number of eggs laid on the plants within strains across all three treatments, using one-way ANOVA with a Tukey adjustment for multiple comparisons. To assess whether the generation times significantly differed between the two strains, we conducted a two-tailed Student's t-test. All analyses were performed using SAS software version 9.1 (SAS Institute 2002-2003).

3.3 Results

The total number of eggs laid on the plants differed significantly between strains and across the three treatments (no-choice pea plant, no-choice cabbage plant, choice of both) (Table 1). Overall, DBM-C_j females laid significantly more eggs than DBM-P females (Figure 1, Table 1). There was a suggestive but non-significant strain × treatment interaction (P = 0.07, Table 1). Under no-choice conditions, DBM-Cj females deposited on average 82.8 eggs (±25.9 SD) on cabbage plants and 1.5 eggs (±3.7 SD) on pea plants (Figure 1). DBM-P females laid on average 28.3 eggs (±14.4 SD) on cabbage and 0.8 eggs (±1.6 SD) on pea plants (Figure 1). Thus, under no-choice conditions, both strains oviposited significantly more eggs on cabbage than on pea plants ($P_{adj} < 0.001$ for DBM-Cj, $P_{adj} < 0.01$ for DBM-P).

When given a choice between a pea and a cabbage plant, both strains preferred to oviposit on the cabbage plant (Figure 1). DBM-Cj females oviposited on average 82 eggs (±23 SD) on cabbage and 4.5 eggs (±10.1 SD) on pea (Figure 1). DBM-P females oviposited on average 55.5 eggs (±19.6 SD) on cabbage and 1.7 eggs (±4.0 SD) on pea (Figure 1). Thus, almost all eggs (> 90%) were laid on cabbage plants, irrespective of DBM strain identity. However, DBM-P females laid significantly more eggs on cabbage when given the choice between pea and cabbage plants than when offered a cabbage plant alone (55.5 (±19.6) versus 28.3 (±14.4)) ($P_{adj} < 0.01$; Figure 1). No such difference across treatments was observed for DBM-Cj females (82±23 SD eggs under choice conditions; 82.8±25.9 SD eggs under no-choice conditions; $P_{adj} = 0.99$).

The generation times between the two strains significantly differed between the two strains (P < 0.0001); the average generation time for DBM-Cj was 24.5±3.5 days, while the average generation time for DBM-P was 27±2.5 days (Figure 2).

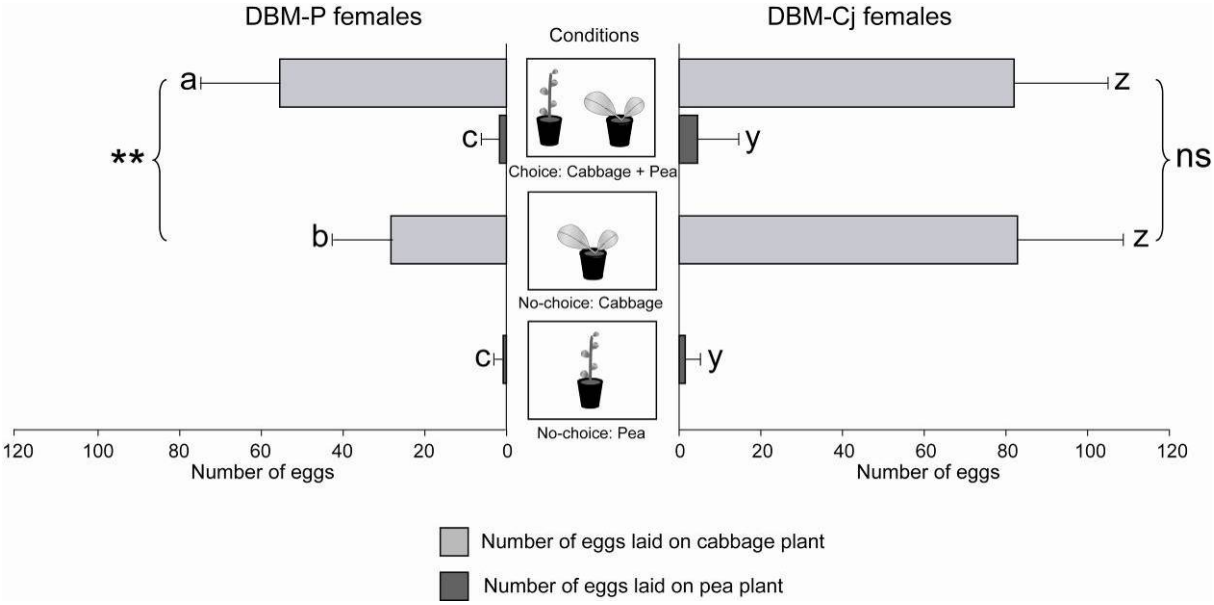


Figure 1 Number of eggs laid by *P. xylostella* pea (DBM-P) or cabbage strain (DBM-Cj) females on cabbage and pea plants under choice and no-choice conditions, respectively (n = 6; mean ± SD). ns = not significant, ** P < 0.01. One-way ANOVA, Tukey's adjustment. Bars marked with same letters are statistically not significant different.

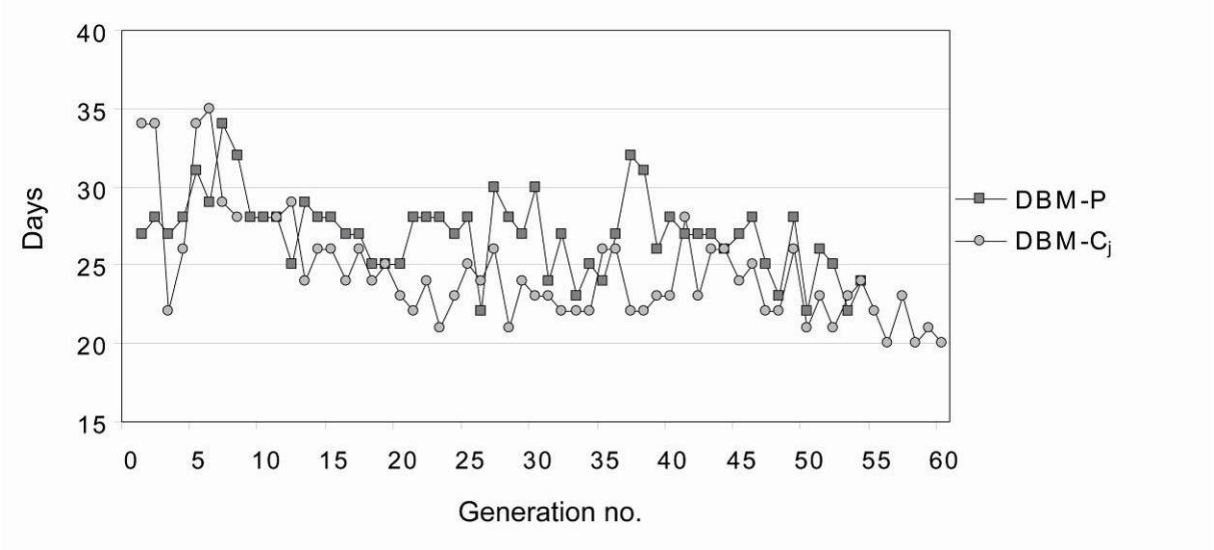


Figure 2 Generation times in days of the two strains DBM-P on pea and DBM-Cj on kale over a period of 4 years (from September 2006 until January 2010). The graph starts at "generation 0", which we set after the strains had stabilized in our rearing at MPICE in Jena. Since DBM-Cj consistently developed faster than DBM-P over the 4 years, in January 2010 DBM-Cj was in the 60th generation and DBM-P was in the 54th generation

3.4 Discussion

Since a host range expansion implies the acceptance of the new host as well as the original host plant, and host shift an exclusive preference for the new host, we expected DBM-P either to oviposit similarly on pea plants and on cabbage plants, or to prefer pea plants. However, DBM-P females still preferred cabbage, although unlike DBM-Cj they laid more eggs on the cabbage plant in the presence of pea plants. Three possible explanations for these findings are that: (i) field females had a genetic preference to oviposit on pea, which has been lost in the laboratory because it was not selected for; (ii) field females never had a genetic preference to oviposit on pea, but pea plants generally stimulated oviposition also in the original population; and/or (iii) early-adult experience affects oviposition preference.

Traits promoting larval vs. adult adaptation to pea may have a different genetic basis and may respond differently to selection, both in the field and in laboratory culture. Larval ability to grow and complete development on pea would have been strongly selected for, among offspring of females that had oviposited on peas in the field. That genetic variation for the ability to survive on pea existed at a low level before the first infestation was shown by Löhr and Gathu (2002), who gradually increased the proportion of pea survivors over several generations starting with a population collected from cabbage. This strong selection on initially rare genetic variants is maintained in the laboratory as well, as DBM-P is reared exclusively on pea. If there was also a pre-existing genetic variation for an active oviposition preference on pea, and if initially rare genetic variants with this preference were responsible for the first infestation, this trait would also have been strongly selected for during the first few generations in the field. However, an oviposition preference for pea is not being selected for in our rearing of DBM-P, since eggs deposited on the sides of the rearing containers as well as on the pea plants are used to produce the next generation. Thus, any genetically-determined oviposition preference, if not immediately fixed in the newly established lab population, could have been subsequently lost over many generations of rearing.

Alternatively, the first females to oviposit on pea may not have carried a genetic preference for pea over cabbage, but instead may have been generally stimulated to oviposit on any available surface by volatiles or other cues presented by the proximity of peas. This tendency would have been adaptive in Kenya in the outbreak year of 1999 after most crucifers had been consumed and were no longer available for oviposition, and it could have also contributed to the continued infestation of the pea crop afterwards. If this tendency had a genetic basis, it

would be maintained by selection under our rearing regime; females who lay more eggs in the oviposition containers in the presence of pea do contribute more to the next generation.

A third explanation for our finding that DBM-P females did not oviposit on pea plants may be the absence of preconditioning in our experiments; individuals were kept from pupation onwards in plastic tubes without exposure to any plant material. Zhang and Liu (2006) compared laboratory oviposition of DBM females collected from cabbage near Hangzhou with or without prior adult exposure to pea plants. Oviposition on pea without pre-exposure was 1-2% in their experiments, similar to our pea-reared DBM-P and cabbage-reared DBM-Cj (Table 2). The Hangzhou cabbage strain females pre-exposed to pea plants laid relatively more eggs on them subsequently, although they still preferred to oviposit on cabbage (Table 2). Zhang et al. (2007) showed that adult exposure to pea odor for three days, even if adults did not emerge in the presence of pea, also increased subsequent oviposition on pea by the Hangzhou cabbage strain. Moreover, Liu et al. (2005) and Wang et al. (2008) demonstrated that pre-exposure of adult females to odors of the non-host plant *Chrysanthemum morifolium* increased acceptance by ovipositing females.

If larval experience on host plants promoted future adult acceptance, DBM-P adults that fed on pea as larvae would be expected to accept pea more readily than the Hangzhou strain raised on cabbage, but this was not the case (Table 2). Zhang and Liu (2006) argued that adult experience, but not larval experience, conditions future adult choices in DBM. The greater importance of adult experience was also seen in earlier bioassays with cabbage strain *P. xylostella*. Whereas larval feeding experience on a neem-based oviposition deterrent did not affect oviposition response in adult females to that deterrent, conditioning after emergence and during the early adult stage altered oviposition preference significantly (Liu and Liu 2006). These findings are in accordance with the neo-Hopkins principle, that adult behavior is influenced not by larval but by early adult experience (Jaenike 1983; Corbet 1985; Cunningham et al. 1999, 2001).

Thus, DBM-P females might have laid more eggs on pea plants in our experiments if they had emerged from the pupa in the presence of pea and experienced the plant before mating and oviposition. However, lack of pre-oviposition exposure to cabbage plants did not prevent either strain from ovipositing heavily there. This is probably due to an innate attraction to cabbage, mediated by the presence of glucosinolates (Hopkins et al. 2009). In our experiments, no insects were pre-exposed to either pea or cabbage, so that neither the overall preference for cabbage over pea, nor the increase in cabbage oviposition in the presence of pea by DBM-P

but not of DBM-C_j can be explained by a difference in pre-oviposition adult exposure to the plants.

Selection for oviposition on a new host would be strengthened if it offered growth advantages to the larvae, but that situation has not yet been attained by DBM-P. Over 50 generations of rearing on pea, the developmental time of DBM-P was longer than DBM-Cj reared concurrently on cabbage (Figure 2), while DBM-P reared on cabbage developed as fast as DBM-Cj (Löhr and Gathu 2002; Knolhoff and Heckel, unpublished data). This indicates that, even after 50 generations, pea is not an optimal host plant for DBM-P larvae nor readily accepted by DBM-P females for oviposition, consistent with our hypothesis that this strain represents a very early stage in adaptation to the new host.

Even though we do not think that presence or absence of early-adult experience significantly affected our experimental outcome, it may explain the continuation of infestation in pea fields in Kenya. Since the offspring of the first pioneering females emerged as adults in the presence of the new host (unlike their mothers), their early exposure may have increased their oviposition there and enabled continuation of the infestation, without requiring a genetic oviposition preference for pea. Thus, a type of phenotypic plasticity (Agrawal 2001; Price et al. 2003) could have facilitated adult adaptation to the new host. A similar effect mediated by early experience has contributed to the well-known host-shift of *Rhagoletis* fruitflies from hawthorn to apple (Bush 1969), where prior exposure was shown to greatly increase oviposition on apple compared to flies that emerged in the absence of fruit (Prokopy et al. 1982).

In conclusion, our findings suggest that the DBM-P strain represents the early stages of a host range expansion, with genetic adaptations enabling larvae to feed on the new host plant pea, as well as the original host cabbage, while adult females still prefer to oviposit on the original host. Larval adaptation has resulted in comparable survivorship with slightly delayed development on the new host plant, while adult adaptation is incomplete in that there is increased oviposition in the presence of the new host, despite the absence of fidelity to it.

Acknowledgements

This study was supported by the Max-Planck-Gesellschaft. We thank Bernhard Löhr for providing the DBM strains from Kenya, Francisco R. Badenes-Pérez for advice on the set-up of the oviposition experiments and two anonymous reviewers for helpful comments.

Table 1 Summary of two-way analysis of variance (ANOVA) results on egg numbers deposited on different host plants by *P. xylostella* pea and cabbage strain females. ANOVA on the three different treatments: no-choice cabbage, no-choice pea, choice cabbage and pea.

Source of variation	d.f.	Sum of squares	Mean square	F value	P	
Strain	1	5852.03	5852.03	6.77	0.0128	*
Treatment	2	18449.56	9224.78	10.67	0.0002	***
Strain × Treatment	2	4847.56	2423.78	2.80	0.0720	n.s.

n.s. = not significant, * $P < 0.05$, *** $P < 0.001$.

Table 2 Relative percentages of eggs laid by *P. xylostella* Hangzhou cabbage strain females on plants in each of the nine plant × experience treatments of Zhang and Liu (2006) compared to the percentages laid by DBM-Cj and DBM-P females (in bold) under choice and no choice conditions.

	Strain	Hangzhou [1]	Hangzhou [1]	Hangzhou [1]	DBM-C_j	DBM-P
Adult pre-exposure to pea		None	After emergence	During and after emergence	None	None
No choice	Cabbage	98.3%	74.3%	83.3%	**98.2%**	**97.3%**
No choice	Pea	1.7%	25.7%	16.7%	**1.8%**	**2.7%**
Choice [2]	Cabbage	98.0%	94.1%	86.1%	**94.7%**	**97.0%**
Choice	Pea	2.0%	5.9%	13.9%	**5.3%**	**3.0%**

[1] Recalculated from Figure 1 of Zhang and Liu (2006) to eliminate eggs laid on the pot and inner surface of the cage in their experiment, so that the percentages of eggs laid on cabbage and on pea plants sum to 100%. Zhang and Liu (2006) used 4 females overnight for 12 h, while we used 5 females for 3 consecutive days.

[2] Oviposition on cabbage in the choice experiment of Zhang and Liu (2006) was estimated by subtracting the proportion of eggs laid on pea plants from the total, because a separate count of eggs laid on pots and inner cage surface was not provided for this experiment.

4. Manuscript II

Preference of diamondback moth larvae for novel and original host plant after host range expansion

Kathrin Henniges-Janssen, Astrid T. Groot and David G. Heckel

Preference of diamondback moth larvae for novel and original host plant after host range expansion

For submission to Entomologia Experimentalis et Applicata

This manuscript addresses the feeding preference of fourth instar larvae of the newly evolved pea-adapted host strain of diamondback moth (DBM-P) for novel and original host plant, sugar pea and cabbage, respectively in comparison to feeding preference of cabbage-adapted larvae and cabbage-reared pea-adapted larvae. DBM-P larvae preferred to feed on the novel host plant pea.

KHJ conceived the study, conducted the experiment and performed data analysis. The manuscript was written by KHJ and revised by ATG and DGH.

Abstract

This study explores the changes in larval preference behavior that accompanied the recent host range expansion of the diamondback moth (DBM), *Plutella xylostella* L. (Lepidoptera: Plutellidae) to sugar pea. In larval choice assays, larvae of the newly pea-adapted host strain were offered the novel host plant sugar pea and the original host cabbage. These larvae significantly preferred the novel host plant pea. However, larval preference of a cabbage adapted DBM strain and of cabbage reared pea-adapted larvae were also tested, and both significantly preferred the original over the novel host. These findings indicate that both genetic differences and previous exposure affect larval host choice, and are of practical as well as of evolutionary relevance.

4.1 Introduction

In herbivorous insects the successful use of a host plant depends on specific behavioral and physiological adaptations in the adult and larval stage. In other words, existence of an insect-host plant association requires that adult females find and accept a plant for egg laying and larvae accept it and are able to fully develop on it (Bernays and Chapman 1994). Accordingly, the acquisition of a novel plant as host is presumably governed by changes in behavior by adults or larvae or both. Feeding and/or oviposition behavior have to change from "not being attracted to" or "being repelled from" to "preference for" or "acceptance of" the novel host. This was the case in the checkerspot butterfly *Euphydryas editha*, which incorporated the introduced plant *Plantago lanceolata* into its diet approximately 100 years ago (Thomas et al. 1987). Oviposition preference for the novel and rejection of the native host has since evolved in populations that reside in regions where the introduced plant grows (Thomas et al. 1987; Singer et al. 1993). Similarly, hatchling preference in the soapberry bug *Jadera haematoloma* has also changed. Ancestral populations of *Jadera haematoloma* have a preference for feeding and reproducing on the original host plant balloon vine (*Cardiospermum halicababum*) while populations that shifted to the introduced ornamental goldenrain tree (*Koelreuteria* sp.) prefer feeding on the novel host (Carroll and Dingle 1996).

We have learned that behavior plays a central role in the acceptance of a novel host plant and changes in this behavior might be the driver for establishment of novel insect-plant relationships. The colonization by an herbivore of a novel host plant can proceed via a host range expansion, the addition of a novel host plant to the diet, or a host shift, addition of a novel plant to diet with concurrent loss of the ability to use the original host. A thorough under-

standing on the exact changes in feeding and oviposition behavior after a change in host plant is of evolutionary as well as practical relevance. The acquisition of a novel host plant is an evolutionary important step towards host race formation that might ultimately lead to formation of a novel species. Attacks of novel host plants by herbivorous insects raise the question if the subpopulations, one specialized on the novel and the other on the original host plant, may evolve into host races, a process which might ultimately result in a speciation event. Two major factors are needed for host race formation: host preference and host-associated fitness (Maynard Smith 1966; Bush 1975). Host preference initiates the process of host race formation and close association with the hovel host plant will reduce gene flow between specialized subpopulations (Feder et al. 1994). Gene flow will be reduced even more if this host preference is accompanied by fitness consequences becoming evident in larval performance on the novel host plant. In DBM-P adult females oviposition preference is still towards the original host plant (Henniges-Janssen et al. 2011) and larval fitness, measured as survival rate, is the same on novel and original host; however, developmental time is increased and pupal weight reduced when feeding on pea reduced on the non-parental host family (Löhr and Gathu 2002; Henniges-Janssen et al. in press). A preference of the novel host plant in adult and larval stages of the herbivore will most likely lead to host race formation whereas an incomplete change in these behaviors, e.g. with one stage still preferring the original host plant or a continued acceptance of original and novel host plant, increases the chance of a backshift to the original host and thus renders the chance for a speciation event impossible. In cases of a pest insect switching to an economically important novel plant species, a thorough understanding of the underlying behavioral changes in larval and adult stage is important for implementation of adequate control measures and improves the ability to predict unwanted host range expansions of this species.

Recently the highly specialized crucifer-feeding diamondback moth (DBM) *Plutella xylostella* L. (Lepidoptera: Plutellidae) expanded its host range to sugar pea (Löhr 2001). This event provides a unique opportunity to investigate behavioral changes underlying a contemporary event of adaptation to a chemically and evolutionary unrelated novel host. The natural host range of DBM encompasses wild crucifers (Brassicaceae), and it is a significant worldwide pest of cultivated crucifers, a plant family characterized by the glucosinolate-myrosinase defense system against herbivore attack. DBM has the ability to deactivate this defense system using a highly active glucosinolate-sulfatase and thus, is specifically adapted to brassicaceous plants (Ratzka et al. 2002). It was very surprising to find DBM feeding on sugar snap peas, *Pisum sativum* L. var. macrocarpon, cultivar Oregon Sugar Pod (Fabaceae), in the field

in the area south of Lake Naivasha in the Rift Valley, Kenya, in 1999 (Löhr 2001). That year, DBM densities on the original cabbage hosts were extremely high, and a neighboring pea field became infested. Because of its well-known status as a crucifer specialist, the identity of the pest as DBM was doubted until confirmed by an entomologist. In 2000, this local population even expanded to an adjacent field of mangetout peas (*Pisum sativum* L. var. macrocarpon, cultivar Snow Green). Because the population persisted as an uncontrollable pest on the pea crop in the following two years, the farmer stopped growing peas, so that this population either became extinct in the field or rejoined the populations feeding on the neighboring cabbage (B. Löhr, personal communication). Larvae were collected from the pea crop in 2000 and 2002, and have been reared on Oregon Sugar Pod peas in the laboratory since then (Löhr 2001). This DBM population represents a unique example for a very recent expansion from the original plant family (Brassicaceae) to a new and dissimilar host plant family (Fabaceae) in the field.

So far, behavior of the newly evolved DBM-P strain has been analyzed in the adult stage whereas studies on the larval stage have yet only focused on larval performance. Laboratory populations of DBM that we (Janssen et al. 2008) and others (Zhang et al. 2007) have tested cannot survive on pea plants, however this population can complete development on a pea host alone and is now referred to as DBM-P (Löhr and Gathu 2002), the pea-adapted strain of the diamondback moth. A study investigating the larval stage of DBM-P showed that DBM-P larvae readily accept sugar pea as host plant and are able to feed and develop to pupal stage on it (85% survival on kale and 83% on peas), with results similar to DBM-P feeding kale or DBM-Cj feeding on cabbage (88% survival on kale and 2% on peas) (Löhr and Gathu 2002). Despite this similar overall survival rate on pea plants, developmental time of DBM-P larvae was still significantly longer on the new host plant peas than on the original kale host plant; and pupal weight was significantly lower for DBM-P reared on pea than on kale. We have subsequently shown that the ability of DBM-P larvae to complete development on pea has a polygenic genetic basis, which does not diminish the ability to complete development on kale (Henniges-Janssen et al. 2011).

Adult host choice behavior in the newly evolved DBM-P strain, i.e. female oviposition acceptance and preference, was investigated in oviposition assays (Henniges-Janssen et al. 2011). The results from these assays were surprising as DBM-P females laid most eggs on cabbage and very few on peas and they laid significantly more eggs on the cabbage plant when pea plants were present. This suggests that host range expansion in DBM-P is still in the early stages, and also raises the question about larval preference, which clearly is the missing piece

to complete the picture on putative changes in host choice behavior in this strain, and therefore was the purpose of this study.

A larval feeding preference assay was set up with fourth instar larvae (L4) of the DBM-P strain. Their feeding preference was recorded by offering pea and cabbage leaf discs and recording the position of the larvae and amount of consumed material per disc after certain time increments in comparison to the choice of larvae from an original cabbage-adapted host strain (DBM-Cj). Using mature L4 larvae introduces the complication that these larvae are already experienced and conditioned to feed on their rearing host plant, i.e. DBM-P on pea and DBM-Cj on cabbage. Neonate larvae are naïve and thus their host plant choice is less constrained, however due to their small size they are regarded as quite sessile and often initiate feeding on the first plant they encounter; moreover, they are difficult to handle in experiments. To test for the effect of prior conditioning, a group of DBM-P larvae were additionally reared on cabbage and then tested for their feeding preference on the two host plants, sugar pea and cabbage.

The results on DBM-P's larval preference, i.e. either for the original or novel host plant, will complete the characterization of the behavioral phenotype of this novel strain. Furthermore, the results will have an impact on our understanding of the stage of the current host range expansion, the potential of host race formation in DBM-P and on the pest status of this insect and will be discussed in these contexts.

4.2 Materials and methods

Insects

Two strains of *P. xylostella* were used in the larval feeding choice assay: the cabbage feeding strain (DBM-Cj) and the pea adapted strain (DBM-P). Both strains originate from Kenya and were kindly provided by Bernhard Löhr from the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya. DBM-P was originally collected from the infested pea field in Naivasha in 2000, and was repeatedly replenished with additional field-collected material from the same site for the next two years (Löhr and Gathu 2002). It has been maintained as a laboratory culture since then at ICIPE in Kenya. DBM-Cj derived from a field population from the semi-arid areas about 40 km south east of Nairobi. Both strains were sent to the Max Planck Institute for Chemical Ecology (Jena, Germany) in May 2005, where they have been reared for more than 50 generations since then. Population sizes of the strains maintained in Kenya are unknown to us, but averaged about 400 adults per generation in Jena. Insect cultures of both strains were reared from egg to adult stage on intact plants in mesh cages

(60×60×60 cm) at 21°C, 50% RH, and 16:8 L:D photoperiod, with DBM-Cj reared on cabbage and DBM-P reared on pea. For mating and oviposition, adult moths were collected with an aspirator (BioQuip Products, Rancho Dominguez, CA, USA) and transferred from cages to mating boxes (15×15×5 cm). Each box contained at least 30 individuals, and per generation 12 to 15 boxes were set up. The bottom of the boxes was covered with tissue paper on which leaves of the respective host plants (cabbage or pea) were placed as oviposition sites. Adult moths were fed with 5% honey solution. After eggs were deposited on leaves and the tissue paper, these were transferred to cages and fresh leaves were added to the plastic box. For this study, we additionally reared DBM-P larvae on cabbage, referred to as DBM-Pc, under same conditions as DBM-P and DBM-Cj.

Plants

Seeds of pea, *Pisum sativum* L. var. *macrocarpon*, cultivar Oregon Sugar Pod, were obtained from Agri-Saaten GmbH (Bad Essen, Germany). Cabbage seeds, *Brassica oleracea* var. *capitata*, cultivar Gloria, were obtained from B and T World Seeds (Aigue-Vives, France). Plants used for rearing of insects were grown in trays (58×32×11.5 cm) in Klassmann Tonsubstrat under greenhouse conditions at 21–23°C, 50–60% RH and 14:10 L:D photoperiod. For larval choice experiment, five week old pea and cabbage plants were used.

Larval feeding choice assay on leaf discs

The set-up of the larval feeding choice experiment is depicted in Figure 1. We used pea and cabbage leaf discs of 1 cm in diameter that were punched from the leaves of plants grown under the above-described conditions. The discs were placed in a Petri dish of 12 cm in diameter. Larvae crawling on the bottom of the Petri dish could easily reach a disc and begin feeding on its rim. A filter paper moisturized with 1 ml of H_2O was placed on the bottom of the Petri dish to prevent desiccation of the plant material. Three leaf discs from the two host plants tested, pea and cabbage, were arranged in alternating order (i.e. in total 6 leaf discs for each arena). This kind of arrangement increased the probability that a larva leaving a disc would encounter the other plant before coming again to the first plant. Prior to the experiment larvae were starved for two hours. At the beginning of the test, one fourth instar larva (L4) of *P. xylostella* was placed in the middle of each arena using a fine paint-brush. An assay consisted of two recordings the behavioral choice of the larva (i.e. location of the larva) and visual estimates of the amount of surface eaten (the percentage of one disc consumed = 1/3 of the total amount of that particular treatment = ca. 33%) at four time points (after 5 min, 30 min,

6 h and 12 h). The bioassay was replicated 40 times (total of tested larvae = 40) with each DBM strain, DBM-P, DBM-Cj and DBM-Pc. Replicates in which larva left the arena and escaped from the Petri dish were not recorded and excluded from data analysis.

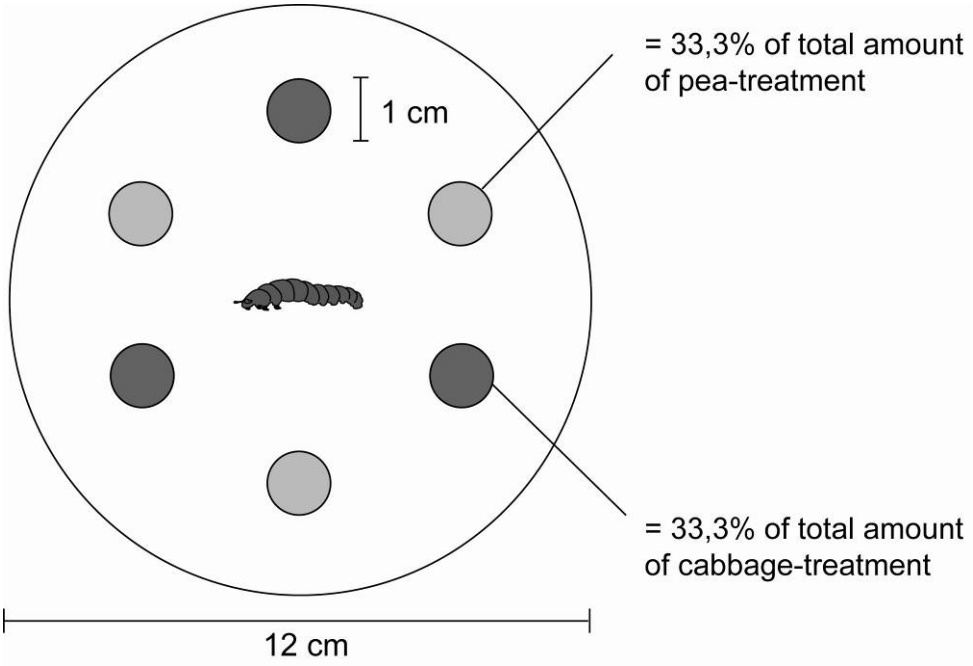


Figure 1 Experimental set-up of larval feeding choice assay. Pea and cabbage leaf discs were offered in an alternate order and a fourth instar larva was placed in the middle of the Petri dish. Position of larva and percentage of consumed leaf area was recorded after 5 min, 30 min, 6 h and 12 h and 30 min, 6 h and 12 h, respectively.

Data analysis

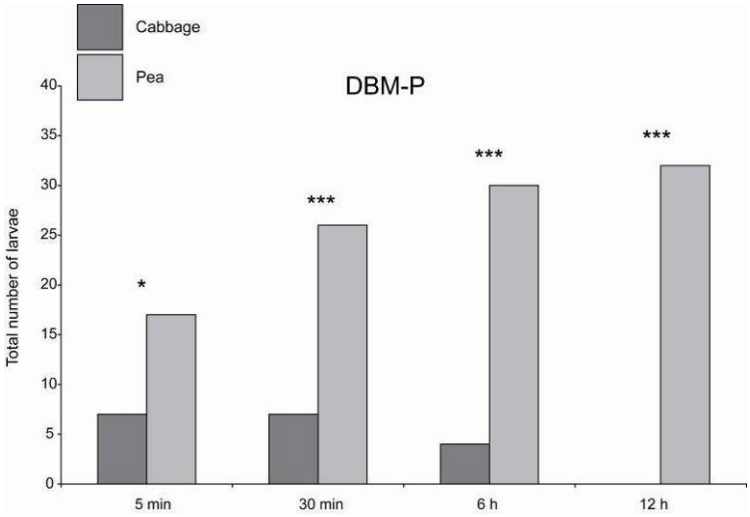
Data was analyzed using R (R Development Core Team, 2010). The number of larvae choosing either of the two host plants, cabbage and pea, respectively, was counted and compared with a Fisher's exact test. The percentage of consumed leaf area per strain and time point were compared using a non-parametric Wilcoxon signed rank test. One-way analysis of variance (ANOVA) was used to test for significant differences in the percentages of consumed leaf area between strains after 6 h and 12 h, respectively. The Tukey's honestly significant difference (HSD) test was used for multiple comparisons.

4.3 Results

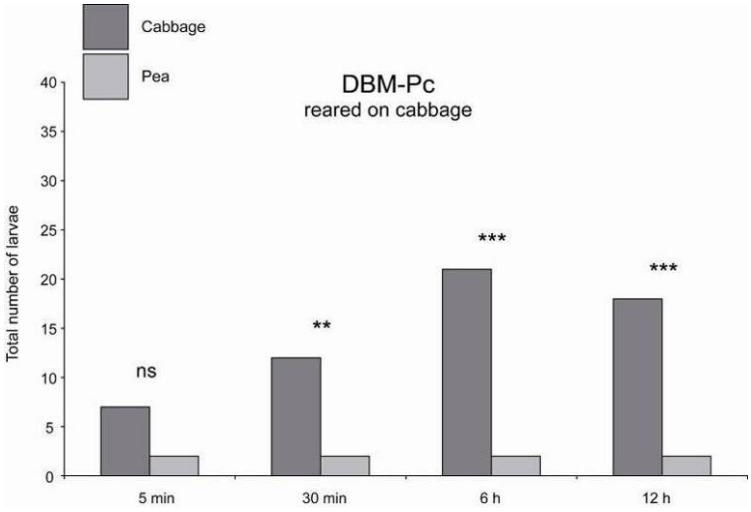
At all observation times larvae of DBM-P were recorded significantly more often on their novel host plant pea (Figure 2; Fisher's exact test $P = 0.024$ at 5 min and $P < 0.001$ at 30 min, 6 h and 12 h) and consumed significantly more pea than cabbage (Figure 3; Wilcoxon signed

rank test $P < 0.001$ at all observation times). In contrast, DBM-Cj larvae consumed exclusively cabbage and occurred significantly more frequently on cabbage leaf discs (Figure 2; Fisher's exact test and $P < 0.001$ at all observation times) and the percentage of consumed cabbage was significantly higher than that of pea (Figure 3; Wilcoxon signed rank test $P = 0.005635$ at 30 min and $P < 0.001$ at 6 h and 12 h). DBM-Pc spent significantly more time on cabbage for the last three time points (Figure 2; Fisher's exact test $P = 0.0017$ at 30 min and $P < 0.001$ at 6 h and 12 h). Accordingly, the percentage of larval feeding preference for cabbage (Figure 3) was not significant at the first time point after 30 min (Wilcoxon signed rank test $P = 0.7728$) and significant at the later time points 6 h and 12 h (Wilcoxon signed rank test $P <0.001$ at 6 h and 12 h). The amount of pea leaf area consumed by DBM-P larvae did differ significantly from the amount of cabbage leaf area consumed by DBM-C larvae ($P_{adj} = 0.02$) but not from DBM-Pc ($P_{adj} = 0.15$) after 6 h. The amount of pea-leaf area consumed by DBM-P larvae was not significantly different anymore to the amount of consumed cabbage leaf area by DBM-Cj larvae ($P_{adj} = 0.27$) after 12 h but to the amount of cabbage consumed by DBM-Pc ($P_{adj} = 0.01$).

(a)



(b)



(c)

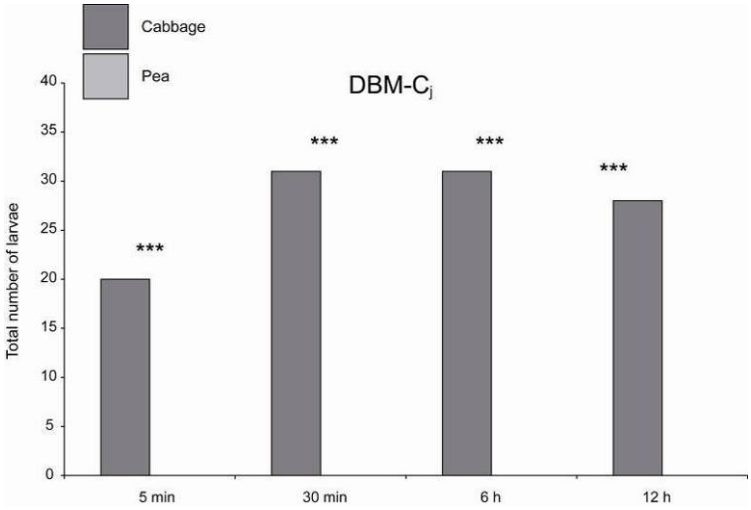


Figure 2 Feeding choice of L4 larvae of DBM-P (n = 38) (a), DBM-P reared on cabbage (n = 30) (b) and DBM-Cj (n = 40) (c) after 5 min, 30 min, 6 h and 12 h. ** $P < 0.01$, *** $P < 0.001$, ns = not significant.

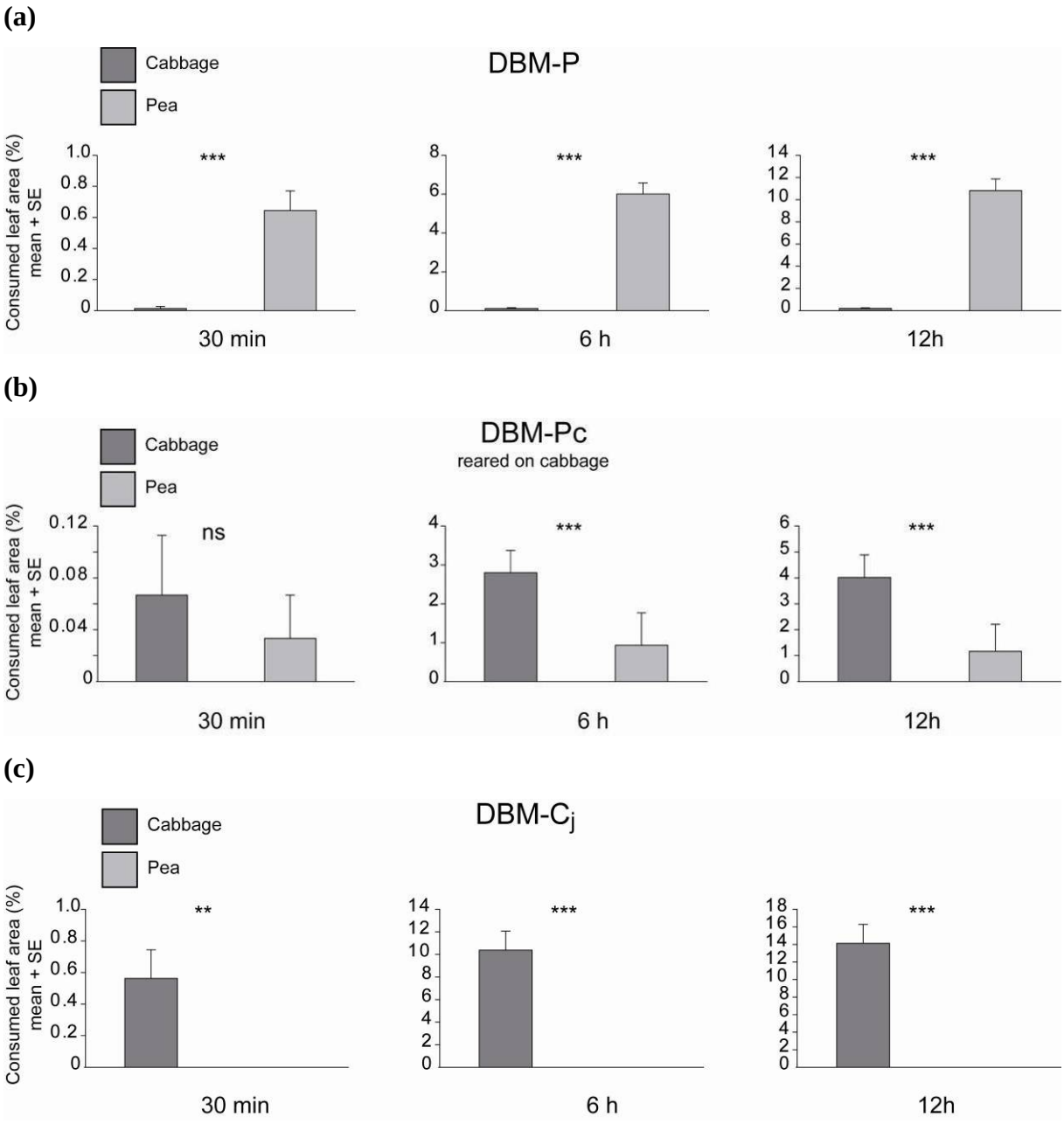


Figure 3 Percentage of consumed leaf area per larva of DBM-P (n = 38) (a), DBM-P reared on cabbage (n = 30) (b) and DBM-Cj (n = 40) (c) after 30 min, 6 h and 12 h. Symbols and abbreviations as in Figure 2.

4.4 Discussion

As expected DBM-P larvae preferred their novel host plant sugar pea. The genetic basis of larval ability to survive on sugar pea has previously been demonstrated (Henniges-Janssen et al. in press). Thus preference in the larval stage differs from that in adult stage, as females of DBM-P did not show any preference for oviposition on pea except for an increased oviposition rate on cabbage in the presence of pea (Henniges-Janssen et al. 2011). DBM-P has expanded its host range to sugar pea in the field and is raised on its novel host in the laboratory.

Despite this adaptation we have previously shown that DBM-P still develops slower on pea than does a cabbage strain on cabbage and pupal weight of DBM-P also is lower on pea (Löhr and Gathu 2002). In the behavioral tests, the amount of pea consumed by DBM-P is less than the amount of cabbage consumed by DBM-Cj after 6 h but was not different anymore after 12 h. Thus the choice that allows DBM-P larvae to utilize a resource that DBM-Cj cannot, seems to result in a moderately reduced performance relative to the original host plant. Whether this reduced performance is due to an insufficient physiological adaptation to be able to cope with defense and secondary plant compounds of sugar pea is not known.

DBM-Cj larvae also behaved as expected and significantly preferred cabbage. Two larvae were observed on pea leaf discs but no feeding was initiated. Although this was not surprising, one could have expected to find more larvae on pea and to observe a few cases of larval feeding initiation on pea. It has been shown that a cabbage-adapted strain can be selected for survival on pea within six generations, a selection that results in extremely high mortality in the early generations (Löhr and Gathu 2002). Thus some genetic variation for larval preference may exist, even in typical cabbage-adapted strain. Genetic analysis of the ability to complete development on pea has further shown that even DBM-P possesses genetic variation for this ability (Henniges-Janssen et al. in press). The host range expansion likely evolved from existing standing genetic variation instead from a spontaneous mutation, which allowed for such a rapid switch to an unrelated host plant. Thus, we had the opportunity to detect any DBM-Cj larvae that initiated feeding and survived on pea in the choice experiment due to genetic variation. However, even if DBM-Cj larvae existed that were genetically predisposed to feed on pea, their prior conditioning on cabbage may have masked this preference in the choice experiments, as suggested by results on DBM-Pc larvae. Thus the phenotypically-observed preference for cabbage may provide an overestimate of the genotypically-based preference for cabbage in the DBM-Cj strain.

Although fourth instar larvae are more convenient in the choice assay, their behavior might be affected by prior experience; so DBM-P larvae raised on cabbage, (DBM-Pc) were also tested. Because of 100% mortality of DBM-Cj neonates provided only pea, the reciprocal test could not be performed. Preference behavior of DBM-Pc larvae approached that of DBM-Cj: Larvae were recorded significantly more often on cabbage and also consumed a significantly higher amount of cabbage than pea. Preference induction through learning is a well known phenomenon studied in a range of phytophagous insects and usually produces a long-lasting behavioral effect (see Bernays and Chapman 1994; Jermy 1987; but also Pszczolkowski and Brown 2005). In addition, choosing cabbage as host is beneficial due to the previously de-

scribed fitness constraints on pea. However, preference of DBM-Pc for cabbage was not as strong as that of DBM-Cj, suggesting a conflict between innate preference and experience. In contrast to DBM-Cj, a few DBM-Pc larvae choose pea and fed on it. Moreover, DBM-Pc's significant preference for cabbage was only recorded after 30 min, for DBM-P and DBM-Cj a significant preference for pea and cabbage, respectively, was recorded already at 5 min; and DBM-Pc consumed smaller amounts of cabbage than did DBM-Cj of cabbage and DBM-P of pea and significant differences in pea vs. cabbage consumption were only observed at 6 h. This indicates that despite pre-conditioning on cabbage, preference for pea was maintained in some DBM-Pc larvae and those larvae with a preference for cabbage took longer for decision-making and showed a restricted consumption.

To further investigate the interaction of pre-conditioning with innate preference, newly hatched first instar larvae with no prior host plant experience should be tested. A possible complication in interpreting those experiments is the possibility that because of their restricted mobility, neonate larvae may have a tendency to accept the first encountered host plant on which they initiate feeding and which they rarely leave. Thus second and third instar larvae should also be tested to see whether a conditioning effect increases with the total exposure time to a given host.

The available information on larval fitness and female preference together with the herein obtained results on larval preference provides the necessary data to address the question whether the behavioral changes that governed the host range expansion are sufficient for host race formation in DBM. Host race formation has been studied in a variety of insect species, and so far the most thoroughly studied example of host race formation is that of the apple maggot fly *Rhagoletis pomonella* (Bush 1969), which quite recently switched to apple (*Malus* sp.) from the native hawthorn (*Crataegus* sp.). Among other traits, host races on apple and hawthorn differ in phenology (Smith 1988) and host preference (Feder et al. 1994) on the two hosts but not in survival rates under laboratory conditions. DBM-P has just initiated the first steps in a range expansion towards sugar pea, and with the original host still preferred by DBM-P for oviposition and supporting better larval performance, a backshift is very likely. Adult females are the mobile life stage and predominantly determine the host plant that the larvae must use, or die. Moreover, although larvae may prefer the novel host they are unlikely to leave a cabbage plant and move to a patch with sugar pea plants due to their limited dispersal ability. Host race formation in theory could result from a host range expansion but DBM-P currently appears to lack the complete set of necessary features for this transition.

DBM's recent host range expansion to pea also has implications for its pest status. This insect is already known to be able to thrive in almost every climate as well as to develop resistance against every insecticide. With these characteristics it is considered one of the most severe pest insects worldwide (Talekar and Shelton 1992; Shelton 2004). Sudden host range expansions to economic important crop species unrelated to the original host crucifers, such as to sugar pea in Kenya, have so far not been reported. The ability to rapidly adapt to unrelated plant species is thus a novel feature in this pest insect and has an important impact on its pest status. Understanding the mechanisms and behavioral changes that were necessary for this range expansion is important to understand the host range expansion, to prevent further spreads on the novel host and to estimate the potential for range expansions to other important crops. An intensified agriculture and increased numbers of introductions of crop species has increased the opportunities for such host switches in recent years. It seems that in DBM larval preference was altered more rapidly than adult preference, which appears to be more conservative. In addition, although DBM-P larvae preferred the novel host plant, they have not yet lost the ability to survive equally well on the original host and even prefer it when raised on it. Thus, although opportunities for range expansions may increase, it is not expected that range expansions followed by host race formation pose a threat. Moreover, host range expansions are likely to be only short-term and for maintenance of the population. The availability of the (preferred) original host will presumably lead to a backshift. A thorough understanding of the underlying behavioral changes in DBM-P might have prevented the farmer from completely stop growing sugar pea but rather implementing control measures that precisely eradicate the larval stage with a preference for pea. With females not ovipositing on sugar pea the pest would then have shifted back to the original host plant itself.

Conclusions

To conclude, the stage of host range expansion differed in adult and larval stage. While DBM-P females still do prefer to oviposit on cabbage and presence of sugar pea just increases the number of eggs laid on cabbage, DBM-P larvae significantly prefer their novel host. Although cabbage reared DBM-Pc larvae prefer their rearing host cabbage, at least a few larvae showed a preference for pea and still fed on it. These behavioral changes enabled DBM to establish on a novel host plant pea but are not likely to be sufficient for host race formation.

5. Manuscript III

Complex inheritance of larval adaptation in *Plutella xylostella* to a novel host plant

Henniges-Janssen, K., Reineke, A., Heckel, D. G. and Groot, A. T. (2011).
Complex inheritance of larval adaptation in *Plutella xylostella* to a novel host plant. Heredity (in press).

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This manuscript describes the deciphering of the genetic basis of the trait 'larval adaptation to pea' to understand the underlying genetic mechanisms by which herbivores colonize and are able to adapt to novel host plants. Feeding assays with backcross progeny and a linkage analysis using AFLP markers revealed a complex genetic basis of the trait and a still genetically heterogeneous pea-adapted DBM strain.

DGH and KHJ conceived the study. KHJ carried out crosses, feeding assays and molecular analysis. KHJ, ATG, DGH and AR discussed results. KHJ, DGH and ATG analyzed data. KHJ wrote manuscript that was revised by ATG and DGH.

Abstract

Studying the genetics of host shifts and range expansions in phytophagous insects contributes to our understanding of the evolution of host plant adaptation. We investigated the recent host range expansion to pea, in the pea-adapted strain (P-strain) of the crucifer specialist diamond-back moth, *Plutella xylostella* (Lepidoptera: Plutellidae). Larval survivorship on the novel host plant pea and a typical crucifer host (kale) was measured in reciprocal F_1, F_2 and back-crosses between the P-strain and a strain reared only on crucifers (C-strain). Reciprocal F_1 hybrids differed: offspring from P-strain mothers survived better on pea, indicating a maternal effect. However, no evidence for sex-linkage was found. Backcrosses to the P-strain produced higher survivorship on pea than C-strain backcrosses, suggesting recessive inheritance. In a linkage analysis with AFLP markers using P-strain backcrosses, two, four and five linkage groups contributing to survival on pea were identified in three different families respectively, indicating oligogenic inheritance. Thus the newly evolved ability to survive on pea has a complex genetic basis, and the P-strain is still genetically heterogeneous and not yet fixed for all the alleles enabling it to survive on pea. Survivorship on kale was variable, but not related to survivorship on pea. This pattern may characterize the genetic inheritance of early host plant adaptation in oligophagous insect species.

5.1 Introduction

Host plant adaptation traits are of fundamental importance to herbivorous insects. Most herbivores are adapted to a specific host plant or a narrow range of host plants on which they rely for food or other resources. Some host plant-insect associations are evolutionarily ancient, while others result from recent colonization events due to introduction of either plant or insect to the range of the other (Fox 2006; Tabashnik 1983). Herbivorous insects may broaden their host range and include new hosts, shift to a novel host, or narrow the range to exclude a former host (Via 1990; Thompson and Pellmyr 1991). Recent host shifts or range expansions provide interesting scenarios for the study of the genetics of adaptation. Although this field has been studied and discussed intensively over the past decades (reviewed in Orr and Coyne 1992; Orr 2005) there is still much controversy about the genetic basis of adaptation. Much debate centers on whether adaptation primarily arises from a few genes with large effect each (i.e. mono- or oligogenic) or from many genes with small effect each (i.e. polygenic) (reviewed in Orr 2005). Similarly, questions remain as to whether adaptation arises from new mutations or standing genetic variation, whether novel adaptive alleles are generally dominant

or recessive, and whether adaptations arising from human disturbance differ in their genetic architecture from those adaptations that arise under natural conditions. An understanding of the genetic architecture (i.e. mode of inheritance, the number of genes involved) of a trait that allows for survival on a novel host plant is important because it dictates its evolutionary potential.

Successful adaptation to a host plant requires that the adult female accepts it for oviposition, and that larvae are able to feed on and develop to maturity on the host. The genetics of host plant adaptation or host-associated performance have been studied in several insects and a range of genetic architectures has been observed: Hawthorne and Via (2001) detected polygenic inheritance in pea aphids, whilst in other cases evidence for the influence of a limited number of genetic factors (oligo- to monogenic inheritance) was found (Jones 1998; Sezer and Butlin 1998; de Jong et al. 2000). For the mode of inheritance a general pattern seems to apply: genes affecting larval performance consistently map to the autosomes (Tang et al. 2006). Genes controlling oviposition preference are less consistent: they are sex-linked in *Papilio* butterflies (Thompson 1988), but autosomal in moths, *Heliothis virescens* and *H. subflexa,* (Sheck and Gould 1995) and *Yponomeuta* species (Hora et al. 2005).

A major limitation in studying the genetic basis underlying novel host plant colonization in herbivorous insects is the difficulty of identifying systems in which adaptation is a recently-completed or still-ongoing process. A suitable system would be one that enables crosses between individuals that recently specialized on different hosts, such as two strains of a species that vary in host use. The crucifer-specialist diamondback moth (DBM), *Plutella xylostella* (Lepidoptera: Plutellidae) provides a unique opportunity to study the genetic basis of a very recent host plant colonization. DBM feeds on crucifers (Brassicaceae), a plant family characterized by the glucosinolate-myrosinase defense system toxic to most herbivores but harmless to *P. xylostella* larvae who circumvent this defense system with a specific enzyme, glucosinolate sulfatase (Ratzka et al. 2002). Due to the success of this detoxicative strategy, DBM is a major pest of cultivated cruciferous vegetables in tropical and semi-tropical regions worldwide. However, in 1999 a population in the Kenyan Rift Valley was reported to feed on sugar snap pea (*Pisum sativum* L. var. *macrocarpon*, cultivar Oregon Sugar Pod (Fabaceae)) (Löhr 2000; Löhr and Gathu 2002). This surprising discovery led to further studies; larvae from this population were collected in 2001 and 2002, and reared on the same pea plant cultivar in the lab since then (Löhr 2001). As this population can survive and develop fully on pea plants, it is referred to as the pea host-strain (P-strain). Löhr and Gathu (2002) showed that P-strain larvae can develop equally well on both host plants, cabbage and pea. In a separate selection

experiment with a typical crucifer-feeding strain of DBM, they were able to increase larval survival on pea from 2.4% to 49.7% within six generations (Löhr and Gathu 2002), suggesting one or a few major pre-existing genes being responsible for adaptation to pea.

So far, nothing is known about the genetics of DBM's P-strain recent larval adaptation to sugar pea. A first step in studying the genetic basis of host plant adaptation involves deciphering of the genetic inheritance. Considering the rapid evolution of the trait, we hypothesized a simple genetic basis (e.g. single dominant or recessive gene). To understand the genetic basis of larval adaptation to pea, we hybridized the P-strain with a C-strain (cabbage-feeding but not pea-adapted strain) of *P. xylostella*, examined the survival rates of F_1 hybrids, F_2 hybrids and backcrosses on pea plants and performed linkage analysis using AFLP markers in female-informative backcross families in order to establish a linkage map. Being a non-model organism so far no homologized genetic linkage map exists for DBM; developing such a map would help in identifying genomic regions contributing to host performance. We found that the trait is mainly autosomal and oligogenic, with additional maternal but not sex-linked effects, and with a surprising degree of genetic heterogeneity still present in the P-strain. We discuss our results in the light of other studies on host plant adaptation and the genetics of adaptation in general.

5.2 Materials and methods

Insects

Two *Plutella xylostella* strains, Waite and DBM-P, were used for crosses. The Waite strain is a cabbage-adapted strain that is unable to survive on pea. It was originally obtained from Waite Campus, Adelaide, South Australia and derived from a field collection in South Australia and was maintained as a laboratory culture for many generations in the laboratory of Dr. Nancy Endersby, Victorian Department of Natural Resources, from whom it was obtained. At the Max Planck Institute for Chemical Ecology (MPICE) in Jena it has been reared since 2005 for more than 50 generations. The DBM-P strain (P-strain) originates from Kenya and was sent to MPICE by Bernhard Löhr from the International Centre of Insect Physiology and Ecology (ICIPE) in Nairobi, Kenya, in 2005. Originally, DBM-P was collected from the infested pea field in Naivasha in 2002, where the host shift was observed in 1999. It was maintained as a laboratory culture ever since at ICIPE in Kenya. At MPICE in Jena, the DBM-P strain has subsequently been reared for more than 40 generations. Both strains are kept under

the same conditions in a Viessmann climatic room at 21°C, 55% RH and a photoperiod of 16:8 LD.

Rearing of the Waite C-strain of *P. xylostella* took place in a rearing cage (40×40×40 cm) with kale leaves (*Brassica napus*) as stimulus and substrate for egg-laying and 5% honey solution provided as adult food source. The leaves and the eggs laid thereon were transferred to smaller plastic containers (18×18×6 cm), and hatched larvae were provided with freshly cut *B. napus* leaves. Pupae were collected from plastic containers and relocated to rearing cages for emergence, mating and oviposition.

Rearing procedure for the DBM-P was similar to the Waite C-strain except that the mating occurred in plastic containers (18×18×6 cm) with cut pea plants offered as egg laying substrate and 5% honey solution as adult food source. For larval development, pupation and emergence of adult moths plant material together with the eggs laid thereon was transferred to a rearing cage (60×60×60 cm) with fresh leaves of *Pisum sativum* var. Oregon Sugar Pod. Plants used for maintenance of insect colonies were reared in the greenhouse at 21-23°C, 50-60% humidity and 14:10 LD.

Crossing strategies

To assess survival on pea, matings between the cabbage adapted Waite C-strain (C) and the pea adapted DBM-P strain (P) were performed to generate F_1 progeny. We chose to use a C-strain from a different continent than the African P-strain to maximize amount of the AFLP polymorphism segregating in the backcrosses. All crosses were single pair matings between virgin males and females, therefore individuals were confined in small tubes and kept therein until emergence. In the first crossing design, from now on referred to as cross 1 (*C1*), C and P males and females were crossed in each direction. Cross-types are abbreviated by writing the paternal strain first, e.g. CP utilized a C-strain male and a P-strain female. The resulting F_1 offspring of each family was divided equally on kale and pea plants and reared to adulthood. The sexes of F_1 adults were determined upon emergence, and these were backcrossed with the respective backcross partner from the Waite strain producing a backcross generation. F_1 adults were also intercrossed producing a F_2 generation.

Since in the *C1* crosses the number of offspring that survived on pea was very low, we conducted a second crossing design, referred to as cross 2 (*C2*). In this case the parental generation (C- and P-strain) was raised on kale to eliminate host-associated maternal effects and derived from intrastrain single pair matings to reduce the genetic heterogeneity within subse-

quent backcrosses. This strategy was used instead of inbreeding parental lines, to avoid inbreeding depression known to cause severe effects (infertility, inviability etc.) in Lepidoptera (e.g. Roush 1986). Similar to *C1*, C- and P-strain males and females were crossed in each possible direction to establish a F_1 generation. However, this time F_1 offspring were exclusively raised on kale. Resulting F_1 adults were either backcrossed to the P-strain males, P-strain females (these backcross partners derived from intrastrain crosses also reared on kale), or intercrossed in single pair matings so that all parents and grandparents were known. An overview of the mating design of both *C1* and *C2* is given in Figure 1.

Feeding assay

A feeding assay was conducted with F_1, backcross and F_2 progeny from *C1*, and backcross and F_2 progeny from *C2* in order to assess the phenotype 'survival on pea'. Ten to 15 eggs were transferred with a fine brush on a leaf of a potted kale or pea plant and the survival rates per family and host plant were determined by assessing the number of emerging adults. In *C1*, progeny from each family (in F_1, backcross and F_2 generations) were divided equally on kale and pea plants. In *C2* the feeding assay was slightly modified. The whole F_1 generation was raised on kale to minimize any maternal host plant effect, and offspring from backcross and F_2 families were unequally apportioned to host plants, with ⅔ on pea and ⅓ on kale (Figure 1b). This served to increase the number of surviving offspring on pea for later genetic analysis. Three backcross families showed high survivorship on pea and were chosen for AFLP analysis; these were BC_01 with 23 of 37 individuals surviving on kale and 35 of 74 on pea, BC_02 with 33 of 46 surviving on kale and 45 of 94 on pea, and BC_03 with 56 of 56 surviving on kale and 59 of 110 on pea (Figure 2). The Pearson's product-moment correlation was calculated to assess correlation between survival rates on kale and pea for each F_1 and backcross family using R (R Development Core Team 2010).

AFLP template preparation and analysis

For genetic analysis genomic DNA was extracted according to a modified protocol from Reineke et al. (1998), using CTAB and a TissueLyser (QIAGEN, Hilden, Germany) for mechanical disruption. The quality of genomic DNA was verified using agarose gel electrophoresis and concentration was measured using a Nanodrop ND1000 (Thermo Scientific, Delaware, USA).

AFLP analyses were performed according to Vos et al. (1995). Extracted genomic DNA (±200 ng) was digested with *Eco*RI and *Mse*I (New England Biolabs, Schwalbach, Germany).

Sequence information of adapters and primers used for AFLP analysis can be found in Supplementary Table 1. In each PCR reaction, two differently labeled *Eco*RI selective primers, one labeled with IRDye™ 700 and the other labeled with IRDye™ 800, respectively, were used in each PCR reaction. This multiplexing allowed detection of two different AFLP reactions simultaneously on the same gel due to the detection of fragments in two different channels of the LI-COR DNA Analyser 4300 (LI-COR Biosciences, Bad Homburg, Germany). For isolation of AFLP band for sequencing, gels were re-run using only one *Eco*RI primer.

Amplified products were separated based on size with a LI-COR DNA Analyser 4300. A formamide-dye stop solution was added to the AFLP reactions and samples were heat-denatured prior to electrophoresis. For separation, a 6.5% polyacrylamide gel (KB-PLUS, LI-COR) was chosen. A labeled size standard was loaded at each end. The gels were run for 2.5 h and the images were collected automatically in a computer file. Gels were scored using the image analysis program Saga^{MX} Version 3.3 (LI-COR).

Families analyzed consisted of grandparents, parents and F_1 progeny that survived on pea and kale. Bands present in the F_1 female, absent in the recurrent backcross father and segregating in the backcross progeny were scored. In this way, only female-informative AFLP bands were used to identify linkage groups. From *C1*, four families were analyzed and from *C2*, three families were analyzed (BC_01, BC_02 and BC_03). The establishment of linkage groups was only performed on *C2* derived backcross families. Because there is no crossing-over in meiosis in Lepidopteran females (Heckel 1993) all markers on the same chromosome will co-segregate as a single unit. A Pascal program written by D.G.H. (DBM3Lnk.p) was used to identify groups of co-segregating AFLPs in those female-informative backcross families. After linkage groups were identified, we determined the source of the chromosome passed on from the F_1 mother (i.e. inherited from the grandfather or grandmother) to her progeny for each of the linkage groups.

We used a two-step procedure to estimate the contribution of chromosomes to differential survival on the two hosts. First, for each linkage group in each backcross family, Fisher's Exact Test was computed from the 2×2 contingency table showing the numbers of backcross progeny feeding on pea carrying alleles from the P-strain (*a*) or C-strain grandparent (*b*) in the first row, and numbers feeding on kale carrying alleles from the P-strain (*c*) or C-strain grandparent (*d*) in the second row. Because of the expectation that alleles from the P-strain with differential survival would confer an advantage on pea-feeding individuals, we computed

the one-tailed probability corresponding to tables with the observed value of *a* or greater, using the FREQ procedure in SAS version 9.1.

In the second step, data from homologous chromosomes were pooled over as many families as possible. Homologous chromosomes were identified by bulked segregant analysis using AFLPs as described below, and assigned numbers in sequence. For Chromosome 1, corresponding linkage groups could be identified in all three families, using an AFLP that was segregating in two families and scored as a codominant marker in the third. Chromosome 3 likewise could be identified in all three families, and Chromosomes 2, 4 and 6 in two families by bulked segregant analysis using AFLPs. Only Chromosome 5 which was identified in BC_01 in the first step failed to be matched with linkage groups from either of the other two families. Data for each chromosome was pooled across all the families for which linkage groups could be identified, and subjected to Fisher's Exact Test as before. This time the criterion for significance was $P < 0.0083$, by applying the Bonferroni correction for six independent tests.

Excision, reamplification and sequencing of AFLP fragments

To develop markers that could be used to homologize linkage groups between backcross families and/or to assess whether any of the AFLP fragments are of specific coding region(s), AFLP bands from the four linkage groups of BC_02 with the smallest P-values by Fisher's Exact Test were excised from AFLP gels for sequencing and further analysis. For this purpose, the selective AFLP reaction that gave rise to the band of interest was repeated as a non-multiplex selective AFLP reaction and the PCR product was loaded on the gel. Recovery of the bands of interest followed the LI-COR protocol (LI-COR AFLP manual 2007). PCR conditions were 35 cycles of 94°C for 30 s, 56°C for 1 min and 72°C for 1 min with a final extension step of 2 min. The amplified fragments were analyzed by agarose gel electrophoresis. When reamplification was successful, 30 ng of the PCR products were treated with ExoSAP (USB Corporation, Cleveland, USA) to remove primers and nucleotides. In cases where agarose gel electrophoresis showed only a faint band of the PCR product a second reamplification was performed with 5 µl of the first reamplification as template (20 cycles of 94°C for 30 s, 56°C for 1 min and 72°C for 1 min). Reamplified AFLP fragments were directly sequenced on an automated sequencer (Applied Biosystems 3730/XL/96 capillary DNA analyzer, PE Applied Biosystems, California, USA). Sequences were deposited in GenBank (Accession Nos. GU594729-GU594732).

Amplification of fragment M-CGA_E-AAG in BC_03

AFLP fragment M-CGA_E-AAG_483 occurred in one linkage group of BC_01 and one of BC_02; therefore, these linkage groups were considered to be the same and named Chromosome 1. However, this fragment did not appear as an AFLP marker in BC_03. Specific primers (PxCG10501-F1 and PxCG10501-R2; Supplementary Table 1) were designed to amplify a portion of this fragment in BC_03. PCR was carried out in a total reaction volume of 20 µl with 50 ng template DNA, 10x mi-Taq buffer, 2.0 mM dNTPs, 20mM forward and reverse primer, respectively, and 5U/ µl Taq polymerase. PCR conditions were 2 min at 95°C for denaturing, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 1 min with a final extension step of 2 min. The PCR product was cleaned with QIAquick PCR purification kit (QIAGEN) and directly sequenced, which identified a SNP polymorphism that was used to map the fragment in BC_03.

Bulked segregant analysis

In order to efficiently identify homologous linkage groups from the three female-informative backcross families of *C2*, we used a modified form of bulked segregant analysis (Michelmore et al. 1991). The basic strategy was to create bulks separately for each family-chromosome combination by pooling DNA from backcross individuals receiving P- versus C-chromosomes from their F_1 parent, and then to screen these bulks simultaneously with many AFLP primer pairs. We created two bulks per family for each of several selected linkage groups: one bulk consisting of individuals where the AFLP marker for this linkage group band was present (plus) and another in which this specific band was absent (minus). Linkage groups (LGs) chosen for bulking were LG2, LG3, LG4, LG5, and LG6 of BC_01; LG2, LG3, and LG4 of BC_02, and LG6 and LG7 of BC_03. To create each bulk, the pre-amplification products of eight individuals (half males and half females; half pea- and half kale-survivors) were combined. All bulks were subjected to selective AFLP amplifications and run together on a polyacrylamide gel. Those pairs of bulks that showed the same presence-absence pattern for the same-sized AFLP band in two or more families were scored as the same linkage group between these families and assigned the same chromosome ID.

5.3 Results

Crossing experiment

Interstrain crosses yielding F_1, F_2 and backcross progeny are depicted in Figure 1 and the survival rates on pea and kale per family are shown in Figure 2. The number of families per type of cross and the overall survival rates of larvae in cross 1 (*C1*) and 2 (*C2*) are given in Table 1. In the F_1 generation we would expect a 100% survival rate on pea under dominant inheritance of the trait 'survival on pea', and no survival if the trait is inherited recessively. Overall, in the F_1 generation (of *C1*) the survival rate on kale was higher than on pea, irrespective of the direction of cross whether C-father × P-mother (CP) or P-father × C-mother (PC). The survival rates on pea of the two crossing types differed: CP-type offspring, with a P-strain mother, had higher survivorship on pea plants (up to 30%; Figure 2a) than F_1 progeny from PC-type, i.e. with a C-mother, where almost no survival occurred on pea. Thus, the offspring of P-strain mothers performed better on pea, indicating a maternal effect. (Possible maternal effects include those due to DBM strain origin or host plant consumed by the larva, which are confounded in this cross since all P-strain mothers consumed pea in this cross.) The survival rates on kale were highly heterogeneous in the CP- as well as PC-type, ranging from 30% to 90% survival, with no obvious pattern. The fact that survivorship in the F_1 generation occurred at all led us to first investigate the assumption of a partially dominant inheritance of the trait 'survival on pea' and thus, directed our choice of the C-strain as backcross partner in *C1*.

Table 1 Number of families of cross 1 (*C1*) and 2 (*C2*) per type of cross (male × female), generation and corresponding overall survival rates on kale and pea. In *C2* the entire F_1 generation was reared on kale.

Cross	Generation	Type	No. of families	Survival rate	
				kale	pea
Cross 1 (*C1*)					
	F_1	C × P	13	0.57	0.14
	F_1	P × C	5	0.5	0.02
	BC	C × CP*	11	0.80	0.03
	BC	CP* × C	4	0.71	0.02
	BC	CP** × C	5	0.89	0.01
	BC	C × CP**	4	0.87	0.01
	F_2	CP** × CP**	1	1.00	0.38
Cross 2 (*C2*)					
	BC	P × CP	4	0.76	0.47
	BC	P × PC	7	0.77	0.35
	BC	CP × P	4	0.64	0.34
	BC	PC × P	3	0.82	0.26
	F_2	CP × CP	3	0.65	0.10
	F_2	PC × PC	4	0.52	0.11

BC = backcross

** F_1 reared on pea

* F_1 reared on kale

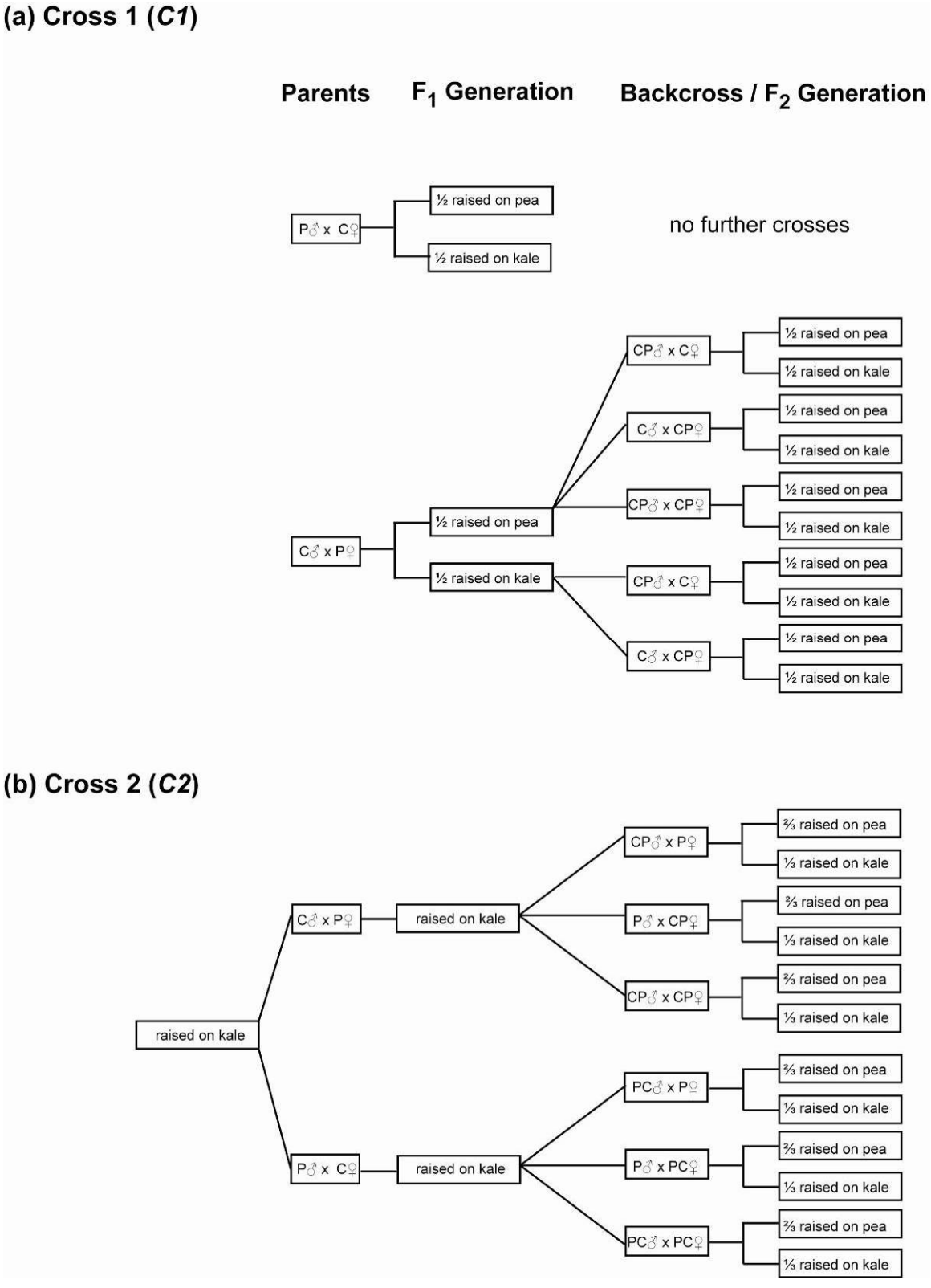

Figure 1 Mating design for cross *C1* (a) and cross *C2* (b). Males and females of the pea-adapted host strain (P) and cabbage-adapted strain (C) were crossed in every possible direction. In *C1*, F_1 and backcross progenies were distributed equally on pea (½) and kale (½) plants. In cross *C2*, all F_1 and P-strain individuals used in the backcross were reared on kale. F_2 and backcross progenies were distributed in a 2:1 ratio (⅔ on pea and ⅓ on kale).

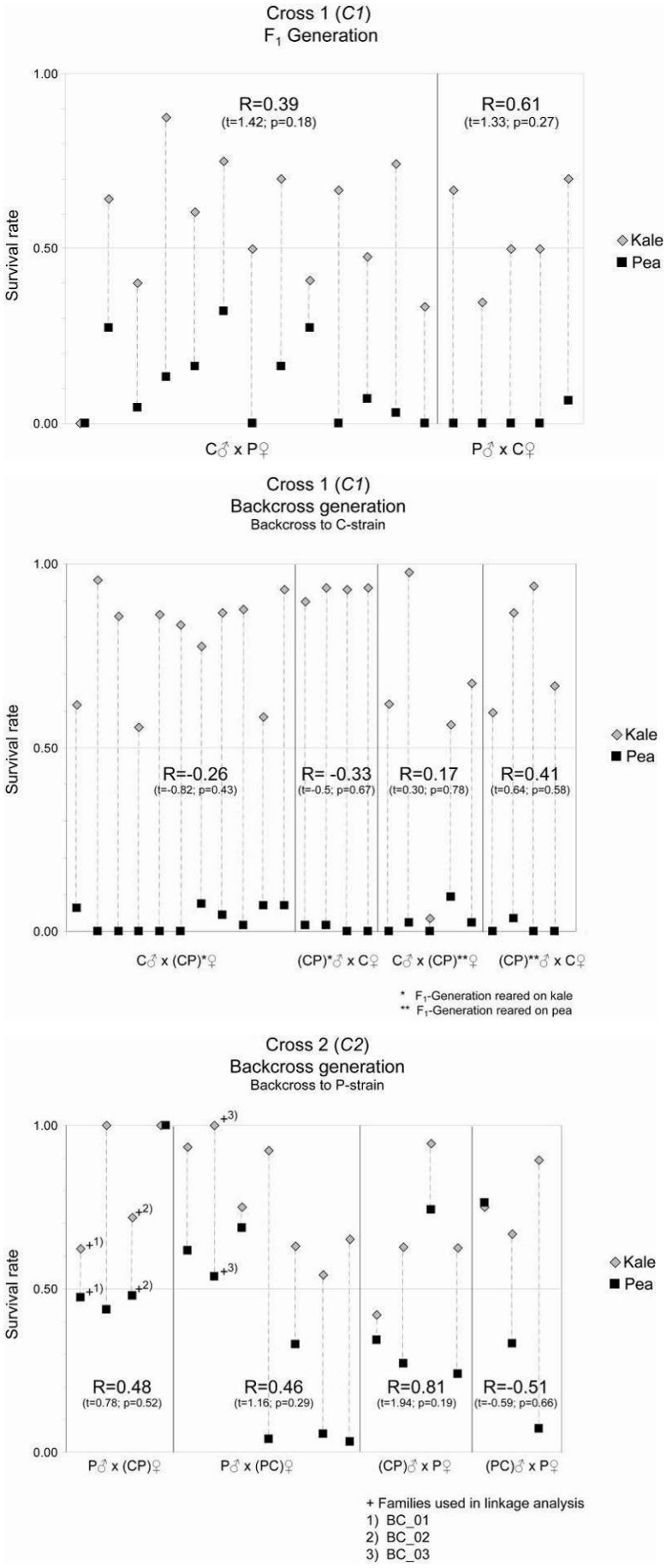

Figure 2 Survival rates of larvae per family in (a) F_1 generation of cross *C1*; (b) backcross generation of cross *C1*; and (c) backcross generation of cross *C2*. Diamonds: survival rates on kale plants; Squares: survival rate on pea plants. Each vertical pair of a square and a diamond, connected with a dotted line, represents the survival rate of larvae from one family on pea and kale, respectively.

Cross 1, backcross and F_2 generation

F_1 survivors descending from the CP cross (*C1*) that survived on kale and pea were either backcrossed to the C-strain to obtain a backcross generation, or intercrossed to establish a F_2 generation. The survival rates of backcross and F_2 progeny were assayed on the two host plants, kale and pea. Under the assumption of a single locus, the expected ratio of offspring from the backcross would have been 50% homozygous for kale-feeding and 50% heterozygous for pea-feeding. Under our predicted (partial) dominant inheritance of the trait 'survival on pea' we would expect a 50% survival rate among backcross progeny and 75% survivorship in F_2 families. But when assaying the survival rates of the (CP) × C and C × (CP) backcross progeny, the survival rate on pea plants was uniformly low (≤ 10%; Figure 2b). The survivorship on kale was not as heterogeneous as seen in the F_1 generation and could be divided into two classes: one in the range of ±90% and the other clustering at ±60% of surviving offspring. However, the single F_2 family (CP × CP) showed a higher survivorship on pea (38%), which was not significantly different from 25% ($\chi^2 = 0.05$, d.f. = 1). The survival rate in the F_2 resembling a 1:3 ratio and the overall low numbers of pea-survivors in the backcross generation suggested a recessive inheritance of the gene(s) responsible for the trait 'survival on pea' rather than the previously assumed dominant inheritance.

Cross 2, backcross and F_2 generation

In *C2* the two parental strains (C and P), the entire F_1 progeny (from PC and CP), and the P-strain individuals used in the backcross as parents were reared on kale to minimize maternal effects that may be generated by the type of host plant the mother consumed (for the mating design of *C2* see Figure 1b). To further investigate the putative recessive inheritance indicated from the first cross (*C1*), F_1 progeny of *C2* were backcrossed to the P-strain as well as intercrossed. Overall, the survival rates on pea plants among backcross progeny of *C2* were higher (Figure 2c) than in backcross progeny of *C1*, which had been backcrossed to the C-strain, supporting a recessive type of inheritance of the trait 'survival on pea'. Apart from a general higher survival rate on kale than on pea, the survival rates on both plants were highly heterogeneous with no obvious pattern of positive or negative correlation in survivorship on the two hosts (Figure 2c).

Overall, we did not detect a significant correlation for survival on kale vs. pea, i.e. a high survival rate on kale was neither positively nor negatively correlated with a high survival on pea (Figure 2a-c), the strongest correlation was shown by the (CP) × C-type of cross but was not significant ($R = 0.81$; $t = 1.94$, $P = 0.19$).

Linkage analysis

The segregation patterns of AFLP markers were used to identify linkage groups in both sets of female-informative backcrosses, in which the mother was always an F_1. Because of the absence of crossing-over in female Lepidoptera (Heckel 1993), linkage groups can be identified as non-recombinant blocks of AFLPs with the same segregation pattern across backcross progeny sets. Four female-informative backcross families of *C1* (C × (CP)-type; BC_07, BC_09, BC_12 and BC_19) were scored for AFLP bands inherited from the F_1 mother that were absent in the C-father. Each family consisted of grandparents, parents and 12 backcross progeny. Previously published AFLP analyses for *P. xylostella* utilized one large family that was scored per AFLP gel (Heckel et al. 1999; Baxter et al. 2005); however, due to the small number of offspring in any given family, offspring of the four families were scored in parallel on a single gel. This limitation together with the high genetic diversity within each of the *P. xylostella* strains (as revealed in genetic analyses; unpublished data), led to the fact that a polymorphic band scored in one family was generally not polymorphic in the other three families. AFLP analysis with 23 primer combinations resulted in the scoring of 239 different AFLP markers which were assembled into a 0/1 matrix. The number of markers per primer combination ranged from 19 loci (E-ATG_M-CTA) to three (E-ATG_M-CTT). Out of the 239 scored markers we found only two markers that were informative in all four families (E-AAC_M-CGA at 465 bp and E-ACA_M-CTG at 229 bp). Eighteen informative markers were present in three families, 115 informative markers in two families and the remaining 104 markers were only present in one of the four families. The low fraction of co-informative markers made it impossible to combine data from the families into a single linkage map. Based on previous studies (Heckel et al. 1999; Baxter et al. 2005), about 250 AFLP markers informative in all four families would have been required to identify all 31 chromosomes present in *P. xylostella*.

The number of surviving backcross individuals per family in *C2* was higher and therefore female-informative families could be scored individually for the establishment of linkage groups. Two families of type P × CP and one family of type P × PC were analyzed. Per family, > 200 female informative AFLP markers were identified and these were grouped according to common segregation patterns to identify linkage groups. Family BC_01 was scored with 44 primer combinations that resulted in 247 informative markers and 31 linkage groups, BC_02 with 48 primer combinations resulting in 199 informative AFLP markers grouped into 31 linkage groups, and BC_03 with 29 primer combinations resulting in 203 informative

markers and 29 linkage groups (the two missing linkage groups are likely marked by a single marker each).

Association of linkage groups with the trait 'survival on pea' in C2

Backcross progeny that survived on pea were expected to inherit alleles promoting the survivorship on pea from the P-strain to a greater degree than backcross progeny that survived on kale. For each linkage group a P-value was calculated using a one-tailed Fisher's Exact Test, to compare P-strain-derived vs. C-strain-derived homologues among pea- and kale-survivors. In this approach, the frequency of P-alleles among pea survivors vs. the frequency of P-alleles among kale survivors was compared; this measures the relative contribution of the P-alleles towards 'survival on pea'. Over-representation of a P-derived linkage group among pea-survivors thus yields a positive association with the trait 'survival on pea' indicated by a significant P-value. In this case, a factor (gene) conferring adaptation to pea found in the P-strain is likely to be located on this chromosome, enabling larvae to feed and survive on the new host plant pea. Under-representation among pea survivors of the P-strain homologue with concomitant over-representation of the linkage group inherited from the cabbage-adapted C-strain produces a negative association with the trait 'survival on pea' and non-significant P-values. BC_01 had five linkage groups and BC_03 had two with $P < 0.05$ (Figure 3). To test the significance of these linkage groups overall, we first needed to identify homologous linkage groups in all three backcross families.

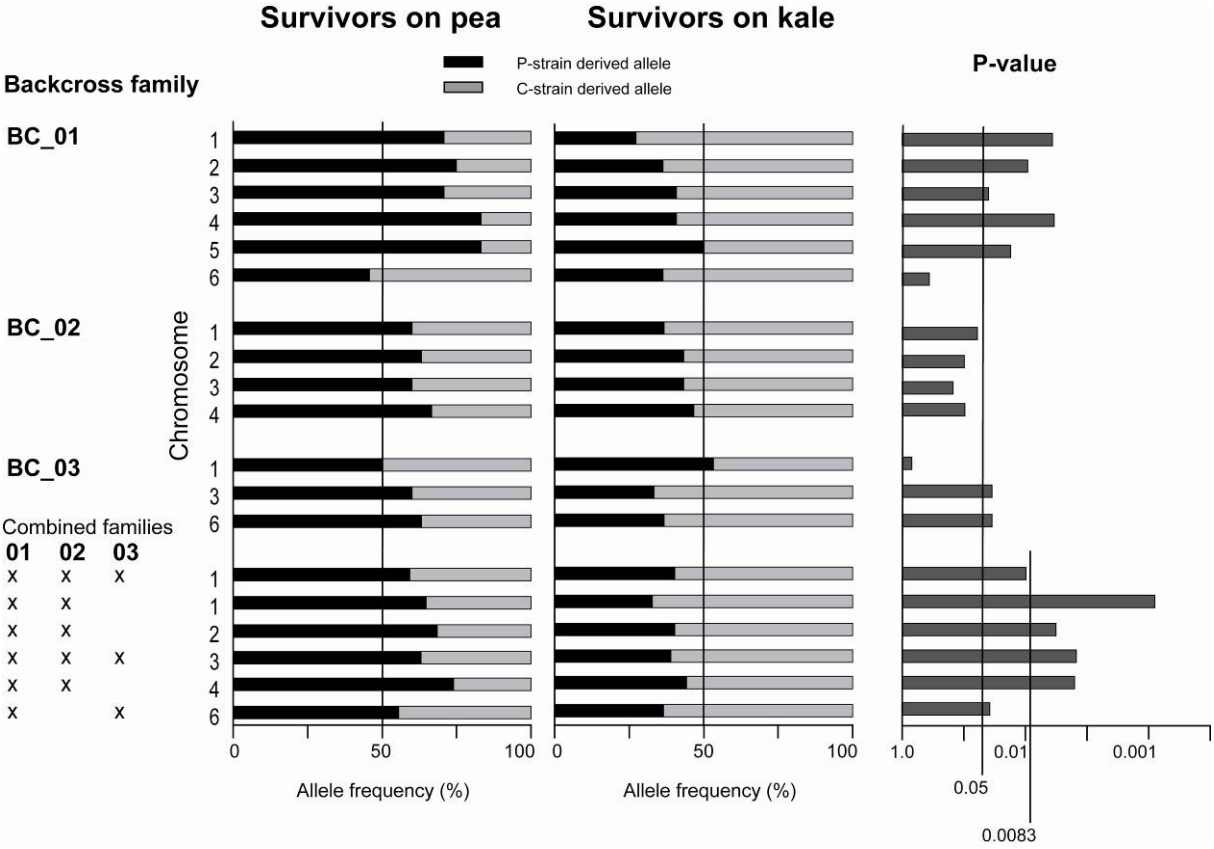

Figure 3 Association between chromosomes of the three analyzed backcross families BC_01, BC_02 and BC_03 in cross *C2* and the trait 'survival on pea'. The P-values were calculated using Fisher's Exact Test. Significant P-values indicate over-representation of chromosomes inherited from the P-strain grandparent via the F_1 female in pea-survivors relative to kale-survivors. P-values corresponding to chromosomes based on pooling homologous linkage groups over families are shown below, with the families that were combined indicated with an X. The Bonferroni-corrected probability value for 6 independent tests is shown as P = 0.0083.

Assignment of linkage groups - Common AFLP fragments

AFLP fragments belonging to the four linkage groups in BC_02 with the lowest P-values were sequenced (GenBank Accession Nos. GU594729-GU594732). AFLP fragment M-CGA_E-AAG_483 (GenBank Accession No. GU594729) showed similarity to a dopa-decarboxylase-like gene (*Drosophila melanogaster amd* CG10501; E = 1e-11). The same fragment (same primer combination at same size) was also found in one of the linkage groups of BC_02. Since individuals of both families showed an AFLP band at the same size for the same primer combination, these linkage groups were considered to be homologous and named Chromosome 1. In BC_03 this AFLP fragment was not present; however specific primers designed from the AFLP sequence successfully amplified the corresponding gene fragment from this family. Sequencing this PCR product revealed that at one nucleotide position, the F_1 mother and some backcross progeny were heterozygous, while the P-strain father and other

offspring were homozygous. The pattern of hetero- and homozygous individuals was the same as the pattern of one of the linkage groups in BC_03, which was therefore considered to be homologous to Chromosome 1 of BC_01 and BC_02. Based on sequence similarity of the AFLP to the *Bombyx mori* predicted protein BGIBMGA002958 (Xia et al. 2008), this would correspond to Chromosome 4 of *B. mori*.

Assignment of linkage groups - Bulked segregant analysis

To find additional homologies among linkage groups between families BC_01, BC_02 and BC_03, we used a modified form of bulked segregant analysis (Michelmore et al. 1991), using AFLP segregation patterns of selected linkage groups within families to define the bulks. We created two different bulks for each such linkage group (Figure 4): one bulk with individuals showing the band (plus) and another bulk from individuals in which the band was absent (minus). If the bulks of two or three families showed the same presence-absence pattern, for a given primer combination and band size, these two linkage groups were considered homologous, thus representing the same chromosome. With this approach we were able to assign the following linkage groups (LG) to the same chromosome: LG2 of BC_02 and LG2 of BC_01 (Chromosome 2), LG3 of BC_02, LG3 of BC_01 and LG6 of BC_03 (Chromosome 3), LG4 of BC_02 and LG4 of BC_01 (Chromosome 4) and LG6 of BC_01 and LG7 of BC_03 (Chromosome 6) (Figure 4, Supplementary Table 2). LG5 of BC_01 represented an additional chromosome that we were unable to homologize to linkage groups in the other two BC families (Chromosome 5, Figure 4).

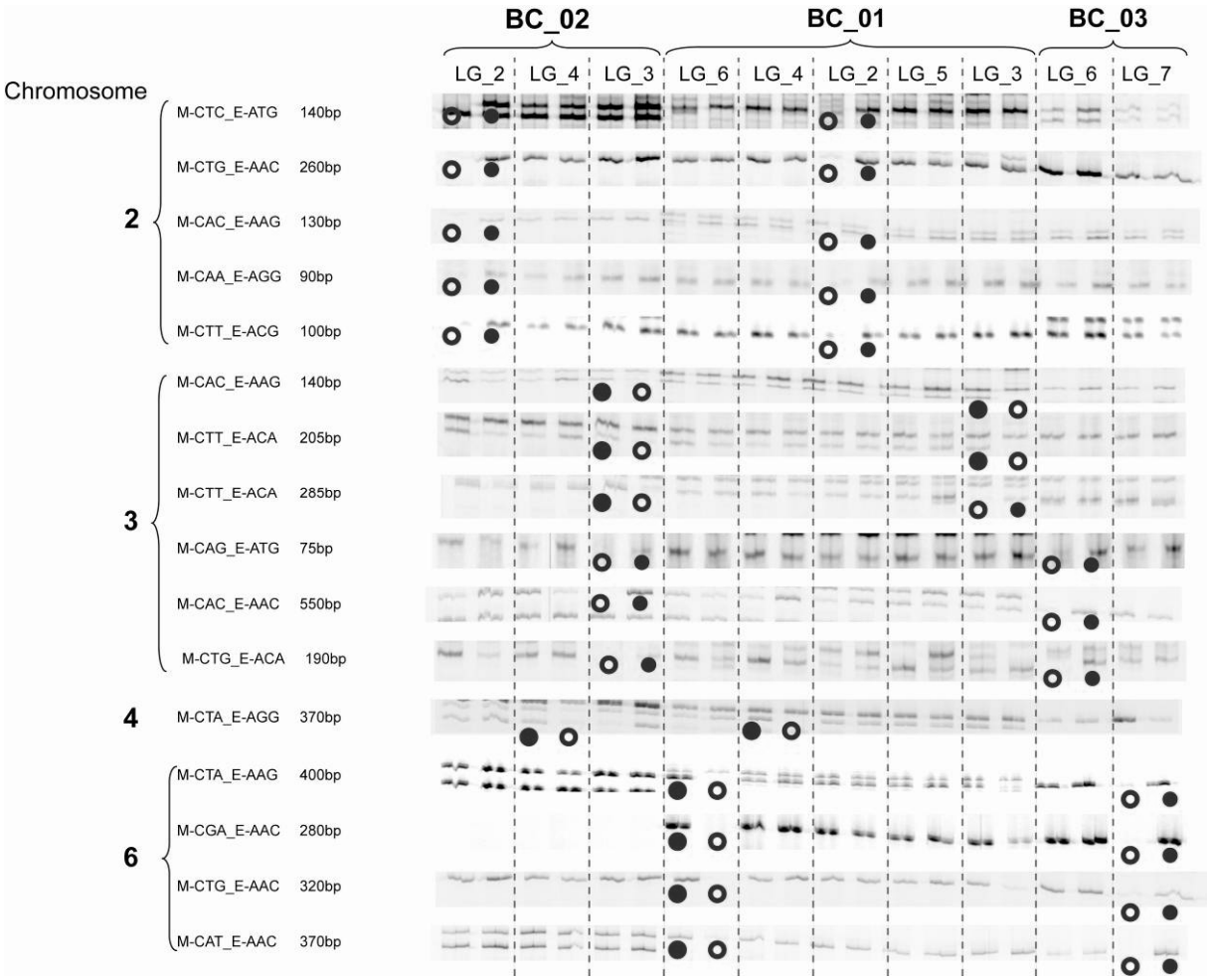

Figure 4 Bulked segregant analysis of AFLPs to identify homologous linkage groups. Each linkage group (LG) in each family is represented by two bulks: one with individuals that carry the AFLP band and the other where the band is absent. Linkage groups within families are listed across the top of the figure. AFLP markers with concordant patterns in two of the bulks are listed down the side, grouped according to chromosome. Primer combinations and band sizes (in bp) are given along with a slice of the gel image showing the AFLP pattern. Circles placed immediately below the relevant band denote where a specific presence/absence pattern (filled circle vs. open circle) occurs, indicating the correspondence between the same linkage group in two different families. Only LG5 in BC_01 fails to match with any of the other patterns shown.

Tests on data pooled over families

We calculated Fisher's Exact Test for the five chromosomes for which homologous linkage groups could be identified, to assess an overall significance with the larger sample sizes obtained by pooling the families (Figure 3). For Chromosomes 2, 3, 4, and 6, pooling the data confirmed the trends shown by individual families, i.e. the P-derived homologues were over-represented in pea survivors relative to kale survivors; and the overall significance increased as data were combined from separate families. For Chromosome 1, pooling BC_01 and BC_02 also greatly increased the significance, but adding BC_03 reduced significance be-

cause BC_03 showed no effect of this linkage group (Figure 3). Chromosomes 1, 2, 3, and 4 are significant at the level of P = 0.0083 (Bonferroni correction for six independent tests).

5.4 Discussion

This study provided a first approach towards understanding the genetic basis of the recent adaptation to sugar pea in the newly evolved P-strain of the diamondback moth, *P. xylostella*. The heterogeneity of larval survivorship on pea as well as on kale between and within the different types of crosses eliminates any simple genetic hypothesis, and this new trait appears to have a complex genetic basis. Our data show that the genes significantly affecting the trait 'survival on pea' in the P-strain of DBM are all autosomal. Differences among reciprocal crosses indicate the presence of maternal effects, not sex-linkage. Survival on pea also shows characteristics of a recessive rather than a dominant mode of inheritance. Further, the trait is not inherited monogenically but under multi-locus control, with the involvement of two to five different loci, depending on the family, which thus contributes to its complex nature.

Recessive inheritance

The conclusion of recessive inheritance of the trait 'survival on pea' is based on crosses and backcrosses between the newly evolved P-strain of DBM and a cabbage-adapted strain and the assessment of the survival rates of F_1, F_2 and backcross progeny on pea. The occurrence of some survivorship on pea in the F_1 generation in the first series of crosses (*C1*), although low, suggested a partially dominant inheritance. However, the low numbers of survivors in the backcross generation together with the higher survivorship of F_2 progeny of *C1* on pea contradicted this assumption, and is instead consistent with the hypothesis of one or more recessive genes being responsible for the trait 'survival on pea'. Additionally, the survival rate on pea in the F_1 generation was significantly higher among F_1 progeny from pea reared P-strain mothers in comparison to those from C-strain mothers. Thus, the survivorship in the F_1 generation on pea that we found in the first cross appears to be due to a maternal effect (discussed below) rather than to partial dominance.

In the second backcross (*C2*), where F_1 progeny were hybridized with the P-strain, we found a much higher but still an overall low survival rate on pea. Given the P-strain's rapid adaptation to pea, we initially assumed dominant inheritance of a recently-derived mutation; yet the current picture is more consistent with recessive inheritance of standing variation. Interestingly, Orr and Betancourt (2001) have shown that the traditional principle of Haldane's sieve (Turner 1981), i.e. recessive alleles having a lower chance of fixation because they predominantly

occur in heterozygotes where they are shielded from selection, holds when fixation of an adaptive trait comes from new mutations but not when it results from standing genetic variation for which the degree of dominance is marginal. In host races of *Mitoura* butterflies the ability to successfully use cedar as a host in the larval stages was expressed as a recessive trait as well (Forister 2005). The recessive mode of inheritance of loci controlling adaptation in DBM might be a common mechanism in this insect, as resistance to Bt toxins was also found to be completely or partially recessive in DBM (Hama et al. 1992; Tabashnik et al. 1997; Tang et al. 1997). Strong selection was exerted for survival on pea since no other food source was available at that time, a scenario comparable to the development of insecticide resistance.

Autosomal inheritance

We conclude that the trait 'survival on pea' is autosomally inherited because all predictions of sex-linkage were rejected. Differences between reciprocal F_1 families in the first crosses (*C1*) may be caused in principle either by maternal effects or by sex-linked genes. Taking into account the fact that *P. xylostella*, like most Lepidoptera, has a ZZ/ZW type of sex determination and female Lepidoptera are the heterogametic sex (ZW), effects of loci on the W-chromosome are confounded with extra-chromosomal maternal effects. Under the hypothesis of W-linkage, since only females carry the W-chromosome, any W-linked genes responsible for pea-adaptation can only be passed from mother to daughter, and consequently, females would have a higher survivorship on pea, which we did not find. The chance of detecting a W-linked trait in Lepidoptera seems very low as the W-chromosome harbors almost no (known) genes (Traut 1999). The only published examples are W-linked copies of the *period* gene in the silkmoth *Antheraea pernyi* (Gotter et al. 1999).

We also considered the possibility of Z-linked sex-linkage, again taking into account that the trait 'survival on pea' is inherited recessively but still evolved quite rapidly in the field. A rare recessive allele has a lower chance of fixation when autosomal because it is shielded from selection when heterozygous, than when occurring on the sex-chromosome, where it is completely exposed to selection in the hemizygous sex (Haldane 1922). Furthermore, many Z-linked traits in Lepidoptera are known to account for inter- and intraspecific differentiation (Prowell 1998; Janz 2003). However, this is not what we found. Under Z-linkage, all F_1 females descending from the PC-cross would carry a pea-adaptation-conferring Z-chromosome (Z^PW^C) inherited from their father, but F_1 females from the CP-cross (with C-strain father; Z^CW^P) would lack it. The fact that CP-females survived on pea plants, and moreover, were

used in single pair matings for back- and intercrosses in which segregation for the trait occurred, rejects the hypothesis of a Z-linkage of the trait 'survival on pea'.

Maternal effect

Besides the genetic inheritance of pea-adaptation, in *C1* maternal effects were evident. F_1 progeny descending from a pea-fed P-strain mother had a higher survival rate on pea than did F_1 hybrids with a kale-fed C-strain mother. An effect due to the origin of the maternal strain cannot be separated from an effect due to the maternal diet in this cross; one or both may be operative. It would have been interesting to assign the mothers of the P- and C-strain to each others' host plant thereby taking the maternal effect into full account. However, this is not possible because the cabbage-adapted strain cannot survive on pea. Instead, we aimed at minimizing possible maternal effects by rearing the P- and the C-strain on kale prior to the F_1 cross, as well as the F_1 generation and all P-strain backcross partners in cross *C2*.

Although their mechanisms are poorly understood, non-genetic maternal effects on offspring phenotype appear to be widespread and are often of profound importance (Kirkpatrick and Lande 1989; Agrawal 2001). They may be adaptive for organisms in heterogeneous environments, such as phytophagous insects, whereby mothers produce offspring that are physiologically “acclimated” to her rearing host (possibly via induction of enzymatic activity in the offspring) (Fox et al. 1995; Bernardo 1996). Maternal effects were frequently only treated as a troublesome nuisance in quantitative genetic studies that need to be overcome by experimental design, rather than a target of experimental studies (Falconer and Mackay 1996; Wolf et al. 1998; Andersen et al. 2005). Only recently have the pervasiveness and ecological and evolutionary significance of maternal effects become appreciated (Bonduriansky and Day 2009; Mousseau and Fox 1998). Fox et al. (1995) found that maternal rearing host affected offspring in the seed beetle *Stator limbatus*: mothers reared on *Cercidium floridum* produced larger offspring that developed faster than offspring of mothers reared on *Acacia greggii*, due to a non-genetic maternal effect. Although we know that pea-adaptation has a genetic basis, the maternal-effect might have contributed to the rate of population adaptation. Considering the recessive nature of the trait, presence of a maternal effect could have accelerated the rate of evolution. Exploring the possible mechanism behind the maternal effect (e.g. transmission of secondary plant compounds or mRNA via the eggs that enhance activity/transcription of digestive gut enzymes) would contribute to the newly evolving field of maternal effect studies.

Oligogenic basis

The complex pattern of larval survival rates in the backcrosses and the results of the linkage group analysis indicate an oligogenic inheritance. Thus, inheritance of pea-adaptation neither involves a very large number of genes of small effect, as it would be seen if loci contributing to pea-adaptation were spread over a large number of chromosomes, nor is it controlled by a single gene. At least two genes are involved, because we found that factors contributing to pea-adaptation are located on at least two chromosomes. It is very likely that more than a few genes are involved in larval adaptation to a new host plant, because the successful development of a larva on its host plant, which was recorded as survivorship in our study, requires a suite of mechanisms. The larva must be able to recognize, digest and fully develop on the newly acquired host plant (Thomas et al, 1987), and failure to do so can have multiple causes. Some larvae died because they did not initiate feeding, others initiated feeding but died before molting to the next larval stage, and still others passed through three larval instars but were not able to pupate successfully (Henniges-Janssen, personal observation). The chance of a single gene controlling such complex patterns seems unlikely, whereas it seems plausible that each of these steps is under complex genetic control. Therefore, adaptation to pea is likely to be oligogenic and our finding of multiple chromosomes associated with pea-adaptation is not surprising. There has been a long-standing debate concerning the number of genes involved in adaptation. Historically, it has been argued that most adaptations result from numerous small changes (Fisher 1930). This view was challenged by more recent laboratory studies (Orr and Coyne 1992; Orr 2005) and theoretical models (Orr 1998), which led to the conclusion that a few major genes account for a large portion of adaptation. The genetic basis of pea-adaptation is probably best explained as a mixture of a few genes (detected in our linkage analysis) with major effects plus the influence of many more loci with minor effects as reflected in the complex pattern of inheritance. Earlier we made the comparison to the genetic basis and inheritance of insecticide resistance, thus, adaptive changes to human-disturbed environments. Insecticide resistance arising in over-sprayed field populations, however, is a typically monogenic trait in most cases (Roush and McKenzie 1987). In contrast, the multiplicity of factors that govern the genetics of host-adaptation has been stressed by several other authors (Bernays and Graham 1988; Lu et al. 2001). Sheck and Gould (1996) showed that different host-associated feeding behaviors in *Heliothis virescens* and *H. subflexa* were controlled by multiple and likely different loci.

Trade-offs in adaptation

Our evidence that a particular chromosome harbors genes contributing to successful growth and development on pea is based on the over-representation of alleles from the P-strain in surviving backcross progeny feeding on pea. To control for possible segregation distortion, i.e. the preferential transmission of an allele during meiosis (Sandler et al. 1957), it would have been ideal to also score backcross progeny feeding on pea that failed to survive. However this was not feasible due to the inability to obtain sufficient DNA from tiny larvae that died at various times throughout the experiment. Therefore we were confined to comparisons among groups of individuals that had survived at least to the pupal stage, and we reared additional backcross progeny on kale to provide a control group for each family. Significance in the Fisher's Exact Test comparing these two groups is sensitive to two effects: selection for P-strain alleles on pea and selection against P-strain alleles on kale. With sufficiently large sample sizes, these two effects could be distinguished statistically by comparison to the 1:1 segregation ratio expected in the absence of any selection or segregation distortion; but even with pooling over families our sample sizes were too small to detect any but the most extreme deviations. However, qualitative comparison of the segregation ratios of pea- vs. kale-survivors (Figure 3) shows that for each of the four chromosomes with the P-strain allele over-represented among pea survivors, it was also under-represented among kale survivors. This provides some evidence of a tradeoff in adaptation to different hosts at the gene level, even though there was no correlation among families for performance on pea vs. kale.

Heterogeneity in P-strain

In comparing the overall survival rates of the different types of crosses, obvious patterns were a generally higher survival rate on kale than on pea, irrespective of generation or type of cross, and a higher survivorship on pea in the P-strain backcross than in the backcross to C-strain. Furthermore, almost no larval survivorship on pea was detectable in the PC-type F_1 offspring from *C1* but up to 30% survived in the CP-type. However, no such pattern was obvious when studying the survival rates of the individual families within and between the types of backcrosses. Moreover, we found no significant positive or negative correlation between the survival rate on pea and kale of families from the same direction of cross, i.e. a high survival rate on kale was not associated with a low survival rate on pea or vice versa. These findings allow for several conclusions: (1) High survival on pea was not just due to general vigor, whereby some families simply survived better regardless of the host plant. (2) Loci affecting survivorship on kale are not linked to loci affecting survivorship on pea. (3) There is no evi-

dence for a trade-off at the family level, such that adaptation to one host results in a relatively poorer performance on alternative hosts (Agrawal 2000).

We conclude that the highly variable survivorship on pea among replicate backcross families is evidence that considerable genetic variation still exists in the P-strain; with those families showing higher survivorship on pea segregating for more pea-adapted genes than families with lower survivorship. The set of chromosomes showing significant effects was also variable across the three high-surviving backcross families. Low-surviving backcross families, which were not analyzed due to lower sample sizes, would be predicted to be segregating even smaller combinations of these chromosomes. Therefore the P-strain is not yet homozygous for all the genes that contribute to pea adaptation but likely in a transition phase to complete host expansion and homozygosity for the trait 'survival on pea'. We hypothesize that an individual does not have to be homozygous at all pea-adapted loci but being homozygous for a core number of alleles enables successful development on pea.

The potential to adapt to a novel host plant in a short evolutionary timescale, as seen in the example of the P-strain's rapid spread to sugar pea, is increased when it results from standing genetic variation whereas more time is needed for awaiting a beneficial mutation (Barrett and Schluter, 2008). Adaptation from standing genetic variation arises faster because the advantageous allele is already present in multiple copies and not only as a single mutation. There is evidence that some standing genetic variation for the ability to feed on legumes and on other host plant species exists in DBM populations: DBM has occasionally been found on plants other than Brassicaceae, among them plants from the Fabaceae family (Robinson et al. 2010). Gupta and Thorsteinson (1960a) showed that some DBM larvae were able to survive on legumes under laboratory conditions. Moreover, an unrelated C-strain responded to laboratory selection on pea to increase its survivorship to nearly 50% over six generations (Löhr and Gathu 2002). This suggests that sufficient pre-existing genetic variation existed in Kenya for the unusually strong and extended selection pressure for survivorship on pea, after destruction of suitable crucifer hosts, to result in the observed sudden host range expansion.

Conclusions and perspective

Our results show that adaptation to sugar pea in DBM P-strain larvae cannot be explained by simple Mendelian inheritance but instead by an intricate genetic pattern composed of an autosomal oligogenic inheritance with a maternal effect and can thus be considered a complex trait. Considering the suite of mechanisms necessary for successful larval host plant adaptation (e.g. host perception, adequate digestion and detoxification), it seems not surprising to

find more than one underlying gene responsible. Now the challenge is to identify candidate genes underlying the P-strain's mechanisms of adaptation to sugar pea. In our follow-up study on the transcriptional response to pea-feeding in DBM larvae (Henniges-Janssen et al. in preparation b), we have identified transcripts with roles in gustation and perception (e.g. odorant binding proteins), detoxification (e.g. cytochrome P450 monooxygenases, glutathione S-transferases), digestion (proteinases), and stress responses. Future mapping efforts will assess whether any of the linkage groups identified here harbor genes that affect expression changes, providing a genetic mechanism of the host expansion of *P. xylostella* to pea.

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Supplementary information

Supplementary Table 1 Sequences of adapters, preamplification primers and selective AFLP-reaction primers used in the AFLP analysis as well as primer sequence for amplification of fragment M-CGA_E-AAG_483 in BC_03.

Adapter/ Primer	Sequence
Adapters	
Eco RI F	5`-CTCGTAGACTGCGTACC-3`
Eco RI R	5`-AATTGGTACGCAGTCTAC-3`
Mse I F	5`-GACGATGAGTCCTGAG-3`
Mse I R	5`-TACTCAGGACTCAT-3`
Preamplification primers	
Eco RI+0	5`-GACTGCGTACCAATTC-3`
Mse I +0	5`-GATGAGTCCTGAGTAA-3`
Selective AFLP-reaction primers	
Eco RI+ 3 selective nucleotides	AAG*, AAC*, ATG*, AGG**, ACG**, ACA**
Mse I+ 3 selective nucleotides	CTC, CAA, CAG, CTT, CGA, CCT, CAC, CAT, CTA, CTG
Primers for amplification of M-CGA_E-AAG-483	
PxCG10501-F	5'-AGGGATGTCCTACCATCAGTGGA-3'
PxCG10501-R	5'-ACCCCAGGCATAATAGCTTGATT-3'

F = forward; R = reverse; * = IRDye 700 labeled; ** = IRDye 800 labeled.

Supplementary Table 2 Assignment of linkage groups (LG) conferring pea-adaptation in the three female-informative backcross families BC_01, BC_02 and BC_03 of cross *C2*.

Chromosome	BC_01	BC_02	BC_03	Assigned by
1	LG_1	LG_1	LG_1	AFLP fragment
2	LG_2	LG_2		Bulk
3	LG_3	LG_3	LG_6	Bulk
4	LG_4	LG_4		Bulk
5	LG_5			Not assigned
6	LG_6		LG_7	Bulk

6. Manuscript IV

Transcriptional changes in diamondback moth larvae feeding on original and novel host plants

Henniges-Janssen, K., Groot, A.T., Heckel, D.G. and Vogel, H.

Transcriptional changes in diamondback moth larvae feeding on original and novel host plants.

For submission to BMC Biology.

This manuscript describes the transcriptional response of pea-feeding larvae of the newly evolved pea-adapted DBM strain in order to identify candidate genes that accompany and mediate the host range expansion to pea.

HV, DGH and KHJ conceived the study. KHJ performed feeding assays. HV and KHJ performed molecular analyses. HV and KHJ analyzed data. KHJ wrote manuscript with revisions from HV, ATG and DGH.

Abstract

Understanding the mechanisms and identifying the genes that underlie adaptation to a novel host plant has been a longstanding aim for evolutionists and ecologists. Recent advances in transcriptome profiling methods and their applicability to non-model organisms have made it possible to unravel the mechanisms and genes that underlie such evolutionary and ecological important processes. The availability of adequate methods and a recent case of host range expansion of the crucifer-specialist diamondback moth from its original host plant cabbage to sugar pea in Kenya, now offer the opportunity to provide insight into transcriptional changes and to identify candidate genes accompanying adaptation to this novel host plant. Using two different transcriptome profiling techniques, cDNA-AFLP (amplified fragment length polymorphism) and a SSH (suppression subtractive hybridization) library, the foremost goal was to identify candidate genes that enable larvae of the newly evolved pea-adapted DBM strain (DBM-P) to feed on pea. Our data suggest a global transcriptional response in pea-feeding DBM-P larvae involving a large number of genes, affiliated with a variety of different functional classes, such as olfaction, metabolism, detoxification, response to stimulus, response to stress, that may be involved in mediating DBM's host range expansion to peas. Additionally, the overall changes in the expression pattern profile of DBM-P larvae feeding on original crucifer and novel host plant as well as after being shifted to related and unrelated host plant was compared to the transcriptional response in two other DBM strains. This revealed a similarly high number of altered transcripts in larvae of all three strains and all kinds of host shifts.

6.1 Introduction

Identifying the genes underlying evolutionary and ecological important processes, such as population divergence and adaptation to changing environments, has become one of the foremost goals for scientists in recent years (Jones 1998; Stinchcombe and Hoekstra 2007). The recent development of powerful transcriptomic and genomic tools enables scientists to meet the challenge of studying the genetic basis of such processes even in non-model-organisms. Not only can these methods provide a powerful tool for the identification of candidate genes, moreover, altered transcriptional regulation itself can be a driver of adaptation and evolution.

Host plant adaptation is a key process that has shaped today's insect diversity. However, present insect-plant associations are not steady-state but constantly coevolving: insects exploit new niches and colonize novel host plants and plants invent novel defenses to escape herbivory. Host shifts and range expansions to novel host plants have been the driver for the evolu-

tion of novel insect-plant relationships. Successful adaptation to another host requires a suite of complex processes: A new plant can be incorporated into the insect's diet when the adult accepts it for oviposition and larvae accept it for feeding and are able to complete their life cycle (Bernays and Chapman 1994). In most cases, phytophagous insects acquire new but biochemically or phylogenetically related plants of the usual host for which they already possess the necessary set of enzymes to meet the requirement of sufficient nutrient uptake and to cope with the plant's defense system (Ehrlich and Raven 1964; Feeny 1992; Murphy and Feeny 2006); whereas sudden host range expansions to distantly related host plants are rare (Strong 1979; Winkler and Mitter 2008).

A recent case of adaptation to a novel host plant and of ongoing evolution is the host plant range expansion of the crucifer-specialist diamondback moth (DBM), *Plutella xylostella* (Lepidoptera: Plutellidae), onto sugar peas in Kenya in the late 1990's (Löhr 2001). DBM is one of the most devastating pest species worldwide (Talekar and Shelton 1993), well known for its intricate detoxification mechanism. DBM larvae have a specific detoxifying enzyme, the glucosinolate sulfatase (GSS), which allows them to feed with relative impunity on brassicaceous plants, toxic to most other herbivores due to the specialized "glucosinolate-myrosinase" defense system (Ratzka et al. 2002). Although DBM relies on glucosinolates as stimulants for feeding and oviposition, a Kenyan population of DBM was reported to have shifted from cabbage to sugar snap peas (Fabaceae) (Löhr 2001). This host range expansion occurred in an outbreak situation when moths were highly abundant and the original host plant cabbage highly infested. In a following year, DBM also invaded neighboring mangetout pea fields (Löhr 2001). DBM has incorporated sugar pea as a novel host plant to its diet, a plant taxonomically and phytochemically unrelated to its original host plant crucifers.

So far, studies aiming in understanding and elucidating the underlying mechanisms of DBM-P's host range expansion have focused on analyzing larval performance (Löhr and Gathu 2002) as well as adult and larval preference for original and novel host plant (Henniges-Janssen et al. 2011; Henniges-Janssen et al. in preparation a) and determining the genetic basis of larval pea-adaptation (Henniges-Janssen et al. in press). It has been found that the newly evolved pea-adapted host strain of DBM (DBM-P) survives on pea as well as on cabbage plants, whereas a cabbage strain (DBM-C) suffered high mortalities on the non-host pea (Löhr and Gathu 2002). In our laboratory, we performed an oviposition assay in which we showed that DBM-P females still prefer to oviposit on their original host plant cabbage (Henniges-Janssen et al. 2011), indicating an early stage of host range expansion. However, in a feeding choice assay, DBM-P larvae readily preferred pea over cabbage (Henniges-Janssen et al. in

preparation a). Backcrosses with DBM-P and cabbage strain individuals followed by a linkage analysis revealed an autosomal, recessive and oligogenic basis of the novel trait 'larval survival on pea' (Henniges-Janssen et al. in press).

Here, we aim to provide insight in the transcriptional changes in pea-feeding DBM larvae and identify genes accompanying and promoting the recent host range expansion. Transcriptional profiling techniques have so far been only rarely applied to identify candidate genes accompanying host plant adaptation or dietary shifts in herbivorous insects. Moreover, DNA microarrays, the standard tool for genome-wide expression analysis, are only applicable to organisms for which a large collection of known transcript sequences is already available. Since transcript sequences were not available for the non-model organism DBM, we had to find an alternative method. We chose to employ two different transcriptome profiling techniques, I) construction and analysis of a SSH (suppression subtractive hybridization) library and II) comparisons of cDNA-AFLP (amplified fragment length polymorphism) in different experimental treatments. An analysis of expression levels of GSS was also included to help to characterize the transcriptional response.

The SSH technique (Diatchenko et al. 1996) enables one to compare two types of mRNA pools and obtain genes that are expressed in higher levels in one mRNA type (DBM-P larvae feeding on pea) than in other (DBM-Cj larvae feeding on cabbage). This method has already been proven to be efficient in identification of e.g. differentially expressed immune-induced genes in insects where it led to the identification of a large number of transcripts that were assigned to genes that might have a function in the studied process (Zhu et al. 2003; Altincicek and Vilcinskas 2007a; Altincicek and Vilcinskas 2007b).

The cDNA-AFLP technique (Bachem et al. 1996) allows for the comparison of more than two sets of mRNAs. As already mentioned, transcriptome analyses have only rarely been applied in herbivores and hence our knowledge on herbivore transcriptional responses to host plant feeding is limited. To understand and interpret expression changes in DBM-P larvae when feeding on pea, we need a general understanding of the transcriptome of host plant feeding larvae. With the cDNA-AFLP method we were able to study and compare i) changes on the transcriptome level in DBM-P larvae as well as in larvae from other strains and the transcriptional response, ii) upon feeding on original and novel host plant as well as iii) after a shift to a related and unrelated host plant.

For the transcriptional analyses, we assessed the larval feeding performance of three DBM strains on their original and novel host plant, cabbage, kale and pea, respectively. These three

strains were: the newly evolved pea-adapted host strain (DBM-P), an original cabbage-adapted host strain (DBM-Cj) and G88, a DBM strain adapted to and reared on a glucosinolate-free diet. G88 larvae do not depend on glucosinolates for feeding stimulation and initiation. In the feeding assay, we confirmed the results from Löhr and Gathu (2002) that DBM-P thrives on both host plants, while survival of DBM-C is restricted to kale. Additionally, we assessed the feeding of G88 strain larvae, which similarly to cabbage-adapted strains could only survive on kale, although a few larvae initiated feeding on pea plants.

As described above, GSS is the key enzyme in DBM that allows larvae to feed and survive on crucifers, whereas most other herbivores cannot feed on crucifers due to the toxic break-down products of the glucosinolates. Since the constitutive synthesis and allocation of an enzyme is energetically expensive, we predict that DBM adjusts its GSS level to the glucosinolate content in the food source. Moreover, we speculate that reduced GSS levels in DBM-P larvae compensate for other trade-offs that are supposed to result from adaptation to two phytochemically diverging host plants. The newly evolved DBM-P strain provides the opportunity to test this hypothesis.

6.2 Materials and methods

Plants

Seeds of pea, *Pisum sativum* var. Oregon Sugar Pod were obtained from Agri-Saaten GmbH (Bad Essen, Germany), cabbage seeds, *Brassica oleracea oleracea* var. Gloria, from B & T World Seeds (Aigue-Vives, France), and kale, *Brassica napus* mixed varieties. Plants used for rearing of insects or in experiments were reared in 50×30×8 cm trays in Klassmann Tonsubstrat under greenhouse conditions at 21-23°C, 50-60% RH and 14:10 L:D photoperiod.

Insects

Three strains of *Plutella xylostella* were used for this study: the newly evolved pea strain (DBM-P) and a cabbage strain (DBM-Cj), as well as the laboratory Geneva 88 strain (G88). DBM-Cj and DBM-P both originate from Kenya and were kindly provided by Bernhard Löhr from ICIPE (International Centre of Insect Physiology and Ecology, Nairobi, Kenya). DBM-P was originally collected from the infested pea field in Naivasha in 2002, where the host shift was observed in 1999. It was maintained as a laboratory culture ever since at ICIPE in Kenya. DBM-Cj derived from a field population from the semi-arid areas about 40 km south east of Nairobi. Both strains were sent to the Max Planck Institute for Chemical Ecology (Jena, Ger-

many) in May 2005, where they have been raised for > 40 generations. Insect cultures of both strains are reared on intact plants at 21°C, 50% RH, and 16:8 L:D photoperiod, with DBM-Cj reared on cabbage and DBM-P reared on pea. G 88 was collected in 1988 from cabbage at the New York State Agricultural Experimental Station, Robbins Farm, Geneva, New York, USA. It has been reared on a wheat germ-casein artificial diet ever since. In 1996 it was kindly provided by A. M. Shelton (Cornell University, Geneva, New York, USA) to the Max Planck Institute for Chemical Ecology (Jena, Germany), where it is reared on diet in a climate chamber at 21°C, 50% RH, and 16:8 L:D photoperiod. Adult moths from all three strains are fed with 5% honey solution.

Feeding treatments for transcriptome analyses

I) SSH

Two different types of mRNAs were generated for the SSH library enriched in host-plant specific genes: from DBM-P feeding on pea and DBM-Cj on cabbage. Larvae were raised on leaves of their respective host plant from egg to 3rd larval instar in 12×12×4 cm plastic boxes in a Snijders climatic chamber at 21°C, 50% RH, and 16:8 L:D photoperiod.

II) cDNA-AFLP and determination of GSS level

For the cDNA-AFLP method and determination of GSS level we set up a complex feeding trial to get a detailed picture on expression changes in DBM upon host plant feeding and upon host plant shifts. Larvae of the three DBM strains were reared on their usual food source until third larval instar (L3), i.e. G88-strain larvae on glucosinolate-free diet, DBM-Cj on kale and DBM-P on pea; additionally, one cohort of DBM-P larvae was reared on kale. Prior to the feeding assay larvae were starved for three hours. After starvation, larvae were either transferred back to their former food source or exposed to a new food source, for a period of 12 and 24 hours, respectively (Table 1). Specifically, diet-reared G88-strain larvae were transferred back to diet and to two novel food sources, kale or pea. DBM-Cj larvae reared on kale were transferred back to kale and additionally exposed to another crucifer, cabbage. DBM-P larvae reared on kale were transferred back to kale and additionally exposed to cabbage and pea. Pea-reared DBM-P larvae were transferred back to pea as well as exposed to kale. Each feeding trial was set up twice with 30 larvae so that larvae could be collected at two time points, 12 and 24 hours. Collected larvae were frozen in liquid nitrogen immediately. The transcriptional responses of the feeding treatments performed with DBM-Cj and G88 larvae

served as controls to unambiguously assign fragments to pea-feeding and to exclude those fragments which are generally expressed upon a diet change in an herbivore.

Table 1 Description of feeding treatments performed with three DBM strains. Three letter abbreviations for each treatment is composed of: DBM strain, rearing host plant or diet until third larval instar (L3) and feeding treatment for 12 and 24 hours.

Strain		**G88**		**DBM-C**		**DBM-P**			**DBM-P**	
Food source until L3		Diet		Kale		Kale			Pea	
Feeding treatment for 12/24h	Diet	Kale	Pea	Kale	Cab	Kale	Cabbage	Pea	Kale	Pea
Treatment abbreviation	G-D-D	G-D-K	G-D-P	C-K-K	C-K-C	P-K-K	P-K-C	P-K-P	P-P-K	P-P-P

Rearing of DBM larvae until third larval instar and 12 and 24 h feeding treatments were carried out in plastic boxes (12×12×4 cm) under the same conditions as the rearing procedure (see above). Instead of using whole plants, the larvae were fed with freshly cut leaves which were kept fresh in floral water picks filled with water.

Isolation of RNA

RNA for the SSH method was extracted from five male and five female larvae of different larval instars from each of the strains, DBM-P and DBM-Cj, respectively. Individuals were frozen in liquid Nitrogen and stored at -80 °C until isolation of RNA. Total RNA was isolated with the NucleoSpin RNA II kit (Macherey & Nagel, Düren, Germany) according to the manufacturers' instructions. Total RNA was further purified with RNeasy columns (Qiagen, Hilden, Germany). Poly(A)+ mRNA was isolated with the NucleoTrap mRNA kit (Macherey & Nagel) according to the manufacturers instructions. After precipitation, the poly(A)+ mRNA was dissolved in RNA Storage Solution (Ambion, Austin, TX), checked for integrity on an agarose gel and spectrophotometrically quantified using a Nanodrop ND1000 spectrophotometer (Thermo, Dreieich, Germany).

For the cDNA-AFLP analysis 15 to 23 L3 larvae from each treatment were pooled for subsequent RNA extraction to avoid the detection of differences in gene expression patterns due to effects of single DBM individuals. Total RNA from larval tissue was isolated using TRIzol Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturers' protocol. Residual DNA was removed by DNase treatment followed by purification using RNeasy Minelute Cleanup Kit (Qiagen, Hilden, Germany). RNA was dissolved in RNA Storage Solution (Ambion, Austin, USA) and checked for its integrity using a Nanodrop ND1000 spectrophotometer (Thermo, Dreieich, Germany), and an Agilent 2110 Bioanalyzer using RNA Nano chips (Agilent, Waldbronn, Germany). RNA from the two time points of each treatment was pooled in a 1:1 ratio for further cDNA synthesis. A total of 800 ng (400 ng from each time point) of

DNA-free total RNA was converted into cDNA. First strand synthesis was performed using the SMART PCR cDNA Synthesis Kit (Clontech, Laboratories Palo Alto, USA) using buffer and reverse transcriptase from TAKARA. For cDNA amplification the Advantage 2 PCR-Kit (Clontech, Laboratories Palo Alto, USA) was used following the instructions of the manufacturer. Resulting double stranded cDNA was purified using the QIAquick purification kit (Qiagen, Hilden, Germany). cDNA quality was checked on a 1.2% agarose gel and concentration determined on a Nanodrop ND1000 spectrophotometer (Thermo, Dreieich, Germany).

I) SSH-Method

Pea-fed and cabbage-fed larval RNAs were reverse transcribed into cDNA. Starting material was poly(A)+ RNA purified from the total RNAs. An oligo(dT)-linker primer was used for first strand synthesis. The resulting S0-cDNAs were then amplified with 18 (tester) and 17 (driver) cycles of LA-PCR (see Fig. 2). ds cDNAs of both, tester and driver, were then digested by restriction enzyme Rae I into narrow fragments.

For subtractive hybridization single stranded (ss) sense cDNA was prepared from the tester and ss-antisense cDNA from the driver. Subtraction was then carried out by hybridization of an excess of driver ss-cDNA with the tester ss-cDNA. Reassociated tester/driver ds-cDNAs were separated from the remaining ss-cDNAs (subtracted cDNA = S1-cDNA) by passing the mixture over a hydroxylapatite column. After hydroxylapatite chromatography, the S1-cDNA was amplified in 14 LA-PCR cycles.

The double stranded cDNA from DBM-P larvae was denatured at 98°C for 60 s and then hybridized at 68°C with an excess of double stranded cDNA from DBM-Cj larvae. The sample was then subjected to two rounds of suppression PCR with PCR-primer 1 and nested primers supplied with the kit. An initial extension at 72°C for 4 min was followed by a denaturation step at 95°C for 1 min and by 25 cycles of denaturation at 95°C for 10 s, annealing at 66°C for 20 s, and extension at 72°C for 90 s. A final 10 min 72°C step was added to allow complete extension of the products. The secondary PCR was performed with nested primer 1 and 2R on the diluted primary PCR products for 12 cycles under the same conditions, except that 68°C was used as annealing temperature.

For cloning, the S1-cDNA was subjected to a limited exonuclease treatment to generate 5’ overhangs at both ends of the cDNA. A cDNA fraction greater than 0.5 kb was obtained by size fractionation on a 1% (w/v) agarose gel. The size-fractionated cDNA was directionally ligated into the *Eco*RI and *Bam*HI sites of the plasmid vector pBS II sk+. The following adap-

ter sequences remain attached to the 5´- and 3'-ends of the cDNAs: 5´-end (*Eco*RI-site) 5'-GAATTCCTCTGGACCTTGGCTGTCACTCAGTT-3'; 3'-end (*Bam*HI-site) 5' GGATCCCCTTACGAGACATCGCCCCGC-(dT25)-3'. Ligations were transformed into T1 Phage resistant TransforMax EC100-T1R (Epicentre) or ELECTROMAX DH5α-E (Invitrogen) electro-competent *E. coli* cells. After transformation, glycerol was added to a final concentration of 15% (v/v). Cells were then frozen at -80° C in aliquots and after a freeze-thaw cycle the titer of the library was determined to be 365 cfu/µl, resulting in a total of 1,430,000 cfu. The percentage of non-recombinant clones in the libraries was determined by religation of the Eco RI / Bam HI digested pBS II sk+ vector in the absence of cDNA and was found to be less than 3%.

Ia) Gene-specific PCR analysis

To analyze the quality of the S0-cDNAs and the success of the subtraction process we performed gene-specific PCR using species-specific primer pairs EF1α and GSS that generate a 180 bp and a 700 bp PCR fragment respectively. The PCR-products obtained after 35 cycles from equal amounts of tester and driver ss-cDNA and the S1-cDNA were checked by agarose gel electrophoresis. The subtraction efficiency was confirmed by quantitative real-time PCR of the EF1 and GSS gene of subtracted PCR products in comparison to unsubtracted PCR products. PCR products were separated on 1% (w/v) agarose gel according to standard procedures.

Ib) Mass colony PCR

To get a comprehensive impression on the distribution of the insert sizes within the S1-library, about 1.000 colonies grown overnight on a Petri dish were suspended in water. With an aliquot of the bacterial suspension, PCR analysis was performed in the presence of the PCR primers used for cDNA amplification. The PCR products obtained after 26 cycles were analyzed on a 1.3% agarose gel. From the mass colony PCR profile, the average insert size in the library was estimated to be about 800 bp.

Ic) Minipreparation, Sequencing and Sequence Analysis

Plasmid minipreparation from bacterial colonies grown in 96 deep-well plates was performed using the 96 robot plasmid isolation kit (Eppendorf) on a Tecan Evo Freedom 150 robotic platform (Tecan). Single-pass sequencing of the 5' termini of cDNA libraries was carried out on an ABI 3730 xl automatic DNA sequencer (PE Applied Biosystems). Contaminants of

vector sequences and rRNA in the data sets were identified and removed. Vector and adaptor sequences were masked and clipped and a series of filtering steps was applied to identify and remove reads that did not contain any or only very short inserts. Vector clipping, quality trimming and sequence assembly was done with the Lasergene software package (DNAStar Inc.). Blast searches were conducted on a local server using the National Center for Biotechnology Information (NCBI) blastall program. Sequences were aligned using ClustalW software.

II) cDNA-AFLP method

For cDNA-AFLP reactions, 200 ng purified cDNA was digested with 5 U *Eco*RI and 3 U *Mse*I for 2 h at 37°C followed by enzyme inactivation for 15 min at 65°C. Accordingly, *Eco*RI and *Mse*I double strand adapters were ligated to the fragments by incubating for 2 h at 16°C. Pre-amplification of cDNA fragments was performed with a 1:10 dilution of the ligation reaction for 20 cycles with 94°C for 30 s, 56°C for 1 min, 72°C for 1 min) using primers corresponding to *Mse*I and *Eco*RI adapters (Table 2). Resulting PCR products were diluted 50× and were subjected to selective amplification with a combination of different *Mse*I and *Eco*RI primers using a touch-down amplification PCR-profile as described by Vos et al. (1995). *Eco*RI primers were labeled with a fluorescent dye (either IRdye 700 or IRdye 800, LI-COR, Germany). *Mse*I and *Eco*RI selective amplification primers had the same sequence as their corresponding pre-amplification primers plus two additional nucleotides (NN) at their 3`-termini. Sixty-four different primer combinations were used for selective amplification: 16 *Mse*I-NN primers (NN is either AA, AC, AG, AT, CA, CC, CG, CT, GA, GC, GG, GT, TA, TC, TG, TT) and 4 *Eco*RI-NN primers (NN is AC, AG, GT, CT). *Eco*RI-AG and *Eco*RI-GT primers were labeled with IRdye 700 and *Eco*RI-AC and *Eco*RI-CT were labeled with IRdye 800, respectively, enabling multiplex PCR as recommended by LI-COR. All amplification reactions were performed in an Eppendorf thermocycler (Eppendorf, Hamburg, Germany). Following amplification, the samples were denatured by adding 10 µl of ddH2O and 15 µ of stop solution (LI-COR, Germany) and heating for 5 min at 94°C. 0.8 µl of the reaction mix were loaded on a 6.5% denaturating polyacrylamide gel (KB Plus Gel Matrix, LI-COR, Germany) and electrophoresed at 1500 V for 2,5 h on a LI-COR DNA Analyzer 4300 (LI-COR, Germany). A digital image of the resulting gel image was collected using the Odyssey scanner ((LI-COR, Germany) and bands were visually scored for presence and absence in the different feeding treatments.

Bands differentially expressed among the 10 different feeding treatments were chosen for further analysis as they might represent candidates for genes involved in host plant adaptation. Scanning of the gels and band excision followed the instructions in the LI-COR manual (LI-COR, Germany). cDNA was eluted from the excised gel piece in 80 µl TE buffer first by incubation over night at 4°C, then at 60°C for 3 h and finally by performing three freeze-thaw cycles and spinning down the left-over acrylamide. To reamplify the differentially expressed fragments, 5 µl of the eluted cDNA were amplified in a total reaction volume of 20 µl with 1 pmol of the respective primers from the selective amplification. PCR parameters were 35 cycles of 94°C for 30 s, 56°C for 1 min and 72°C for 1 min with a final extension step of 2 min. The amplified fragments were analyzed by agarose gel electrophoresis. When reamplification was successful, 30 ng of the PCR product were treated with ExoSAP to remove primers and nucleotides. In cases where agarose gel electrophoresis showed only a faint band of the PCR product a re-reamplification was performed from 5 µl of the first reamplification. Reamplified cDNA-AFLP fragments were directly sequenced on an automated sequencer (Applied Biosystems 3730/XL/96 capillary DNA analyzer, PE Applied Biosystems, USA). Obtained sequences of cDNA-AFLP fragments were compared to GenBank entries using tblastx program and *P. xylostella* cDNA libraries.

IIa) Quantitative real time PCR

To verify the differential expression patterns observed in cDNA-AFLP analysis, selected TDFs were chosen for analyzing their expression pattern by an independent method, qRT-PCR. RNA from those feeding treatments in which certain genes were apparently upregulated was transcribed into single-stranded cDNA using the Thermo Verso cDNA-Kit (Thermo). Gene-specific primers were designed based on the sequence from the selected fragments as well as for the housekeeping genes EIF1 (elongation initiation factor 1) and RPS (ribosomal protein), serving as control genes. qRT-PCR was also carried out to compare GSS expression in larvae exposed to the following feeding treatments: G-D-K, G-D-P, C-K-K, P-K-K and P-P-P (see Table 1). In this case only EIF1 served as control gene because of the space limitation on the 96-well plate. qRT-PCR was done in optical 96 well plates on a MX300P Real-time PCR detection system (Stratagene, Amsterdam, The Netherlands) using the Absolute QPCR SYBR Green Mix (ABgene) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye included in the PCR mix. A dissociation curve analysis was performed for all primer/probe pairs.

6.3 Results

Feeding assay

In a feeding assay the newly evolved pea-adapted host strain (DBM-P), an original cabbage-adapted host strain (DBM-Cj) and G88, a DBM strain adapted to and reared on a glucosinolate-free diet, were assessed for their ability to survive on cabbage and pea (Figure 1). Although feeding on pea was initiated among some of the neonate DBM-Cj and G88 larvae no survival on pea was recorded for these strains. DBM-Cj and G88 had a survival rate of 62.0% and 56.0% on cabbage, respectively. DBM-P survived equally well on both host plants (60% and 70%, respectively; Figure 1).

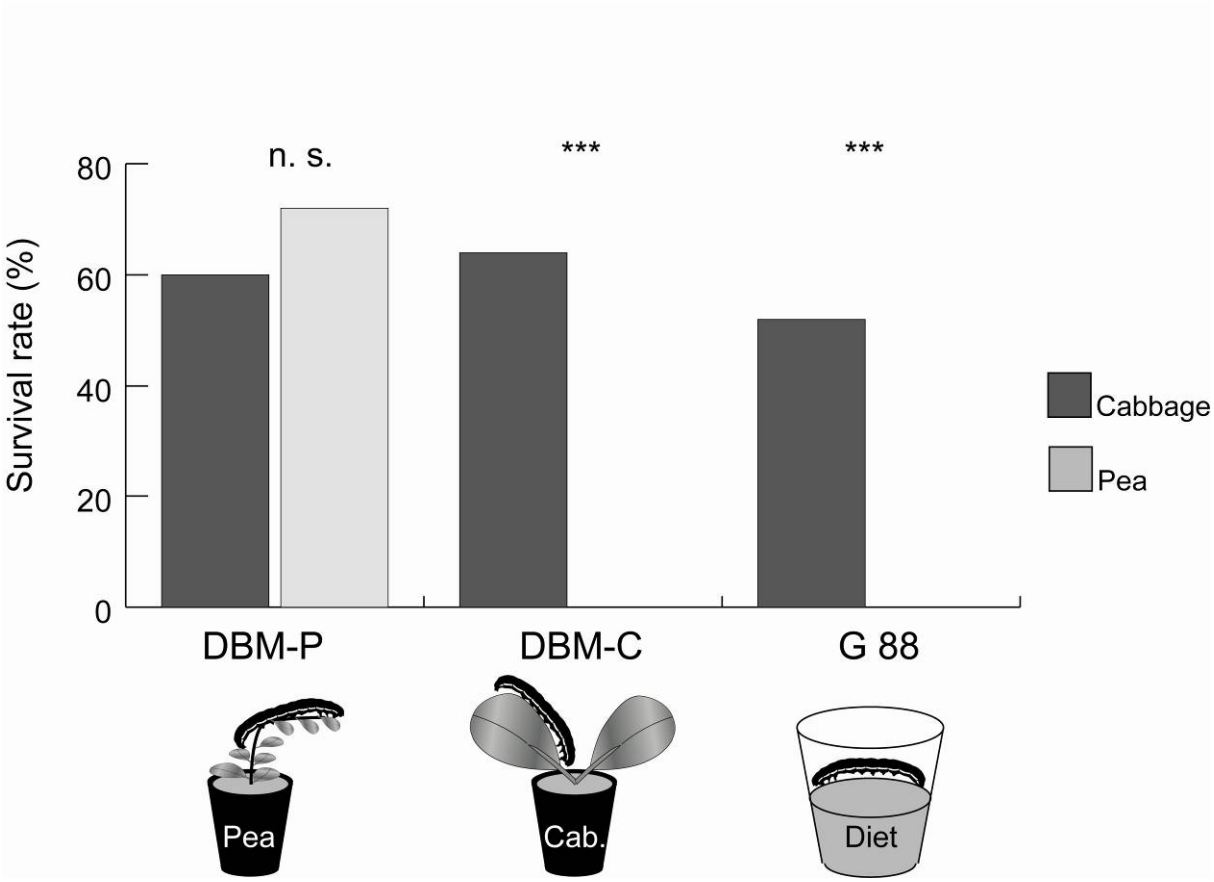
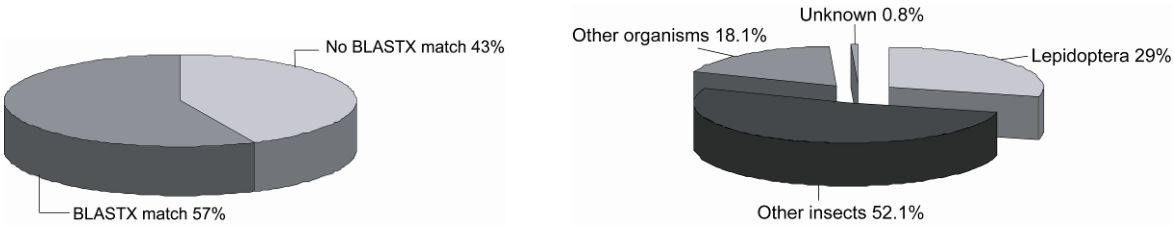


Figure 1 Feeding assay to characterize larval performance on cabbage and pea of the three DBM strains used in transcriptome profiling approaches. DBM larvae are depicted on the food source they are reared on (pea, cabbage or artificial diet).

SSH

Of the 2,800 sequenced colonies, 43% did not return any significant ($E < 0.1$) blastx match (Figure 3a). Of the remaining sequences, > 80% had best matches to insect sequences, specifically 29% to Lepidoptera and 52.1% to other insects (Figure 3b).



(a) **(b)**

Figure 3 (a) Proportion of SSH sequences with and without blastx matches (E < 0.1) in GenBank. (b) Percentages of sequences with matches from blastx classified by organism.

Those sequences returning a significant blastx match were ascribed a putative biological process (Figure 4) and molecular function (Figure 5) using Blast2GO (Götz et al. 2008). Supplementary Tables 2 and 3 summarize the biological processes and molecular functions respectively. In the biological process analysis, nearly 50% of sequences predicted to encode proteins involved in either cellular processes (773) or metabolism (697) (Figure 4). Correspondingly, in the molecular function analysis, the majority of the sequences predicted proteins with either binding functions (722) or catalytic activity (564) (Figure 5).

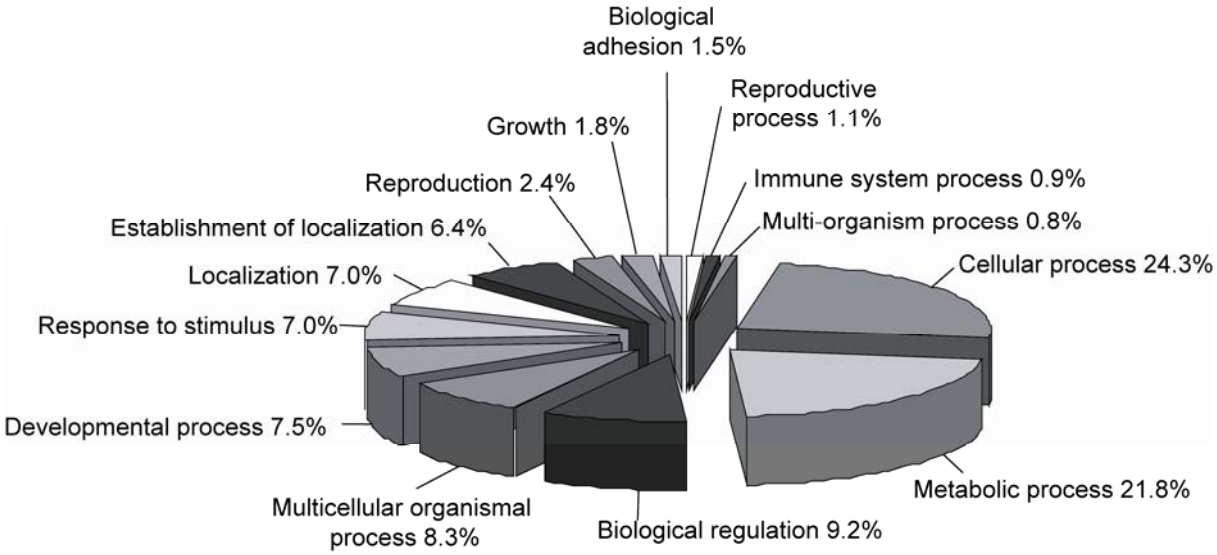


Figure 4 Percentage analysis of the biological processes ascribed to the 2,081 contigs by Gene Ontology and Blast2G0. Only those processes that were ascribed > 30 genes are shown. Genes may be assigned to more than one term.

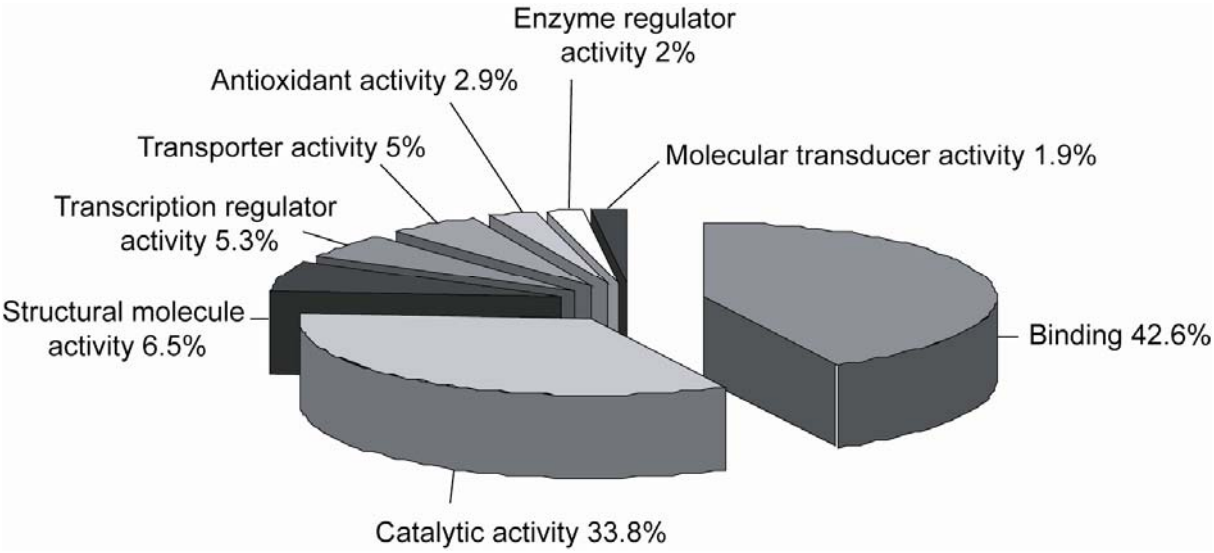


Figure 5 Percentage analysis of the molecular functions ascribed to the 2,081 contigs by Gene Ontology and Blast2G0. Only those functions that were ascribed > 30 genes are shown. Genes may be assigned to more than one term.

cDNA-AFLP

The expression patterns of approximately 11,000 transcripts were monitored with 64 different primer combinations for selective amplification out of which 87% were differentially expressed and 13% monomorphic. For each primer combination, approximately 170 fragments were visualized as bands, varying in size from 50 to 650 bp. The average number of upregulated and downregulated fragments obtained for each treatment ranged from 14.4 to17.0 and 9.1 to 10.0, respectively. A typical picture of a cDNA-AFLP gel image is shown in Figure 6. The numbers of strain and treatment specific transcripts are shown in Table 2.

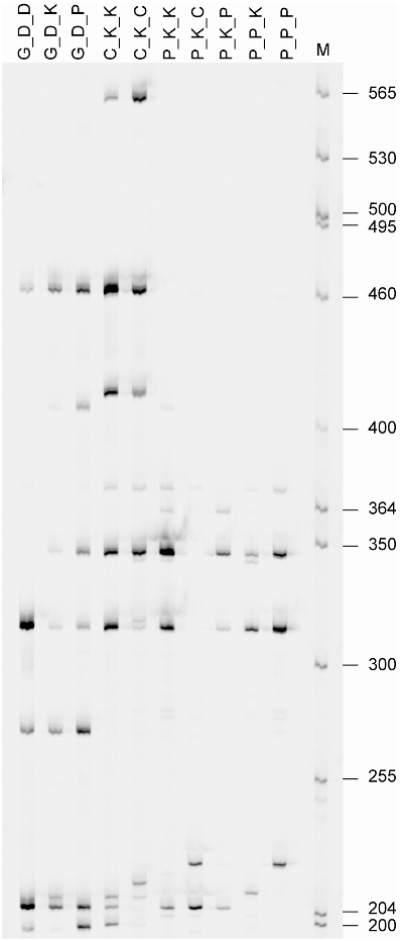


Figure 6 Example of a typical cDNA-AFLP gel image. For abbreviations see Table 1. M = marker.

Table 2 Number of strain and treatment specific bands. For abbreviations see Table 1.

Strain/feeding treatment	Number of unique bands	Number of strain specific bands
G-D-D	19	
G-D-K	20	103
G-D-P	17	
C-K-K	17	44
C-K-C	12	
P-K-K	21	
P-K-C	17	
P-K-P	15	107
P-P-K	15	
P-P-P	17	

All bands were scored visually and assigned as upregulated/overexpressed (band present) or downregulated/underexpressed (band absent). Comparisons were made by comparing one treatment to any other treatment on the respective cDNA-AFLP gel images, i.e. the presence of a band in treatment A and absence in any other treatment, e.g. B, was recorded as overexpression in the former and underexpression in the latter. A numerical overview of the differences between the feeding treatments showing the total number of differentially-expressed genes in each treatment is provided in Figure 7.

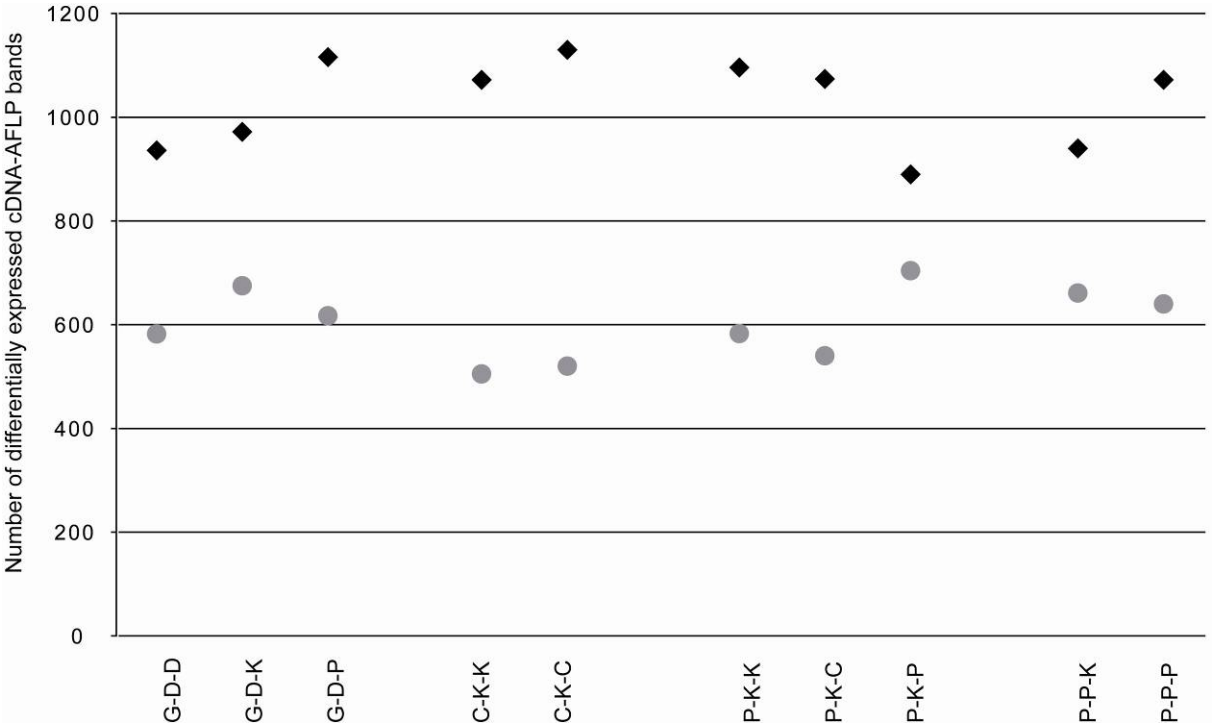


Figure 7 Total number of upregulated (black squares) and downregulated (grey circles) transcripts in each treatment. For abbreviations of treatments see Table 1.

A total of 124 TDFs with differential expression profiles in comparison to another feeding treatment, with a focus on those expressed in pea-feeding DBM larvae were excised from the gels, eluted, and re-amplified with the appropriate cDNA-AFLP primers. For 30 of these fragments, successful isolation from the gels and/or reamplification of the cDNA was not possible, which is not uncommon (Yang et al. 2005). Direct sequencing of the remaining 90 cDNA fragments yielded products that could be used to screen public databases for homologous sequences. The majority of these fragments gave no significant similarity to known sequences. Half of these unmatched sequences were either retrieved in the SSH library (3 sequences), a *P. xylostella* EST database (18), or in both (24) (Figure 8), indicating that these transcripts were in fact derived from *P. xylostella* RNA currently lacking functional annotations and are not artifacts. The remaining unidentified transcripts may be exclusively associated with DBM's feeding response to its different hosts and could represent novel genes. The hits for the cDNA-AFLP fragments resulting from matches to sequences derived from the *P. xylostella* EST database or SSH library are presented in Table 3.

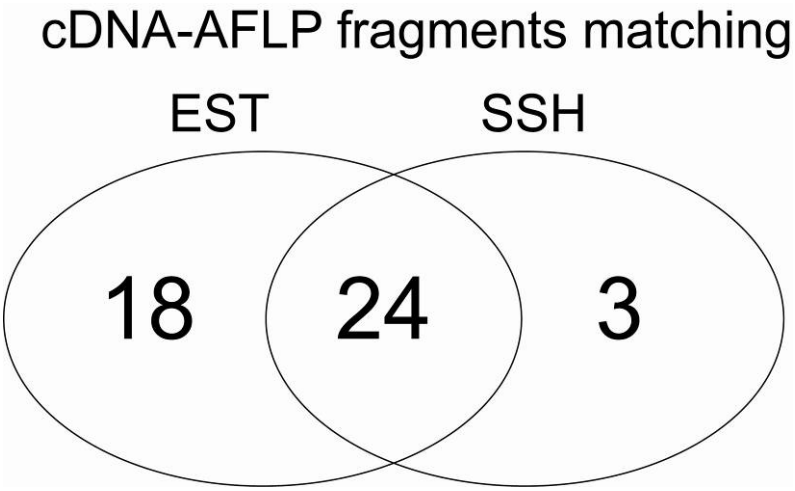


Figure 8 Number of cDNA-AFLP fragments with a match in a *Plutella xylostella* EST database (EST) and/or SSH library (SSH).

Table 3 cDNA-AFLP fragments returning a significant blast hit. Expression patterns are shown for each fragment; with 0 = band absent and 1 = band present. Sequences were derived from grey highlighted treatments.

cDNA-AFLP Contig No.	Banding pattern										Hit against Px complete ESTs	Hit against Px sub lib	Best blast hit matching Px contig	E-value
	G-D-D	G-D-K	G-D-P	C-K-K	C-K-C	P-K-K	P-K-C	P-K-P	P-P-K	P-P-P				
c-AFLP2	0	0	1	1	1	1	0	0	0	1	yes	yes	receptor expression enhancing protein - isoform2	1,00E-48
c-AFLP10	0	0	0	0	0	0	0	0	0	1	yes	yes	DNA polymerase epsilon subunit 2	1,00E-45
c-AFLP15	0	0	0	1	0	1	0	0	0	0	yes	yes	muscle assembly protein	1,00E-30
c-AFLP17	0	0	0	0	0	1	1	0	0	0	yes	no	kinase anchor protein	1,00E-13
c-AFLP18	1	1	1	1	1	1	0	1	1	1	yes	no	ubiquitin family member isoform 1	1,00E-33
c-AFLP20	0	1	0	0	0	1	0	0	1	1	yes	yes	sucrose-6-phosphate hydrolase	1,00E-06
c-AFLP21	0	0	0	0	0	0	1	1	0	1	yes	yes	calponin transgelin	1,00E-08
c-AFLP22	0	1	1	0	0	1	1	0	1	1	yes	yes	aminoadipate-semialdehyde dehydrogenase	1,00E-05
c-AFLP29	0	0	0	0	0	1	1	0	0	1	no	yes	Nadh dehydrogenase subunit 4	0,13
c-AFLP37	1	1	0	0	0	1	1	0	1	1	yes	no	Cytochrome C oxidase subunit III	0,1
c-AFLP39	1	0	0	0	0	0	0	0	0	0	yes	no	ecdysteroid-regulated protein	1,00E-70
c-AFLP40	0	1	0	0	0	1	0	0	1	1	yes	yes	sucrose-6-phosphate hydrolase	1,00E-04
c-AFLP50	0	0	0	0	0	0	0	0	0	1	yes	yes	phosphoserine aminotransferase	1,00E-09
c-AFLP53	0	0	0	0	0	1	1	1	1	1	yes	yes	Chromodomain helicase-DNA-binding protein	1,00E-05
c-AFLP60	0	0	0	0	0	0	0	0	0	1	yes	yes	short chain dehydrogenase/reductase	1,00E-45
c-AFLP75	0	0	0	0	0	0	0	1	1	1	yes	yes	glucosyl-glucuronosyl transferase	1,00E-20
c-AFLP80	0	0	0	1	1	0	0	0	0	0	yes	no	phd finger 5a transcription factor	1,00E-51
c-AFLP83	0	1	0	0	0	1	1	1	1	1	yes	yes	hydroxyacylglutathione hydrolase	1,00E-53
c-AFLP86	0	1	0	0	0	1	1	1	1	1	yes	yes	ubiquitin-conjugating enzyme	1,00E-74
c-AFLP87	0	0	0	1	1	0	0	0	0	0	yes	no	CCAAT-ovary c ebpg transcription factor	1,00E-26
c-AFLP89	0	0	0	0	0	1	0	1	0	1	yes	yes	ubiquitin-conjugating enzyme 2	1,00E-58
c-AFLP90	1	0	0	0	0	0	0	1	0	0	yes	no	ubiquitin-conjugating enzyme 2	1,00E-58
c-AFLP91	0	0	0	0	0	1	0	1	0	1	yes	yes	ubiquitin-conjugating enzyme 2	1,00E-58
c-AFLP130	1	1	1	0	0	1	0	1	0	0	yes	no	profilin	1,00E-04

GSS-level

The mRNA level of the glucosinolate sulfatase (GSS) was determined in a subset of RNA-types of the ten feeding treatments: G88 reared on diet and shifted to kale (G-D-K), G88 reared on diet and shifted to pea (G-D-P), DBM-Cj continuously reared on kale, DBM-P reared on kale and shifted to pea (P-K-P) and DBM-P continuously reared on pea. Changes in GSS level after 12 and 24 h of feeding on the respective food source were compared. As expected, in those larvae that were reared on a crucifer and then shifted to a non-cruciferous plant or diet the GSS-level decreased in the time range of 24 h whereas the GSS-level increased when feeding on a crucifer, i.e. glucosinolate-containing food source, or when being shifted from a non-cruciferous plant/diet to a crucifer (Figure 2).

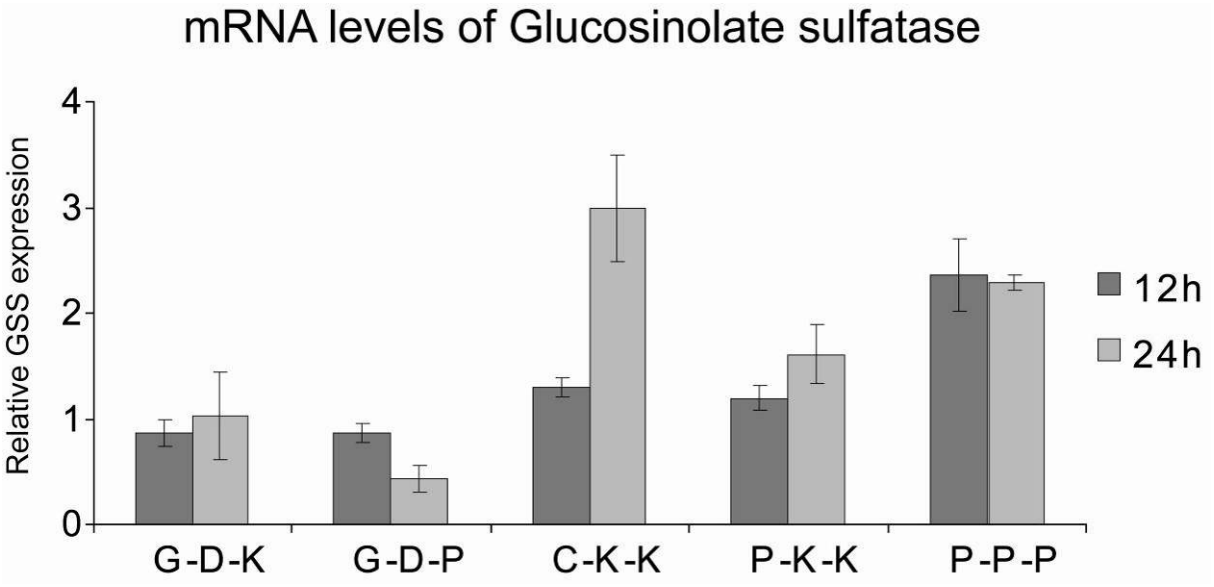


Figure 2 Quantification of glucosinolate sulfatase (GSS) level in the three DBM strains after 12 and 24 hours of feeding on kale or pea using qRT-PCR. For abbreviations see Table 1.

6.4 Discussion

Despite extensive genomic and functional studies on plant responses to herbivory few researchers have investigated transcriptional changes in non-model herbivores upon feeding on their host plant(s) and to our knowledge no one has studied gene expression changes accompanying host shifts or range expansions. The present study provides the first large-scale investigation of gene expression changes upon host plant range expansion and the identification of candidate genes in DBM larvae feeding on sugar pea. We were able to successfully characterize the overall transcriptional response as well as to identify putative candidate genes enabling adaptation to pea.

Feeding assay

A feeding assay preceded the molecular analyses in this study in order to characterize larval performance on original and novel host plant, cabbage and pea, respectively. This information helped in the design of the feeding treatments for the cDNA-AFLP approach. As expected the DBM-P strain was the only strain that was able to complete development on both hosts while the other two strains, G88 and DBM-Cj, could only survive on cabbage. G88 had the lowest survival rate on cabbage which might be due to the rearing regime. The DBM G88 strain has been raised for more than 100 generations on a glucosinolate-free diet, so that glucosinolates and any other crucifer-specific compounds might pose a challenge. Although none of the 25 G88 larvae were able to survive on cabbage, a few larvae initiated feeding on pea. Putatively, larvae from this strain do not rely as much on glucosinolates or other crucifer-derived compounds as feeding stimulant, as do larvae from DBM-Cj. With this information we decided to perform a shift of L3 G88 larvae from diet to pea and study the transcriptional response in the cDNA-AFLP approach.

SSH vs. cDNA-AFLP approach

Each of the two approaches primarily had the aim to characterize DBM's transcriptional response upon pea-feeding and to identify genes that contributed to the host range expansion to pea plants. This now offers the possibility to compare the two techniques with regard to their usefulness in reaching this aim. Both techniques have previously been applied separately in insects to study transcriptional changes and to identify candidate genes associated with a specific treatment or diet. Zhu et al. (2003) used SSH to identify genes up-regulated in response to bacterial infection in the tobacco hornworm *Manduca sexta* and obtained more than 230 differentially expressed genes, half of which were identified as immune-response genes. To detect changes in gene expression between two populations of the brown planthopper *Nilaparvata lugens* feeding on resistant and susceptible rice strains the cDNA-AFLP technique was applied and revealed that out of 61 sequenced differentially expressed fragments thirteen had sequence similarities to known genes, with functions including detoxification, stress response, and signaling (Yang et al. 2005). In our study, the SSH approach likewise yielded a large number of candidate genes (> 2,000 transcripts).

However, from the number of candidate genes obtained we were not able to determine the extent to what the transcriptome was altered upon pea-feeding, and how the transcriptome is altered when DBM is shifted to a similar host plant. One further disadvantage of the SSH is that expression levels must be very great for a fragment to be identified (Diatchenko et al.

1996). These drawbacks could be compensated for by employing a second transcriptional profiling technique, the cDNA-AFLP method. This technique allows for comparison of more than two RNA types and is based on visualizing expression changes on a gel. Thus, we were able to compare different feeding treatments and shifts between similar and dissimilar host plants. In addition, this approach revealed that the transcriptomes of DBM-P larvae feeding on pea were altered in a similar way as transcriptomes of DBM larvae from other strains after being shifted to a different host. In contrast to the SSH approach, most of the cDNA-AFLP derived fragments did not correspond to known sequences in the available databases. This could be due to the fact that these sequences correspond to 3' untranslated regions of genes where the sequences are often less conserved than the sequences of protein-coding regions. For half of the cDNA-AFLP fragments, these could be matched to a larger contig from a *P. xylostella* EST database or from the SSH library and assigned a putative function. Thus, the two techniques complemented each other and helped in gaining a comprehensive picture on the transcriptional response in DBM upon pea feeding and to identify the genes that might govern the novel adaptation to pea.

General transcriptome response in DBM larvae

A general understanding of the changes in the transcriptome upon feeding in DBM is necessary to understand and interpret the transcriptional response in the pea-adapted DBM strain. The high number of altered cDNAs in the SSH approach was indicative of a complex transcriptional response to pea-feeding, whereby many different biological processes are involved and a large part of the larval metabolism needs to adapt to the novel host plant. This result was supported by numerical characterization of the cDNA-AFLP banding pattern. This revealed that responses were similarly altered in all treatments, with numbers of upregulated (1,000) and downregulated (600) fragments in the same magnitude, apart from minor deviations. A similar pattern was observed for host-shifted and not-shifted larvae. In general, larvae that did experience a diet or host plant change had a higher number of upregulated fragments than larvae that continued feeding on the rearing host plant or diet. There was only one exception in kale-reared DBM-P strain larvae, which after being transferred to pea (P-K-P) showed a decrease in upregulated fragments. Thus, a change in food source likewise evokes a change on the transcriptome level, irrespective whether the shift is to a related (e.g. other crucifer) or unrelated (e.g. sugar pea) host plant. This is not surprising as different host plants likely require specific adaptive mechanisms, such as different sets of digestive and detoxification enzymes. It is therefore assumed that upon a shift to another host plant these enzymes have to be

elicited, resulting in an increase of upregulated fragments to meet the biochemically challenges posed by this novel host. Feeding on a different host plant might also elicit stress, as the SSH approach revealed a high number of transcripts related to stress response (see below), demonstrated by an increase in differentially expressed cDNAs. A similar increase in upregulated cDNAs across strains and treatments indicate that pea-feeding and a shift to pea do not elicit more genes than feeding on original host plant or shifts to related host plants. A similar result was obtained by Heidel-Fischer et al. (2009), who found that more genes were concurrently upregulated on plants that shared either ancestry or growth form in larvae of the comma butterfly *Polygonia c-album*.

For the number of downregulated fragments there was not such an obvious pattern in increase or decrease of fragments according to changes in host plant or diet. Interestingly, the highest increase in downregulated fragments was observed in the kale-reared DBM-P strain when shifted to pea. This treatment was the one exception where a decrease in upregulated fragments was observed after host shift.

However, although the transcriptome response might be similar in number of up- and downregulated fragments, the underlying genes and their function are likely to differ between the treatments. A detailed analysis on the putative functions of fragments involved in pea-feeding was provided by sequencing cDNA-AFLP- and SSH-derived fragments will be discussed below.

Candidate genes

The major aim of the study was to unravel the molecular mechanisms that underlie and promote adaptation to pea in DBM-P larvae. According to the known processes that mediate larval adaptation, i.e. finding and accepting as well as successful feeding and development on the host plant, and the phytochemical differences between novel and original host plant, we expected to find genes that underlie the above mentioned processes and those that help in dealing with the challenges posed by the novel host (Figure 9). In the following fragments derived from either of the two approaches are discussed with regard to their putative role in larval adaptation to pea.

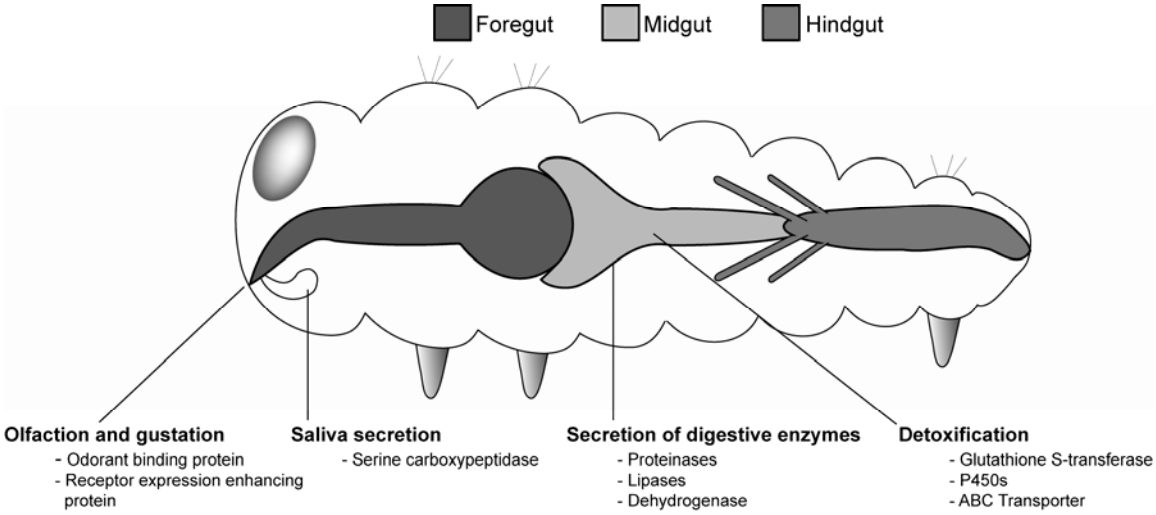


Figure 9 Identified transcripts and their putative location of expression in the larva.

Olfaction

In both approaches we identified fragments corresponding to genes with a role in olfaction and gustation. Olfactory and gustatory systems play crucial roles for insects in finding and accepting food. The adaptation to peas might therefore be governed by the olfaction and gustatory reception and acceptation of its new host plant. Fragment c-AFLP2 (Table 3 and Figure 9) resembled a receptor expression enhancing protein (REEP), and was sequenced from a fragment that was expressed in DBM-P larvae continuously reared on pea. REEPs have been mainly studied in mammals where they have been shown to promote functional cell surface expression of odorant receptors (Saito et al. 2004). In situ hybridization of mouse olfactory epithelium revealed that Reep1 expression in mouse was associated with odorant receptors and enhanced the odorant receptor responses to odorants (Saito et al. 2004). Studies with REEPs in insects are lacking and we can only speculate that REEP might have a similar function in insects and positively enhance the olfactory response of DBM-P larvae to pea.

With the SSH technique we obtained two fragments corresponding to members of the Chemosensory protein (CSP) gene family (Chemosensory proteins 4 and 8) and one that belongs to the class of general odorant binding proteins (GOBPs; Q8WRW8_MANSE Antennal binding protein 4 - *Manduca sexta*). CSPs are a family of small soluble proteins associated with insect sensory organs, including the sensillum lymph. A role in odorant transport, similar to insect odorant binding proteins (OBPs), has been proposed. Odorant binding proteins (OBPs) are the first biochemical step in odor perception (Vogt et al. 1999); they act as carriers of hydrophobic odor molecules by transporting them through the aqueous lymph to transmembrane odor receptors located on the dendritic membrane of olfactory neurons (Krieger and Breer 1999). OBPs may have multiple ways to modulate the olfactory response: (1) selectively bind certain

odorants, (2) eliminate irrelevant or toxic odors, (3) specifically present odorants to olfactory receptor proteins, thereby controlling the range of stimuli to which olfactory receptors are exposed, and (4) selectively inactivate odors (Steinbrecht 1998). Thus, the first step towards pea-adaptation might be mediated by one or several of these mechanisms, e.g. DBM-P larvae have an expanded odor range including the positive response to pea odor. By contrast, DBM-Cj strain larvae might not be able to recognize pea plants as a potential food source or be repelled by them and thus not initiate feeding. A mutation in OBPs was found to be responsible for *Drosophila sechellia*'s attraction to fruits of *Morinda* which repels all other *Drosophila* species (Matsuo et al. 2007).

Metabolism

A majority of the identified transcripts are involved in metabolic processes and catalytic activity, respectively (Figure 4 and 5; supplement Table 2 and 3). In detail these transcripts code for proteins that act in degradation and modification processes such as digestion and detoxification and will be discussed in the following.

Digestion

To ensure complete degradation of nutritional compounds and the presence of diverse nutrient compounds, different to the spectrum found in crucifers, it is reasonable to suspect that a variety of digestive enzymes is required and has to act in concert. Induction and differential expression of several enzymes responsible for degradation of different plant compounds and nutrients were identified.

In chewing insects, such as herbivore larvae, solid food is ground externally in the preoral cavity, where biochemical degradation can already be started (Terra and Ferreira 2005). Saliva is secreted from the mandibular or labial salivary glands and used to solubilize and transport food (Terra and Ferreira 2005). We identified several salivary enzymes (Q6TRX9_CULQU Putative GQP-rich salivary protein - *Culex quinquefasciatus*; Q49B95_9DIPT Salivary/fat body serine carboxypeptidase - *Sitodiplosis mosellana*), one of which, a serine carboxypeptidase, was found to play a role in the extra-cellular digestion of proteins in *Sitodiplosis mosellana* larvae feeding on wheat kernels (Mittapalli et al. 2006). Sugar peas belong to the legumes, a family known for nitrogen fixation and therefore rich in protein content. Possibly, DBM-P larvae deal with the legume specific protein content by initiating protein degradation already outside the gut in the preoral cavity.

The cDNA-AFLP analysis revealed a fragment with similarity to sucrose-6-phosphate hydrolase (cAFLP_20, Table 3). Sucrose-6-phosphate hydrolase is the product of the scrB gene required in sucrose fermentation. It has been intensively studied in the sugar-dependent caries bacterium *Streptococcus mutans* (Hayakawa et al. 1986) and was found to be induced by the presence of sucrose. The high sugar content of sugar pea pods is already documented in the name of the plant and sugar pea leaves contain sugar in higher amounts than crucifer leaves. When insects feed on sucrose-containing diets, it is generally assumed that this sugar is hydrolyzed. Sucrose-6-phosphate hydrolase is therefore likely to play a role in digestion of sucrose-containing sugar pea-leaves and an upregulation of this enzyme might be an adaptive mechanism in sugar pea-feeding DBM larvae. Sucrose-6-phosphate hydrolase has been found to be upregulated in pea- as well as kale-feeding DBM larvae, the sequences however, was obtained from pea-feeding larvae. This enzyme has previously been identified in larvae of Asian longhorned beetles, *Anoplophora glabripennis*, where it is supposed to be involved in lignocellulose degradation (Geib et al. 2010). Other sucrose hydrolases have been detected in the larval midgut of the sugar cane stalk borer *Diatraea. saccharalis* when feeding on its sugar-rich host plant sugar cane (Carneiro et al. 2004).

A cDNA fragment which was exclusively expressed in DBM-P larvae reared on peas showed similarity to a *Bombyx mori* short-chain dehydrogenase/reductase. Its unique occurrence in pea-feeding DBM-P larvae emphasizes the putative importance of this gene for DBM's host range expansion to peas. Short-chain dehydrogenases/reductases (SDRs) were also identified in the SSH approach and are one-domain NAD(P)(H)-dependent enzymes of typically 250 amino acid residues (Joernvall et al. 1995). SDR family members are enzymes of great functional diversity and catalyze a wide range of chemical reactions including oxidation, reduction, epimerisation, dehydration and decarboxylation (Persson et al. 2003).

We expected to find transcripts that help in dealing with the novel phytochemical environment posed by the novel host plant pea. Among the SSH-derived transcripts we found several insect proteinases. Lepidopteran insects have proteinases as major components of their digestive complement to catalyze the release of peptides and amino acids from dietary proteins are found most abundantly in the midgut region (Jongsma and Bolter 1997; Terra and Ferreira 2005). Upon herbivore attack, plants (especially legumes) produce proteins that inhibit insect gut proteinases. These proteinase inhibitors (PIs) enter in the insect digestive tract along with the food and then block the protein digestion, leading to the starvation of the insect for amino acids and resulting in retardation of growth and development (Ryan 1990). In pea-feeding DBM-P larvae we detected a variety of proteinases, possibly as a mechanism to circumvent

the fatal action of the PIs: (A) production of numerous proteinase isoforms varying in their sensitivity to a specific PI or (B) the overexpression of proteinases compensates for the inhibited proteinases. These might be likely mechanisms of DBM-P larvae to cope with PI-containing pea-leaf material and thus, only DBM larvae capable of adapting to these (novel) plant defenses have a chance to survive and to emerge as a pest on the novel host plant.

Detoxification

Overall, the data suggest that several detoxification pathways are utilized when DBM-P larvae are exposed to feeding on pea plants. One possible strategy that DBM-P larva could employ is to have a general elevated detoxification response when exposed to a novel chemical environment as posed by the new host plant pea. Detoxification has classically been divided into three phases and representatives from each of the three phases were among cDNA-AFLP and/or SSH derived fragments.

In phase I of the detoxification process (harmful) endogenous compounds are metabolized (by e.g. oxidation, hydrolysis and reduction) for further deactivation and excretion processes. A range of fragments coding for genes involved in these processes were detected with the SSH technique and can be found under the GO category “catalytic activity” and metabolic process (Figure 4 and 5; supplement Table 2 and 3). Cytochrome P450s are the most prevalent representatives of phase I detoxification enzymes. The Lepidopteran insect genome probably carries about a hundred P450 genes and their induction or constitutive overexpression ensures detoxification and leads to tolerance of toxic secondary plant compounds or insecticides (Feyereisen 1999). Several P450s (homologues to CYP4M6 from the corn earworm *Heliothis zea*, CYP305B1 from *B. mori*, CYP9G2 from *P. xylostella* and CYP4M14v1 from the common cutworm *Spodoptera litura*) were obtained from the SSH approach. In DBM overexpression of P450s is suggested to have an implication in permethrin resistance (Bautista et al. 2007). The role of P450s in host plant adaptation has been studied in detail in two lepidopteran species, the black swallowtail *Papilio polyxenes* and the parsnip webworm *Depressaria pastinacella*. Larvae of these species are able to detoxify toxic furanocoumarin found in their host plants by midgut P450 activities (Berenbaum et al. 1996). DBM-P larvae might deal with xenobiotic metabolites from pea leaves in a similar way, i.e. by detoxifying them with P450s.

Phase I derived metabolites can be excreted if polar enough but usually are further converted by phase II reactions, in which they are conjugated with a variety of endogenous compounds. Typical phase II enzymes are glutathione s-transferases (GSTs) and UDP-glucosyltransferases (UGTs). In the SSH library we found transcripts with homology to members of the GST gene

family: GST omega 1 from the wild silk moth *Bombyx mandarina*, GST 1 and GST omega 2 from the silk moth *Bombyx mori*, as well as with homology to *P. xylostella* GST isozyme 3. GSTs have an oxidoreductase activity and are involved in the detoxification of a wide array of compounds and generally function on hydrophobic organic compounds (Atkins et al. 1993). In DBM (Kao and Sun 1991; Chiang and Sung, 1993) and other insects, GSTs have been implicated in degradation of and resistance to insecticides (Reidy et al. 1990; Clark et al. 1986; Kostaropoulos et al. 2001) and might be of general importance to herbivores that have to deal with toxic compounds. Fragment cAFLP_75 matched to an UGT, an enzyme participating in detoxification processes (Huang et al. 2008). This fragment was only expressed in DBM-P larvae and the sequence obtained from DBM-P larvae continuously reared on pea (P-P-P).

Conjugated forms of xenobiotics, derived from step I and II of the detoxification pathway, can then be recognized by specific membrane-associated transporters. A range of transcripts with similarity to active transmembrane transporters were found in the GO category "transporter activity" (supplement Table 3). Several transcripts with similarity to ATP-binding cassette (ABC) transporters representing the third step in the detoxification pathway were among SSH derived fragment hits. ABC transporters are membrane bound efflux transport proteins that effectively lower intracellular concentrations of toxins or xenobiotics in general (Jones and George 2004). Only very recently has the ABC transporter family been recognized for its importance in mediating dietary selection in herbivores. ABC transporters may be as critical as detoxification enzymes and play a central role as herbivore counter-mechanism to plant chemical defense (Sorensen and Dearing 2006). Gaertner et al. (1998) showed that the active excretion of nicotine and other alkaloids in the tobacco hornworm is mediated by a membrane protein of the ABC transporter family and allows *M. sexta* to adapt to nicotine-containing plants toxic to most other insects. In DBM-P larvae, the active excretion of potentially toxic compounds found in pea leaves could have promoted the adaptation to this new host plant.

With the cDNA-AFLP method we identified three ubiquitin-related genes (ubiquitin family member isoform 1, c-AFLP18; ubiquitin conjugating enzyme, c-AFLP86; ubiquitin conjugating enzyme 2, c-AFLP89-91; Table 3). Ubiquitination, which is associated with proteasome-mediated protein degradation, and alters protein localization, activity and interactions in a proteasome-independent way, is involved in miscellaneous biological processes such as targeting intracellular polypeptides for degradation (Peters et al. 1998). Ubiquitin has already been found to play an important role in DBM's resistance to the pyrethroid Deltamethrin (Luogen et al. 2009). Taking into account the known insecticidal action of pea seeds, mainly caused by lectins and proteinase inhibitors, it follows that pea-feeding DBM larvae should

rapidly degrade and/or excrete these toxic proteins, which in turn might explain expression of genes associated with the ubiquitin pathway.

A hydroxyglutathione hydrolase (c-AFLP83, Table 3) was identified among cDNA-AFLP fragments, an enzyme acting in the glyoxylase system. The glyoxylase system is still not completely defined, but it is evident that it represents a detoxification mechanism (Thornalley 1990). Like the ubiquitin-related genes this fragment was upregulated only in DBM-P strain larvae. An increased machinery of detoxification enzymes might be an adaptive mechanism that allows pea-feeding DBM-P larvae to cope with pea-derived toxic compounds.

Energy metabolism

The above described counter-defense against the dietary threats posed by the novel host pea requires a lot of energy. With the cDNA-AFLP technique we identified two fragments upregulated in pea-feeding DBM larvae with a role in energy metabolism, one with similarity to *B. mori* Cytochrome C oxidase subunit 3 (cAFLP_37, Table 3) and another to NADH dehydrogenase subunit 4 (cAFLP_29, Table 3). Similarly a large number of energy metabolism related genes were identified in the SSH library (GO category "catalytic activity", Figure 5; "oxidoreductase activity", supplement Table 3). The upregulation of Cytochrome C oxidase subunit 3 in pea-reared and pea-feeding DBM-P larvae (P-P-P) could result both from an increased number of mitochondria and/or changes in transcription rate per se. Cytochrome c oxidase (COX) is a large transmembrane protein complex found in bacteria and in the mitochondrion. Subunits 1, 2 and 3 are large and highly hydrophobic proteins encoded in the mitochondrial genome. COX is the last enzyme in the respiratory electron transport chain of mitochondria (or bacteria). It plays a fundamental role in energy production of aerobic cells, and also contributes to the storage of energy in the form of an electrochemical gradient that will be used by the oxidative phosphorylation system for synthesis of ATP (Fontenesi et al. 2008). As COX activity can be modulated according to the energetic requirement of the cell, its increase in P-P-P individuals could be related to an increased need of energy such as for synthesis and allocation of energy-dependent enzymes. NADH dehydrogenase is a key metabolic enzyme in the mitochondrion respiratory chain and its upregulation as well of others members of the energy supply chain might serve to alleviate the energy shortage due to an increased energy demand for upregulation of e.g. detoxification enzymes.

Response to stress

The SSH approach revealed that numerous transcripts with a role in response to oxidative stress are upregulated in pea-feeding DBM-P larvae (supplement Table 2), such as two clones with homology to members of the superoxide dismutase family (Mn superoxide dismutase of *B. mori* (Q65Y02), extracellular Cu/Zn superoxide dismutase of the black garden ant *Lasius niger* (Q5QE66)). The presence of SODs indicates oxidative stress caused by plant derived chemical defense compounds such as prooxidant phytochemicals (Aucoin et al. 1995). Prooxidant phytochemicals are secondary metabolites also found in legume plants and act as defense against microbial, fungal and herbivorous insect attack (Ahmad and Pardini 1990; Pritsos et al. 1991). Prooxidant compounds present in leaves of sugar peas evoke oxidative stress and induce SODs in pea feeding DBM-P larvae. However, in expressing these genes DBM-P larvae are able to deal with and detoxify harmful stress elicitors, a mechanism which might be lacking or expressed at much lower levels in cabbage-strain DBM larvae.

Transcription factors

Another group of overexpressed fragments in pea-feeding DBM-P larvae were transcription factors. Although none of the transcriptional approaches applied herein can address the possibility of point mutations in transcription factors that lead to an over- or underexpression of a certain transcript, such a mechanism may allow organisms to rapidly adapt to a novel environment (Oleksiak et al. 2002). An emerging theme in evolutionary biology recognizes that mutations in regulatory sequences can account for major physiological differences between strains with coding genes remaining relatively unchanged. For example, a transcript resembling a PHD-finger protein 5a (c-AFLP80, Table 3) was identified. PHD-finger 5A protein belongs to a novel murine multigene family that is highly conserved during evolution. The function of PHD-finger 5A is not clear but it may act as a chromatin-associated protein and participate in transcriptional regulation (Trappe et al. 2002). New patterns of gene expression might also be associated with pea-adaptation in DBM-P larvae. A mutation in a transcription factor for a gene belonging to one of the processes discussed above could result in over- or underexpression of its regulatory target and could have facilitated pea-adaptation. A mutation in a transcription factor for example an odorant binding receptor could result in an over- or underexpression of the specific receptor rendering DBM-P larvae to be more attracted to pea or less to cabbage and promote the adaptation to the novel host pea. Similarly, an overexpression of an enzyme needed for successful degradation of (toxic) pea secondary compounds could govern larval adaptation to sugar pea.

Unknown/Orphan genes

In both cDNA-AFLP and SSH approach we found a number of transcripts that gave no hit in the existing databases. We would not expect all these transcripts to be non-coding RNA. Especially transcripts derived from the cDNA-AFLP approach provided no significant similarity to public databases but matched to sequences from a *P. xylostella* library. An explanation might be that sequences of cDNA-AFLP fragments failed to cover critically important sites and domains that might aid in identification. On the other hand, these fragments might represent genes that are unique to DBM or even exclusive to DBM-P feeding on pea plants, putatively representing novel genes. Species-specific protein-coding regions with unknown functions are so called "orphan" genes and are considered to evolve faster than conservative genes (Domazet-Loso and Tautz 2003). Recent studies have shed light on the function of orphan genes and indicate that these taxon-specific genes are involved in important species-specific adaptive processes enabling organisms to adapt to changing environmental conditions and new habitats (Khalturin et al. 2009).

Glucosinolate sulfatase level in crucifer and non-crucifer feeding larvae

We have previously discussed the role of detoxifying enzymes in pea-adaptation and of changes in transcript abundance accompanying changes in host plants, such as host range expansion. The detoxifying enzyme glucosinolate sulfatase (GSS) is known to play a key role in DBM's adaptation to crucifers. Glucosinolates are crucifer-specific defense compounds and have not been found in legumes. Thus, in contrast to the detoxifying enzymes discussed above which might be specifically needed for pea-feeding, GSS is not needed for feeding on glucosinolate-free pea plants. The adaptive tailoring of the enzymatic response of an herbivore towards a host plant is thus not only upregulation of needed enzymes but also the downregulation of an "unneeded" enzyme, such as GSS in case of pea-feeding or on other glucosinolate-free diets because of the trade-off between the benefits of processing food through a machinery of digestive enzymes and the cost of maintaining and carrying these enzymes (Naya et al. 2005). Indeed, we found that DBM larvae generally seem to adjust GSS levels to a certain extent according to glucosinolate content in the food source. DBM larvae increase GSS level when being shifted from glucosinolate-free host plants or diet to a glucosinolate-containing food source and reciprocally decrease GSS level when feeding on a glucosinolate-free food source (artificial diet or sugar pea). We would expect that this adaptation will likely only occur at the transcript level. A genetically manifested constitutive downregulation of GSS would only be advantageous if DBM-P had shifted completely to sugar pea and would not feed on

the original host plant anymore. In the present case of a host range expansion with DBM-P feeding on original and novel host plant the ability to adapt GSS level to glucosinolate content seems of greater advantage. It allows for spontaneous changes in host plant use according to host plant availability which can ensure maintenance of DBM populations in cases when one or the other host is rare. The results also show that ability to adjust GSS levels according to glucosinolate content is not DBM-P exclusive but a mechanism exerted in larvae of all tested DBM strains.

Conclusions and perspective

Taken together our findings showed that feeding on the novel host plant pea had profound effects on the transcriptome level. With global changes affecting the transcriptome in general as well as differential expression of a range of specific genes coding several physiological pathways in DBM-P larvae and caused changes in the regulation of a number of genes, such as olfaction, metabolism, response to stress. Some of these transcripts could be host plant-specific and some of them could play a more general role in larval response to feeding on a novel host plant in general. A future step could be to locate the herein characterized candidate genes on the five linkage groups that were identified to harbor loci that contribute to larval adaptation.

Supplementary information

Supplementary Table 1 Sequences of primers and adapters used for cDNA-AFLPs.

Primer/ Adapter		Sequence
*Eco*RI adapter	forward	5'-CTCGTAGACTGCGTACC-3'
	reverse	5'-AATTGGTACGCAGTCTAC-3'
*Mse*I adapter	forward	5'-GACGATGAGTCCTGAG-3'
	reverse	5'-TACTCAGGACTCAT-3'
*Eco*RI pre-amplification primer		5'-GACTGCGTACCAATTC-3'
*Mse*I pre-amplification primer		5'-GATGAGTCCTGAGTAA-3'

Supplementary Table 2 Biological process (The Gene Ontology Consortium, 2001).

Function	No. of sequences
Biological regulation	
axon ensheathment	6
blood coagulation	16
cell redox homeostasis	8
cellular chemical homeostasis	25
hemostasis	16
homeostatic process	44
negative regulation of biological process	83
negative regulation of cellular process	74
negative regulation of developmental process	30
negative regulation of growth	10
negative regulation of metabolic process	30
negative regulation of multicellular organismal process	8
positive regulation of biological process	107
positive regulation of cellular process	54
positive regulation of developmental process	19
positive regulation of growth	51
positive regulation of metabolic process	30
regulation of action potential	7
regulation of anatomical structure morphogenesis	10
regulation of binding	8
regulation of biosynthetic process	25
regulation of catalytic activity	22
regulation of cell cycle	16
regulation of cell growth	11
regulation of cell proliferation	19
regulation of cell size	11
regulation of cellular component organization and biogenesis	40
regulation of cellular component size	9
regulation of cellular metabolic process	125
regulation of defense response	9
regulation of growth rate	47
regulation of lipid metabolic process	6
regulation of membrane potential	7
regulation of multicellular organism growth	7
regulation of muscle contraction	8
regulation of neurogenesis	7
regulation of neurotransmitter levels	7
regulation of programmed cell death	43
regulation of protein metabolic process	43
regulation of signal transduction	24
regulation of transport	10
signal transduction	93
vitamin metabolic process	16
Cellular process	
alcohol metabolic process	43
aldehyde metabolic process	6
amine metabolic process	52
amino acid and derivative metabolic process	57
aromatic compound metabolic process	19

Function	No. of sequences
Establishment of localization	
hydrogen transport	9
intracellular transport	82
Ion transport	42
nucleobase, nucleoside, nucleotide and nucleic acid transport	7
organic acid transport	8
protein transport	62
regulation of transport	10
RNA transport	7
secretion by cell	30
secretory pathway	22
vesicle-mediated transport	33
Growth	
negative regulation of growth	10
positive regulation of growth	51
regulation of cell growth	11
regulation of growth rate	47
regulation of multicellular organism growth	7
tissue regeneration	7
Immune system response	
innate immune response	20
Localization	
cell migration	12
hydrogen transport	9
intracellular transport	82
Ion transport	42
nucleobase, nucleoside, nucleotide and nucleic acid transport	7
organelle localization	10
organic acid transport	8
protein localization	69
protein transport	62
regulation of transport	10
RNA localization	7
RNA transport	7
secretion by cell	30
vesicle-mediated transport	33
Metabolic process	
alcohol metabolic process	43
aldehyde metabolic process	6
amine metabolic process	52
amino acid and derivative metabolic process	57
aromatic compound metabolic process	19
biopolymer metabolic process	272
carbohydrate metabolic process	69
cellular biosynthetic process	182
cellular catabolic process	119
cellular lipid metabolic process	87
cellular macromolecule metabolic process	301
cofactor metabolic process	30
energy derivation by oxidation of organic compounds	17

Function	No. of sequences
axon ensheathment	6
cell cycle phase	10
cell death	58
cell differentiation	111
cell migration	12
cell redox homeostasis	8
cell-cell signaling	24
cellular biosynthetic process	182
cellular catabolic process	119
cellular chemical homeostasis	25
cellular component assembly	81
cellular component disassembly	11
cellular lipid metabolic process	87
cellular macromolecule metabolic process	301
cellular morphogenesis during differentiation	28
cellular structure morphogenesis	37
cofactor metabolic process	30
cytokinesis	7
dendrite development	7
electron transport	61
energy derivation by oxidation of organic compounds	17
germ cell development	10
glycerol ether metabolic process	10
heterocycle metabolic process	9
hormone metabolic process	20
macromolecular complex assembly	72
membrane organization and biogenesis	15
mitotic cell cycle	21
mitotic spindle organization and biogenesis	11
neurogenesis	30
neuron development	28
nitrogen compound biosynthetic process	21
nitrogen compound catabolic process	13
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	207
one-carbon compound metabolic process	12
oocyte construction	8
organelle localization	10
organelle organization and biogenesis	171
organic acid metabolic process	105
oxidative phosphorylation	29
oxygen and reactive oxygen species metabolic process	23
peptide metabolic process	8
phosphorus metabolic process	46
pigment metabolic process	6
positive regulation of cellular process	54
regulation of action potential	7
regulation of cell cycle	16
regulation of cell growth	11
regulation of cell proliferation	19
regulation of cellular component organization and biogenesis	40
regulation of cellular metabolic process	125
regulation of membrane potential	7
regulation of neurogenesis	7
regulation of neurotransmitter levels	7
regulation of programmed cell death	43
germ cell development	10
glycerol ether metabolic process	10
heterocycle metabolic process	9
hormone metabolic process	20
lipid biosynthetic process	44
lipid catabolic process	28
lipid metabolic process	106
macromolecule biosynthetic process	109
macromolecule catabolic process	71
negative regulation of metabolic process	30
nitrogen compound biosynthetic process	21
nitrogen compound catabolic process	13
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	207
one-carbon compound metabolic process	12
organic acid metabolic process	105
oxidative phosphorylation	29
oxygen and reactive oxygen species metabolic process	23
peptide metabolic process	8
phosphorus metabolic process	46
pigment metabolic process	6
positive regulation of metabolic process	30
regulation of biosynthetic process	25
regulation of cellular metabolic process	125
regulation of lipid metabolic process	6
regulation of protein metabolic process	43
sulfur metabolic process	14
xenobiotic metabolic process	11
Multicellular organismal process	
angiogenesis	8
axis specification	9
axon ensheathment	6
blood coagulation	16
bone remodeling	7
dendrite development	7
development of primary sexual characteristics	6
embryonic morphogenesis	6
gastrulation	6
hemostasis	16
instar larval or pupal development	8
larval development	70
molting cycle, protein-based cuticle	7
negative regulation of multicellular organismal process	8
neurogenesis	30
neuron development	28
organ morphogenesis	20
regionalization	10
regulation of action potential	7
regulation of multicellular organism growth	7
regulation of muscle contraction	8
regulation of neurogenesis	7
regulation of neurotransmitter levels	7
sensory perception	18
smooth muscle contraction	8
system development	119
tissue development	19

Function	No. of sequences
regulation of signal transduction	24
ribonucleoprotein complex biogenesis and assembly	82
secretion by cell	30
secretory pathway	22
spindle elongation	6
sulfur metabolic process	14
transmission of nerve impulse	20
vesicle-mediated transport	33
vitamin metabolic process	16
xenobiotic metabolic process	11
Developmental process	
angiogenesis	8
axis specification	9
axon ensheathment	6
body morphogenesis	12
cell death	58
cell differentiation	111
cellular morphogenesis during differentiation	28
cellular structure morphogenesis	37
dendrite development	7
development of primary sexual characteristics	6
embryonic development ending in birth or egg hatching	81
embryonic morphogenesis	6
gastrulation	6
instar larval or pupal development	8
larval development	70
metamorphosis	6
morphogenesis of an epithelium	6
neurogenesis	30
neuron development	28
oocyte construction	8
organ development	82
organ morphogenesis	20
positive regulation of developmental process	19
regeneration	7
regionalization	10
regulation of anatomical structure morphogenesis	10
regulation of neurogenesis	7
regulation of programmed cell death	43
reproductive structure development	8
sex differentiation	26
system development	119
tissue regeneration	7
tube development	6

Function	No. of sequences
transmission of nerve impulse	20
tube development	6
Reproduction	
development of primary sexual characteristics	6
gamete generation	29
oocyte construction	8
reproductive structure development	8
sex differentiation	26
single fertilization	6
Response to stimulus	
blood coagulation	16
defense response to bacterium	9
germ cell development	10
inflammatory response	13
innate immune response	20
locomotory behavior	9
regulation of defense response	9
regulation of immune response	6
regulation of response to external stimulus	6
response to bacterium	10
response to DNA damage stimulus	22
response to drug	19
response to extracellular stimulus	22
response to fungus	6
response to heat	9
response to hormone stimulus	27
response to hypoxia	6
response to inorganic substance	12
response to nutrient	13
response to organic substance	23
response to oxidative stress	39
response to starvation	10
response to temperature stimulus	12
response to toxin	7
response to wounding	42
response to xenobiotic stimulus	12
tissue regeneration	7
xenobiotic metabolic process	11

Supplementary Table 3 Molecular function (The Gene Ontology Consortium, 2001)

Function	No. of sequences
Antioxidant activity	
Glutathione peroxidase activity *1	9
Binding	
Amino acid binding	6
Calmodulin binding	8
Cation binding	169
Chaperone binding	7
Coenzyme binding	36
Collagen binding	13
Cytoskeletal protein binding	24
DNA binding	61
Enzyme binding	16
Fatty acid binding	7
Heme binding	15
Identical protein binding	35
Iron-sulfur cluster binding	13
Laminin binding	12
Metal ion binding	198
Polysaccharide binding	7
Protein binding, bridging	6
Protein dimerization activity	26
Protein domain specific binding	9
Protein transmembrane transporter activity *3	8
Purine nucleotide binding	99
Pyridoxal binding	8
Receptor binding	24
Ribonucleotide binding	87
RNA binding	99
Transcription coactivator activity *2	15
Transcription corepressor activity *2	6
Transcription factor binding	25
Translation initiation factor activity	18
Unfolded protein binding	18
Catalytic activity	
ATP dependant helicase activity	11
Carbon-oxygen lyase activity	12
Catalase activity	12
Cis-trans isomerise activity	7
Electron carrier activity	49
Hydrolase activity	
acting on acid anhydrids	66
acting on ester bonds	52
acting on glycosyl bonds	26
acting on carbon-nitrogen bonds	19
Intramolecular oxidoreductase activity	9
Ligase activity	
forming carbon-oxygen bonds	8
forming carbon-nitrogen bonds	8
Monooxygenase activity	14
Oxidoreducutase activity	
acting on CH-OH group donors	47
acting on NADH or NADPH	23
acting on peroxide as receptor	24
acting on CH-CH group donors	17
acting on paired donors, with incorporation or reduction of molecular oxygen	14
acting on sulfur group of donors	9
acting on aldehyde or oxo group of donors	9
acting on single donors with incorporation of molecular oxygen	9
Peptidase activity	98
RNA helicase activity	6
Serine hydrolase activity	52
Transferase activity	
transferring acyl groups	21
transferring alkyl or aryl groups	18
transferring glycosyl groups	14
trasnferring nitrogenous groups	6
transferring one-carbon groups	20
transferring phosphorous-containing groups	36
Enzyme regulator activity	
Protease inhibitor activity	9
Molecular transducer activity	
Receptor activity	33
Structural molecule activity	
Structural constituent of chitin-based cuticle	10
Transcription regulator activity	
General RNA polymerase II transcription factor activity	6
Transporter activity	
Active transmembrane transporter activity	32
Oxygen transporter activity	6
Passive transmembrane transporter activity	9
Protein transporter activity	17
Substrate-specific transmembrane transporter activity	46

* also assigns to:
1 catalytic activity
2 transcription regulator activity
3 transporter activity

7. General discussion

The colonization of and adaptation to a novel host plant by an insect fascinates and intrigues scientists. An understanding of how and by what underlying mechanisms herbivores acquire and adapt to novel host plants is essential for the understanding of evolutionarily relevant processes as well as for applied reasons, when pest insects spread to novel crops. Studies that address this question will contribute to the understanding of behavioral changes after host shifts and range expansions, the genetic basis of adaptation and the molecular mechanisms that govern host switches. As examples of recent events of changes in host plant use are limited, the host range expansion of DBM to sugar pea offers an excellent example for such a study. To provide a first comprehensive understanding of DBM's host range expansion and the underlying mechanisms, I employed a behavioral approach with an oviposition assay to assess adult oviposition preference (Manuscript I) and a feeding assay to study larval feeding preference (Manuscript II) of the newly evolved pea host strain (DBM-P), performed backcrosses and linkage analysis to decipher the genetic basis of larval pea-adaptation (Manuscript III) and carried out a transcriptome approach to identify candidate genes and molecular mechanisms that accompany the host range expansion (Manuscript IV). An overview of the results is presented in Figure 1.

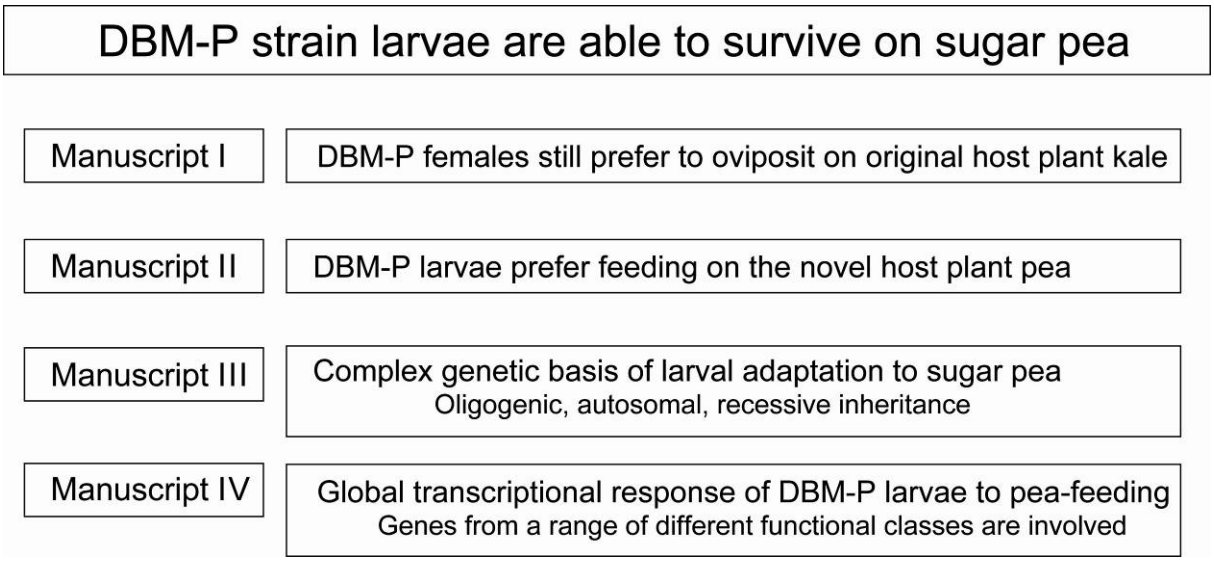


Figure 1 Overview of the results of this thesis.

Adult behavior on novel host plant

The adaptation to a novel host plant is believed to be governed by genetically manifested behavioral and/or physiological changes in the larval and/or adult stages of an herbivore. To better understand the recent host range expansion to sugar pea in DBM and as a source for any further investigations, I first characterized host-plant associated adult behavior in the newly evolved pea-adapted host strain of DBM. Changes in behavior are regarded as the prevalent constraint on host range expansion in phytophagous insects, and several authors have stressed that host-specialized insects sometimes possess the potential for a greater diet breadth physiologically than is realized behaviorally (Bernays and Chapman 1994). In Lepidoptera, such as DBM, this constraint is mainly exerted by the ovipositing female. The small and sessile larvae are not able to move long distances and their diet depends on their mother's host plant choice. Thus, female oviposition behavior is supposed to play an important role and might even be considered as driving force in the recent host range expansion. The oviposition assay yielded a surprising result: oviposition behavior of DBM-P females differs from that of females from an original cabbage strain (DBM-Cj), as they increase their oviposition rate on kale in the presence of the new host. However, they still prefer kale over sugar pea as oviposition site (Manuscript I), confirming the behavioral constraint hypothesis. The question thus emerges how host range expansion in DBM-P proceeded if females do not oviposit on sugar pea plants. Three reasons might account for DBM-P females not ovipositing on sugar pea plants.

The first possible explanation is that field females had a genetic preference to oviposit on pea, but that this genetic preference has been lost under the rearing regime in the laboratory. As DBM-P is reared exclusively on pea, a strong selection for larval adaptation to sugar pea is exerted in the laboratory. However, an oviposition preference for pea is not being selected for in our rearing of DBM-P, because eggs deposited on the sides of the rearing containers as well as on the pea plants are used to produce the next generation. Thus, any genetically-determined oviposition preference, if not immediately fixed in the newly established laboratory population, could have been lost over many generations of rearing.

The second possible reason for not finding an oviposition preference of DBM-P females is that field females may never have had a genetic preference to oviposit on pea and oviposition on sugar pea was just due to a general stimulatory property of sugar pea plants. In the course of infestation of the pea fields in Kenya, this behavior then became adaptive and if it had a genetic basis, it would have been maintained even under our rearing, because females that lay

more eggs in the oviposition containers in the presence of pea would contribute more to the next generation. Therefore this explanation is unlikely.

The third possible explanation is that early-adult experience played a role in oviposition preference and DBM-P females not ovipositing on pea plants in my assay was due to the absence of preconditioning in our experiments; individuals were kept from pupation onwards in plastic tubes without exposure to any plant material. A lack of larval experience cannot account for absence of adult pea acceptance, because each strain experienced its host plant as larvae, and consequently DBM-P should accept pea as oviposition site. The importance of early-adult host experience in DBM seems more important in this context and has been stressed by other authors (Liu and Liu 2006). However, lack of pre-oviposition exposure to cabbage plants did not prevent either strain from ovipositing heavily on these plants, which might be due to an innate attraction to cabbage, mediated by the presence of glucosinolates (Hopkins et al., 2009). In our experiments, no pupae or emerging adults were pre-exposed to either pea or cabbage, so that neither the overall preference for cabbage over pea, nor the increase in cabbage oviposition in the presence of pea by DBM-P but not of DBM-Cj can be explained by a difference in pre-oviposition adult exposure to the plants. Even though a presence or absence of early-adult experience is not believed to have significantly affected the outcome of the oviposition assay, it may explain the continuation of infestation in pea fields in Kenya.

To conclude, oviposition behavior in the newly evolved pea host strain only slightly differs from that of original cabbage strain females in that there is increased oviposition in the presence of the new host, a finding which suggests that the DBM-P strain represents an early stage of a host range expansion.

Larval preference behavior

Contrary to the result of the adult preference test revealing that DBM-P females still prefer to oviposit on the original host plant, DBM-P larvae preferred to feed on the novel host plant pea (Manuscript II). Larvae of a cabbage adapted strain (DBM-Cj) were also tested and showed a significant preference for cabbage, with not a single larva recorded on or feeding on pea. This finding has an impact on the question whether adult and larval behavior are correlated as well as on the stage of host race formation and will be discussed in these contexts below. The significance of the finding that DBM-P larvae prefer sugar pea over cabbage was constrained by the fact that fourth instar larvae, i.e. experienced individuals conditioned by contact with their rearing host (i.e. DBM-P on pea and DBM-Cj on cabbage), were used. However, although host plant choice in later larval instars might be directed towards the host plant they experi-

enced during rearing, neonate larvae are less mobile and often initiate feeding on the "next best" host plant they encounter and do not "chose" their favorite host plant. The mobility of neonate and mature larval instars has been tested in DBM and it was shown that later larval instars have a greater potential to move longer distances and move significantly faster (Eigenbrode and Shelton 1990). A second culture of DBM-P was set up reared on cabbage (DBM-Pc) and in the preference test these larvae preferred their rearing host plant cabbage. However, a small proportion was also found on pea indicating that preference for the novel host plant is stronger in the larval than adult stages of DBM-P.

Genetic basis of larval adaptation to novel host plant

The results of the oviposition assay shows that a strong female oviposition preference for the novel host plant is not an absolutely necessary behavioral trait in host range expansion. Larval adaptation to the novel host plant is however essential and a prerequisite for establishment of novel herbivore host plant associations. That adaptation to sugar pea exists in DBM-P larvae is demonstrated in larval ability to feed and develop on the novel host in the field as well as under laboratory conditions (Löhr 2001; Janssen et al. 2008, Manuscript IV). Feeding assays showed that individuals from other DBM strains fail to feed and develop on sugar pea. Crosses between the newly evolved pea host strain (DBM-P) and a cabbage strain (C-strain) further established the heritability of DBM-P's ability to survive on pea. Therefore, I started with a detailed characterization of the genetic basis of this trait (i.e. mode of inheritance, the number of genes involved), which is described in Manuscript III.

A classical question centers on how many genes influence ecologically important traits, such as host plant adaptation. The genetic basis of pea-adaptation is probably best explained as a mixture of a few genes (linkage analysis in Manuscript III) with major effects plus the influence of many more loci with minor effects as reflected in the complex pattern of inheritance. Historically, it has been argued that most adaptations result from numerous small changes (Fisher 1930). This view was challenged by more recent laboratory studies (Orr and Coyne 1992; Orr 2005) and theoretical models (Orr 1998), which led to the conclusion that a few major genes account for a large portion of adaptation; as it is the case for the herein analyzed trait 'larval survival on pea'.

Concerning the question about dominant or recessive inheritance of pea-adaptation, a primary assumption was partial dominant inheritance due to the occurrence of some survivorship on pea in the F_1 generation in the first series of crosses. However, this conclusion was disproved by the low numbers of survivors of cabbage backcross offspring together with the higher sur-

vivorship of F_2 progeny on pea. Instead, the hypothesis of one or more recessive genes being responsible for the trait 'survival on pea' proved true: progeny from a pea-strain backcross showed higher survivorship on pea. The fact that I found a significantly higher survival rate on pea in the F_1 generation among F_1 progeny from pea reared DBM-P mothers in comparison to those from cabbage-strain mothers appears to be due to a maternal effect rather than to partial dominance.

The trait 'survival on pea' was inherited autosomally because all predictions of sex-linkage were rejected. Under W-linkage only females would carry the W-chromosome, i.e. the genes responsible for the trait 'survival on pea', and W-linked genes would only be passed from mother to daughter. Consequently, only females would survive on peas, which was not the case. Taking into account that the trait 'survival on pea' is inherited recessively but still evolved quite rapidly in the field, Z-linked sex-linkage was considered. A recessive autosomal allele has a low chance of fixation because it is shielded from selection when heterozygous but it is completely exposed to selection when occurring on the sex-chromosome of the hemizygous sex (Haldane 1922). Under Z-linkage, all F_1 females with pea-strain father would carry a pea-adaptation-conferring Z-chromosome (Z^PW^C), but F_1 females with a cabbage-strain father (Z^CW^P) would lack it. The fact that F_1 females from crosses of both directions survived on pea plants and were used in single pair matings for back- and intercrosses, rejects the hypothesis of a Z-linkage of the trait 'survival on pea'. Autosomal inheritance of host plant related traits has also been found in other moth species (Sheck and Gould 1993).

The unexpected oligogenic instead of monogenic basis of larval pea-adaptation that I found seems less surprising if we consider the suite of mechanisms necessary for successful larval host plant adaptation (e.g. host perception, adequate digestion and detoxification), i.e. multiple traits likely controlled by more than one gene. The question then became how the trait larval pea-adaptation, when being so complex, could have evolved and spread within such a short time? The potential to adapt to a novel host plant in a short evolutionary timescale, as seen in the example of the DBM-P strain’s rapid spread to sugar pea, is increased when it results from standing genetic variation whereas more time is needed for awaiting a beneficial mutation (Barrett and Schluter 2008). Adaptation from standing genetic variation arises faster because the advantageous allele is already present in multiple copies and not only as a single mutation. There is evidence that some standing genetic variation for the ability to feed on legumes and on other host plant species exists in DBM populations: DBM has occasionally been found on plants other than Brassicaceae, among them plants from the Fabaceae family (Robinson et al. 2010). Gupta and Thorsteinson (1960a) showed that some DBM larvae were able

to survive on legumes under laboratory conditions. Moreover, an unrelated cabbage-adapted DBM strain responded to laboratory selection on pea to increase its survivorship to nearly 50% over six generations (Löhr and Gathu 2002). DBM's (rapid) adaptive responses are also shown in this insect's ability to develop resistance against every available insecticide and to survive in almost every climate. These adaptive responses and the recent adaptation to the novel host plant sugar pea, suggest that sufficient pre-existing genetic variation existed in these DBM populations and enable (rapid) adaptation to novel and/or changing environments. In Kenya, this standing genetic variation, together with an unusually strong and extended selection pressure for survivorship on pea after destruction of suitable crucifer hosts, most likely resulted in the observed sudden host range expansion.

The identified genetic basis of larval pea-adaptation contributes to the understanding of how species adapt to a novel host plant. The finding of autosomal inheritance of larval host plant adaptation is in accordance with the results from other studies (Sheck and Gould 1995; Tang et al. 2006). The demonstration that pea-adaptation has an oligogenic basis supports the view that adaptations arise from a few major loci accompanied by a number loci of minor importance.

Correlation of adult and larval host plant performance

The result of the oviposition preference test, revealing a preference of DBM-P females for the original crucifer host (Manuscript I), and the feeding assay revealing larval preference for the novel host pea (Manuscript II), as well as larvae being able to thrive on pea (Manuscript III), raises the question how host plant performance traits between adults and larvae correlate. A classical perception is that oviposition preference should correlate with host plant suitability for larval development, as manifested in the so-called "concept of optimal oviposition" (Jaenike 1978). In addition, models of speciation through host shifts often assume that divergence, particularly in sympatry, is most likely if the inheritance of preference and performance are correlated through pleiotropic effects or linkage (Hawthorne and Via 2001). After the establishment of these theories, many studies have investigated the correlation between female oviposition preference and larval performance, with no clear consensus: correlations range from poor to strong (Wiklund 1975; Thompson 1988; Scheirs and De Bruyn 2002; Forister 2005). While a positive correlation between oviposition preference and larval performance was observed in *Polygonia c-album* (Nylin and Janz 1993), these traits are inherited independently in *Mitoura* butterflies (Forister 2005). My studies in DBM suggest an independent inheritance as well: larvae are well enough adapted to develop on sugar pea whereas females

increase oviposition rate in the presence of the novel host plant but still prefer to lay eggs on the original host. However, although DBM-P larvae are able to thrive on the novel host plant pea, they still perform better on crucifers in terms of developmental time (Löhr and Gathu 2002). Even after more than 50 generations of rearing on pea, the developmental time of DBM-P was longer than DBM-Cj reared concurrently on cabbage (Manuscript I). Thus, an oviposition preference for sugar pea plants is not advantageous as long as the original host plant is present and selection for oviposition on a new host would be strengthened only if it offered growth advantages to the larvae, but that situation has not yet been attained by DBM-P. This finding again supports the assumption that DBM-P is still in an initial stage of host range expansion and the insect had insufficient time to completely adapt to the novel host plant, which explains why correlation between these traits is not (yet) positive. In addition, we found that larval adaptation is inherited autosomally. The genetic basis of oviposition preference in DBM was not tested explicitly, but in the majority of Lepidopteran species tested so far oviposition preference was found to be Z-linked (Charlesworth et al. 1987; Sperling 1994; Janz 2003). If DBM fits this common pattern, then this supports the notion that the two traits are not positively correlated nor genetically linked.

Transcriptional profile of pea-feeding DBM larvae

The current lack of knowledge concerning transcriptional responses to plant-feeding in herbivorous insects in general and specifically after a host range expansion together with the proven complex genetic basis of pea-adaptation (Manuscript III) directed the choice for a transcriptome study to reveal putative candidate genes and functional groups of genes that allow for pea-feeding and pea-adaptation in DBM. Manuscript IV provides an example of one of the few studies that investigate large-scale gene expression changes and identification of candidate genes in an herbivore feeding on original and novel host plant(s) and upon a shift to a related and unrelated host plant and aimed to enhance the knowledge on the underlying molecular mechanisms. Two different transcriptome profiling techniques were applied, cDNA-AFLP (amplified fragment length polymorphism) and SSH (suppression subtractive library), which are both particularly appropriate for gene expression studies in non-model species. One major difference between the two methods is that SSH allows for comparison of only two RNA-types whereas in the cDNA-AFLP procedure several RNA-types can be compared simultaneously. Applying cDNA-AFLP and SSH had the advantage that results obtained in one approach could be complemented and supported by those from the respective other method. The SSH approach yielded a large number of differentially expressed fragments

(>2,000 transcripts) and thus provided many candidate genes. The high number of altered fragments was also indicative of a complex transcriptional response to pea-feeding, whereby many different biological processes are involved and the whole metabolism of the larva needs to adapt to the novel host plant. The global alteration of the transcriptome was confirmed in the cDNA-AFLP approach, too. This method also allowed for comparing responses on the RNA level of DBM-P larvae feeding on pea vs. DBM larvae from other strains feeding on their host plant(s) and revealed that gene expression profiles were altered in a similar fashion. With the cDNA-AFLP technique only a small number of candidate genes associated with pea-feeding could be identified and many of these fragments did not give a hit in a public database such as GenBank. These fragments could however be assigned to SSH-derived transcripts indicating that they were not artifacts.

It was the major aim of the differential gene expression approach to identify those genes that are differentially expressed in DBM-P feeding on pea and play a role in adaptation to the novel host plant. Whereas the degree of alteration in DBM feeding on crucifers and non-crucifers and after shifts to related and unrelated host plants appeared similar, the underlying genes and adaptive mechanisms are likely not to be similar, as chemical differences among different plant species may require a very specific subset of genes to be up- and downregulated in the herbivore upon feeding. Both methods indicated that genes coding for diverse biological processes mediate larval pea-adaptation in DBM-P, such as genes involved in olfaction, digestion, metabolism, response to stress and transcriptional regulation.

Crucifers and sugar pea, being taxonomically unrelated, are likely to vary in their spectrum of odors they emit. DBM-P larvae could have adapted to sugar pea by accepting and being attracted to odors from pea plants. Transcripts coding for genes involved in different steps and processes of olfactory perception and processing were upregulated in DBM-P larvae. A mutation in an odorant binding protein has been found to account for the host shift of *Drosophila sechellia* to *Morinda* fruits, from which all other *Drosophila* species are repelled (Matsuo et al. 2007). A similar mechanism could be anticipated for DBM-P larvae.

A predominant group of pea-feeding responsive transcripts were genes involved in metabolic processes. Their increased expression might represent a major part of DBM-P's strategy to adapt to the novel host pea. Several detoxification pathways seem to be utilized by DBM-P larvae when exposed to feeding on pea plants. Detoxification has classically been divided into three phases and representatives from each of the three phases were among cDNA-AFLP and/or SSH derived fragments. Phase I reactions consist of oxidation, hydrolysis and reduc-

tion producing metabolites which can be excreted if polar enough but usually are further converted by phase II reactions. In phase II, the polar products are conjugated with a variety of endogenous compounds and can then be recognized by specific membrane-associated transporters in phase III detoxification. Representatives from each of the three phases could be identified in pea-feeding DBM-P larvae, such as the well studied cytochrome P450s. P450s have been suggested to be involved in permethrin resistance in DBM (Bautista et al. 2007) and enable other lepidopteran species to detoxify toxic compounds found in their host plants (Berenbaum et al. 1996). Additionally, enzymes involved in phase II and transporters for phase III of detoxification were likewise upregulated in pea-feeding larvae and this complex response on the detoxification level might be a mechanism of adaptation.

The transcriptome approach also revealed the upregulation of several digestive enzymes, probably as a response to the different nutritional background of the novel as compared to the original host. An adaptive mechanism in pea-feeding DBM-P larvae could be an overexpression of some general acting digestive enzymes to meet the nutritional requirements as well as an upregulation of specific enzymes that are needed particularly for digestion of pea leaves, e.g. the overexpression of a sucrose-6-phosphate hydrolase required in sucrose utilization. The adaptation to sugar pea might therefore be governed by synthesis and allocation of the above described enzymes which allow DBM-P larvae to cope with pea-specific defense compounds and ensure a sufficient supply of nutrients from the novel host.

Increases in enzymatic activity as described above might be related to energetic costs explaining the increase in energy-metabolism transcripts as documented by the SSH and cDNA-AFLP approach (e.g. cytochrome c-oxidase, NADH dehydrogenase). However, despite this upregulation less energy may still be allocated to fitness-related traits and could serve as an explanation for the retarded development of pea-reared DBM-P larvae and their lower pupal weight (Manuscript I; Löhr and Gathu 2002).

To compensate for trade-offs due to an increased supply of needed enzymes it can be speculated that enzymes not needed in degradation of pea-derived materials are downregulated. An obvious candidate to test this hypothesis is the detoxifying enzyme glucosinolate sulfatase (GSS), which is indispensable for DBM larvae feeding on glucosinolate-containing crucifer host plants but not necessary when feeding on glucosinolate-free plants such as sugar pea. When comparing the GSS-level of DBM larvae feeding on sugar pea vs. cabbage or kale using qRT-PCR we found that DBM larva seems to adjust its GSS level according to glucosinolate content in the food source. A further step in the transcriptional analysis of DBM-P's

host range expansion could therefore be to use qRT-PCR to specifically analyze expression levels of candidate genes which are anticipated to convey pea-adaptation as well as those that have just been identified in the above described approach.

Host race formation and speciation

The question of (ongoing) sympatric speciation in DBM may be a moot point for the DBM-P strain, because it meanwhile has become extinct or indetectable in the field, after the farmer stopped growing sugar peas due to control failures. Nevertheless, such a case of sudden expansion to a dissimilar host plant must be considered as an event of evolutionary significance. The pea host strain still exists in the laboratory, and as such represents a snapshot of the situation in the field back then. Thus, the question of host race formation as an intermediate step in the speciation process can be addressed using this strain and a preliminary conclusion on the extent of host race formation in DBM can be drawn on the basis of the results presented in Manuscripts I to IV.

The following set of experimentally verifiable criteria for host race formation has been established: i) original and derived population occur in sympatry, ii) the two populations feed on different host plants, iii) host preference for either of the two hosts, iv) genetic differentiation between the two populations, v) assortative mating (Maynard Smith 1966; Bush 1975; Jaenike 1981; Abrahamson et al. 2001; Drès and Mallet 2002). The field situation in Kenya as described by Löhr (2001) and Löhr and Gathu (2002) clearly prove the sympatric occurrence of a pea and cabbage-adapted DBM strains and thus the fulfillment of the first criterion. That the two populations of DBM (pea- and crucifer-feeding) feed on different host plants was proven in feeding assays (Manuscripts III to IV) which documented that DBM-P larvae can survive and prefers feeding on pea whereas all other tested DBM strains cannot thrive on this host. However, the DBM-P strain, i.e. the only DBM strain capable of surviving on plants other than crucifers, is not restricted to sugar pea but can still thrive equally well on the original and novel host plant, so that criterion iii) is only partially fulfilled. And although larvae of this strain prefer feeding on the novel non-crucifer host plant pea (Manuscript II), the oviposition assay in Manuscript I revealed that DBM-P females still prefer to oviposit on cabbage, so that the requirement for criterion iii), the preference for one host plant in one population and the respective other host plant in the other population, is only partially met. The DBM-P strain differs in the genetically based trait larval pea-adaptation from other crucifer DBM strains (Manuscript III). A specific approach addressing the extent of genetic differentiation between pea and crucifer strains is pending, and so far no genetic markers for differentiating the pea

host strain from other DBM strains have been identified. The fifth criterion for host race formation is assortative mating, i.e. individuals from the same population should preferentially mate with each other, which will reduce gene flow between populations and favor reproductive isolation. Though no specific experiments have been carried out to test for assortative attraction or mating in DBM (i.e. premating barriers), the successful crosses between pea and cabbage strain individuals producing viable progeny (Manuscript III) did not indicate any postmating barriers. Moreover, females do still oviposit on the original host plant, where putatively previously the mating had occurred. Pea- and cabbage strain individuals are likely to encounter each other on the same host plants, preventing a reduction in gene flow if there is no assortative mating based on premating cues. In conclusion, the DBM-P strain was on the way towards host race formation as some of the criteria were met, but this was not completed. A logical conclusion from the results of Manuscripts I to III is therefore that DBM-P manifested the initial stages of an evolutionary host range expansion, with larvae preferring to feed on the novel host plant. However, the host range expansion is incomplete due to the lack of oviposition fidelity on pea plants and the genetic heterogeneity with alleles for pea-adaptation not being fixed yet in the DBM-P strain.

Human-disturbed environments as a stage for host range expansion

In this thesis I have studied the underlying mechanisms of a host range expansion that occurred in an agricultural setting. In recent years, anthropogenic impact has increasingly driven ecological and evolutionary processes in many species. The case of DBM provides a useful example to study a process where a pest species emerges as a new pest on a novel crop. Several questions can be posed about studying an event that occurred in a non-natural environment: What is the relevance of host range expansions in non-natural environments? Are agricultural settings appropriate for studying host range expansions? Can the results be compared to host range expansions in natural environments? Do the results found here have any practical implications?

The area of agriculturally used land is constantly expanding because of the need for increasing yields to meet the demands for food of the growing world population (World Bank 2008). The process of agricultural intensification is likely to accelerate, and with it the introduction of novel plant and pest insect species occurring in each others' ranges, which concurrently increases the arena of species interactions and thus, the potential of further host shifts and range expansions of pest herbivores. This trend towards an increased and accelerated rate of pest species changing their hosts clearly demonstrates the need for understanding such events.

For applied reasons, the importance of studying host range expansions in agroecosystems is clearly justified. A thorough knowledge of the underlying mechanisms helps to estimate the potential of range expansions and to adequately adjust cultivation and management strategies after a crop has been colonized by a novel pest. The finding that DBM was only at the beginning of host range expansion, revealed in the lack of oviposition fidelity for pea plants in DBM-P females and better larval performance on crucifers (Manuscript I), decreases the risk of DBM evolving as a novel pest on sugar pea. However, the finding that DBM-P larvae preferred to feed on pea when given a choice (Manuscript II) and that despite its complex genetic basis the adaptive change towards sugar pea evolved quite rapidly (Manuscript III) manifests DBM's status as a severe and highly adaptive (e.g. thrives in many climates, insecticide resistance) pest insect.

Agroecosystems are ecologically simple with a few associated species, which has led to the objection that these environments and any processes therein are only of little relevance to more fundamental studies of plant insect relationships. On the other hand, agricultural environments have explicitly been stressed to be important arenas for evolutionary change and because of their relative simple composition of species provide useful models for studying evolutionary processes such as host shifts and range expansion (Via 1990). Agricultural environments are characterized by the feature that they are closely monitored and precisely documented, implying that all ongoing changes are well documented, e.g. the invasion of a novel pest insect by host shift or range expansion. Thus, the availability of background information on the circumstances of host shifts in agroecosystems bears an advantage over studies that evaluate host plant changes from the evolutionary past, which often can only infer range expansions and shifts underlying present host plant relationships. This was also true for DBM's host range expansion, for which was known that it occurred in a situation where the original host plant was extremely rare due to severe DBM attack, so that a neighboring sugar pea field was the only available and reachable green food source for DBM. The shift to the neighboring pea field and adaptation to the novel host occurred rather rapidly, these information would not have been available for a shift in native environments.

Conclusions and future perspectives

This thesis aimed to provide the first comprehensive insight into the background of the host range expansion of DBM from cruciferous plants to sugar pea. The findings indicate that DBM initiated the beginning of a host range expansion which is governed by behavioral changes mostly in larval behavior, which has a complex genetic basis and is accompanied by

broad transcriptional changes. These findings together with several unaddressed issues concerning the recent host range expansion open up new questions in this context and demand for further studies. The finding that DBM-P females are stimulated by the presence of their novel host pea in that they increase their oviposition rate on kale, justifies further study on the detection of putatively stimulating compounds emitted by sugar peas involving e.g. gas chromatography and electroantennograms. The proven feeding preference for the novel host plant pea in mature DBM-P larvae could additionally be studied and compared to feeding preferences in early larval instars. Future genetic mapping efforts should assess whether any of the identified linkage groups harbor genes that affected expression changes revealed in the transcriptome study, so that actual genes can be identified that may be involved in the host expansion of *P. xylostella* to the unrelated host plant pea. As feeding specialization often sets the stage for reduction in mating between host strains feeding on different host plants, a relevant research direction would be a study on the mating behavior between new and original DBM strains, especially possible assortative mating due to premating barriers, such as variation in sexual communication cues. A completely different approach could be a detailed analysis of the novel host plant sugar pea and its secondary compounds with regard to feeding deterrents and stimulants. The work described in this thesis sets the stage for these future studies which will shed additional light on the mechanisms and consequences of host shifts in herbivorous insects.

8. Summary

Much of today's insect diversity has been shaped by herbivores exploiting and colonizing novel host plants. The successful adaptation to a novel host requires a suite of complex processes: the adult must accept it for oviposition, and larvae must accept it for feeding and be able to complete development on the new host. It has been a longstanding aim of biologists to understand the mechanisms that underlie such an evolutionarily and ecologically important process. Recently, the crucifer specialist diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), has expanded its host range in Kenya to a novel host plant, sugar pea (*Pisum sativum*, Fabaceae), that is taxonomically and phytochemically different from the plants of its original host plant range Brassicaceae. This recent event offers the possibility to examine the mechanisms underlying adaptation to a novel host plant and contribute to the understanding of species exploiting novel niches. In order to get a detailed understanding of the underlying mechanisms, the host range expansion was studied at three levels: experiments were carried out to examine changes in larval and adult host behavior, crosses were conducted to determine the genetic basis of larval adaptation to pea, and cDNAs were sequenced to characterize transcriptional changes and identify candidate genes that may enable the newly evolved host strain of DBM (DBM-P) to thrive on sugar pea.

A shift or expansion to a new host plant may be initiated by a change in behavior; therefore adult oviposition and larval feeding behavior were examined on a typical crucifer host and the novel host pea. To assess oviposition acceptance, no-choice experiments were conducted in which DBM-P females or females of a Kenyan cabbage-adapted strain (DBM-Cj) were confined with either a cabbage or a pea plant; to assess oviposition preference, females were offered both plant species at the same time. Surprisingly, DBM-P females laid most eggs on cabbage and very few on pea. However, they laid significantly more eggs on the cabbage plant when pea plants were present. Larvae of the same strains were offered pea and cabbage leaf discs and larval position and percentage of consumed leaf area was recorded after certain time intervals. This revealed that DBM-P larvae preferred their novel host plant pea whereas DBM-Cj fed exclusively on cabbage. Thus, both larval and adult behavior has diverged in the DBM-P strain.

Examining the genetic changes underlying the ability of larvae of the DBM-P strain to feed and complete development on pea requires first determining how many genes are involved in pea-adaptation, their patterns of expression (dominance vs. recessivity), and how they interact. To this end, larval survivorship on the novel host plant pea and a typical crucifer host

(kale) was measured in reciprocal F_1, F_2 and backcrosses between the DBM-P strain and another strain that had been reared only on crucifers (DBM-Cj). Backcross individuals were then genotyped for linkage analysis using AFLP markers. Backcrosses to DBM-P produced higher survivorship on pea than backcrosses to DBM-Cj, suggesting recessive inheritance. No evidence of sex-linkage was found. The linkage analysis using three different DBM-P strain backcross families revealed two, four and five linkage groups contributing to survival on pea respectively, indicating oligogenic inheritance. Thus the newly evolved ability to survive on pea has a complex genetic basis, and variation in number of pea-adaptation conferring linkage groups indicates that the DBM-P strain is still genetically heterogeneous and not yet fixed for all the alleles enabling it to survive on pea.

Finally, in order to understand the molecular mechanisms underlying host plant adaptation and host range expansion to sugar pea, two transcriptome profiling techniques were employed: subtractive suppression hybridization (SSH) and cDNA-AFLP. These complementary methods are appropriate for non-model organisms such as DBM. SSH revealed a globally altered transcriptome profile of pea-feeding DBM larvae involving a large number of genes, affiliated with a variety of different functional classes (e.g. olfaction, metabolism, detoxification, response to stress), which may be involved in DBM-P's host range expansion to pea. The cDNA-AFLP method confirmed this pattern and additionally identified transcripts with altered expression when DBM strains were grown on kale, pea, or an artificial diet not containing glucosinolates.

These findings suggest that the evolutionary host range expansion of DBM-P is still at an initial stage, with larvae preferring the novel host plant pea but adult females lacking oviposition fidelity on it. Genetically, DBM-P's host-range expansion is governed by a number of genes with recessive alleles that are autosomally inherited and not yet fixed. This is surprising given the speed with which the infestation developed on pea in the field in Kenya. Likely candidate gene(s) involved in this expansion are those with a role in host plant perception, digestion and detoxification, which are expressed differently in DBM-P. The main future task is to identify the actual genes underlying this host plant expansion, which will make it possible to trace back genetic variation in these genes among different DBM populations and their potential for alterations in host plant range.

9. Zusammenfassung

Ein Großteil der heutigen Insektendiversität ist dadurch entstanden, dass pflanzenfressende Insekten neue Wirtspflanzen erobert und kolonisiert haben. Die erfolgreiche Anpassung an einen neuen Wirt erfordert eine Folge von komplexen Prozessen: Eine neue Pflanze wird in den Wirtspflanzenkreis aufgenommen, wenn die Adulten sie zur Eiablage akzeptieren, die Larven sie als Nahrung annehmen und ihren Lebenszyklus auf dem neuen Wirt abschließen können. Es ist ein seit langem vorherrschendes Ziel von Biologen, die Mechanismen, die einem solchen evolutionär und ökologisch bedeutenden Prozess unterliegen, zu verstehen. Kürzlich hat die als Kruziferen-Spezialist bekannte Kohlmotte (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) ihren bisherigen Wirtspflanzenkreis um eine taxonomisch und phytochemisch nicht mit dem bisherigen Wirtspflanzenkreis Brassicaceae verwandte Pflanzenart, die Zuckererbse (*Pisum sativum*, Fabaceae), erweitert. Dieses Ereignis ermöglicht es, die Mechanismen zu untersuchen, die der Anpassung an eine neue Wirtspflanze zugrunde liegen und zum Verständnis beizutragen, wie Arten neue Nischen erobern. Um diese Mechanismen im Detail zu verstehen, wurde die Erweiterung des Wirtspflanzenkreises auf drei Ebenen untersucht: Es wurden Versuche ausgeführt, um mögliche Verhaltensveränderungen der larvalen und adulten Tieren auf dem Wirt zu untersuchen. Zudem wurden Kreuzungen durchgeführt, um die genetische Basis der Anpassung an Erbsen im Larvalstadium zu bestimmen. Des weiteren wurde cDNA sequenziert, um transkriptionale Änderungen zu charakterisieren und Kandidatengene zu identifizieren, die es dem neu entstandenen Erbsen-Stamm der Kohlmotte (DBM-P) ermöglichen, auf Erbse zu gedeihen.

Eine Änderung oder eine Erweiterung des Wirtspflanzenkreises kann durch einen Verhaltenswechsel ausgelöst werden. Dafür wurden Eiablage- und larvales Fraßverhalten auf einem typischen Kruziferen-Wirt und auf dem neuen Wirt „Erbse“ untersucht. Um die Akzeptanz zur Eiablage zu bestimmen, wurden No-Choice-Experimente durchgeführt, bei denen weibliche Tiere des DBM-P-Stammes und des kenianischen an Kohl angepassten Stammes (DBM-Cj) entweder Kohl oder Erbse angeboten bekamen. Um die Eiablagepräferenz zu bestimmen, wurden den Weibchen im Choice-Experiment beide Pflanzenarten gleichzeitig angeboten. Erstaunlicherweise legten DBM-P-Weibchen die meisten Eier auf Kohlpflanzen ab, wenn Erbsenpflanzen zugegen waren. Larven des gleichen Stammes wurden Erbsen- und Kohl-Blattscheiben angeboten, und die Larvenposition sowie der Prozentsatz konsumierter Blattfläche wurden in bestimmten Zeitintervallen notiert. Dies

ergab, dass DBM-P-Larven ihre neue Wirtspflanze Erbse bevorzugten, während DBM-Cj-Larven ausnahmslos auf Kohl fraßen. Somit unterscheiden sich das larvale und das adulte Verhalten des DBM-P-Stammes voneinander.

Die Untersuchung der genetischen Basis der Anpassung der Larven des DBM-P-Stammes an und deren vollständige Entwicklung auf Erbsen erfordert zunächst die Ermittlung der Anzahl an Genen, die bei der Anpassung an die Wirtspflanze Erbse involviert sind, ihre Expressionsmuster (dominant oder rezessiv) und Interaktionen. Zu diesem Zweck wurden die larvalen Überlebensraten auf der neuen Wirtspflanze Erbse sowie einem typischen Kruziferen-Wirt (Kohl) in reziproken F_1-, F_2- und Rückkreuzungen zwischen Individuen des DBM-P Stammes und eines Stammes, der an Kohl angepasst ist (DBM-Cj), gemessen. Rückgekreuzte Individuen wurden dann für eine spätere Kopplungsanalyse mit AFLP-Markern genotypisiert. Zu DBM-P rückgekreuzte Individuen wiesen auf Erbse eine höhere Überlebensrate auf als DBM-Cj-Rückkreuzungen, was auf eine rezessive Vererbung schließen lässt. Es wurde kein Beweis für einen geschlechtsgebundenen Vererbungsgang gefunden. Die Kopplungsanalyse mit drei Rückkreuzungsfamilien ergab, dass zwei, vier bzw. fünf Kopplungsgruppen zum Überleben auf Erbse beitragen, was auf eine oligogene Vererbung hindeutet. Die neu erworbene Fähigkeit, auf Erbse zu überleben, hat somit eine komplexe genetische Basis und die variierende Anzahl an Kopplungsgruppen, die für dieses Merkmal verantwortlich ist, deutet an, dass der DBM-P Stamm genetisch noch heterogen ist und die Allele, die ein Überleben auf Erbse gewähren, noch nicht fixiert sind.

Um schließlich die molekularen Mechanismen, die für die Anpassung an die Wirtspflanze und die Erweiterung auf Zuckererbse erforderlich sind, zu verstehen, wurden zwei Techniken zur Transkriptomprofilierung eingesetzt: cDNA-AFLP und subtraktive Suppressions-Hybridisierung (SSH), die auch für Nicht-Modell-Organismen wie die Kohlmotte anwendbar sind. SSH ergab eine weitgehende Veränderung des Transkriptomprofils der auf Erbsen fressenden DBM-Larven, an der eine Vielzahl an Genen mit unterschiedlichen Funktionen (Olfaktion, Metabolismus, Detoxifizierung, Stressreaktion) beteiligt ist, die für die Erweiterung des Wirtspflanzenkreises auf Erbsen verantwortlich sind. Mithilfe der cDNA-AFLP-Methode konnte dieses Muster bestätigt werden, und es wurden weitere Transkripte mit verändertem Expressionsmuster in DBM Stämmen identifiziert, wenn diese auf Kohl, Erbse oder einer künstlichen Diät fraßen.

Diese Resultate lassen vermuten, dass sich die Erweiterung des Wirtspflanzenkreises des DBM-P-Stammes noch im Anfangsstadium befindet, wobei die Larven die neue Wirtspflanze

Erbse bevorzugen, aber die Eiablagefreudigkeit der adulten weiblichen Tiere auf Erbse fehlt. Die genetische Grundlage der Wirtspflanzenkreiserweiterung von DBM-P sind mehrere Gene, deren Allele autosomal vererbbar und noch nicht fixiert sind. Dies war angesichts des innerhalb kurzer Zeit erfolgten Befalls des Erbsenfeldes in Kenia erstaunlich. Wahrscheinliche, die Wirtspflanzenkreiserweiterung beeinflussende Kandidatengene sind solche, die für Wirtspflanzenwahrnehmung, Verdauung und Detoxifizierung verantwortlich sind und in DBM-P differentiell exprimiert sind. Die Hauptaufgabe der Zukunft wird es sein, die tatsächlichen Gene zu identifizieren, die dieser Wirtspflanzenkreiserweiterung zugrunde liegen. Dies wird es ermöglichen, die Variabilität dieser Gene in anderen DBM-Populationen zu erforschen und damit das Potenzial dieser Populationen zu möglichen Veränderungen des Wirtspflanzenspektrums abzuschätzen.

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12. Selbständigkeitserklärung

Die zur Zeit gültige Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität ist mir bekannt. Die vorliegende Arbeit wurde von mir selbst und nur unter Verwendung der angegebenen Hilfsmittel erstellt und alle benutzten Quellen angegeben. Alle Personen, die an der experimentellen Durchführung, Auswertung des Datenmaterials oder bei der Verfassung der Manuskripte beteiligt waren, sind benannt.

Es wurde weder bezahlte noch unbezahlte Hilfe eines Promotionsberaters in Anspruch genommen.

Die vorliegende Arbeit wurde bisher weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch bei einer anderen Hochschule als Dissertation eingereicht.

Jena, den 2011 ……

Kathrin Henniges-Janssen

13. Curriculum Vitae

Personal data

Name:	Kathrin Henniges-Janssen, née Janssen
Date of birth:	24.04.1981
Place of birth:	Ibbenbüren, Germany
Home address:	17, Rue Marie-Adélaïde, 2128 Luxembourg
E-mail:	khennigesjanssen@gmail.com, kjanssen@ice.mpg.de
Marital status:	married, 1 child

Scientific career

since 6/2006	PhD student at the Max-Planck-Institute for Chemical Ecology, Jena and Friedrich-Schiller-University, Jena Advisors: Prof. David G. Heckel and Prof. Astrid T. Groot
01/2009 – 03/2010	Parental leave
10/2004 – 06/2006	Master of Science in Agricultural Sciences at the University of Hohenheim, Stuttgart Major: Crop Sciences, Specialisation in Phytopathology
09/2000 – 09/2004	Bachelor of Science in Agricultural Sciences at the University of Hohenheim, Stuttgart Major: Crop Sciences

Education

2000	Abitur at Grimmelshausen Gymnasium, Gelnhausen

Stays abroad

2004	DAAD scholarship for research training at the University of Adelaide, Australia in the 'Laboratory of Insect Molecular Biology' of Prof. Otto Schmidt (2 months)
2002	Erasmus scholarship for exchange semester at the Royal Veterinary and Agricultural University of Copenhagen, Denmark (1 semester)

Practical trainings

2004	Summer School 2004, Euroleague for Life Sciences "Pathogens, Parasites and their Hosts – Ecology, Molecular Interactions and Evolution" at the University of Hohenheim, Stuttgart (1 month)
2003 – 2005	Student assistant to the academic adviser of the Faculty of Agricultural Sciences at the University of Hohenheim, Stuttgart
2002	Hessen Department of Agriculture, Gelnhausen, Farming extension service for plant protection and dairy (6 weeks)
2001	Kraft-Wald GbR, Bensheimer Hof, Riedstadt, Agricultural cooperation for plant breeding (2 months)
2000	Sonnenhof, Buseck, Organic farm for dairy cattle and cereal crops (3 months)
1999	Frankfurt Zoological Garden, Great apes (2 weeks)

Affiliation	Deutsche Gesellschaft für allgemeine und angewandte Entomologie
Reviewer	Bulletin of Entomological Research

14. Publications

Henniges-Janssen, K., Reineke, A., Heckel, D.G. and Groot, A.T. (2011). Complex inheritance of larval adaptation in *Plutella xylostella* to a novel host plant. Heredity, (in press). Accepted 18 February 2011.

Henniges-Janssen, K., Schöfl, G., Reineke, A., Heckel, D.G. and Groot, A.T. (2011). Oviposition of diamondback moth in the presence and absence of a novel host plant. Bulletin of Entomological Research 101, 99-105.

Janssen, K., Reineke, A., Scheirs J., Zebitz, C.P.W. and Heckel, D.G. (2008). A host shift of diamondback moth from crucifers to peas: Life history traits and genetic mechanisms. In: Shelton, A.M., Collins, H.L. and Zhang, Y. (editors). The management of Diamondback Moth and Other Crucifer Pests: Proceedings of the 5th International Workshop, Beijing 2006. China Agricultural Science and Technology Press, Beijing. pp. 55-62.

Presentations

Henniges-Janssen, K., Heckel, D.G., Vogel, H. and Groot, A.T. (2010). A new taste for pea: Unravelling the genetics of diamondback moth's recent adaptation to sugar pea. Fribourg Ecology & Evolution Days 2010 ‘Evolutionary and ecological genomics of adaptation’ Fribourg, Switzerland. Poster

Henniges-Janssen, K., Groot, A.T., Vogel, H., Heckel, D.G. und Reineke, A. (2008). Genetische Basis des Wirtswechsels der Kohlmotte *Plutella xylostella* von Kruziferen auf Erbsen. 56. Deutsche Pflanzenschutztagung, Kiel, Germany. Poster

Henniges-Janssen, K., Reineke, A., Scheirs, J.A., Groot, A.T. and Heckel, D.G. (2007). Genetic basis of host range expansion in the diamondback moth. 11th Congress of the European Society for Evolutionary Biology, Uppsala, Sweden. Poster

Janssen, K., Reineke, A., Scheirs, J.A., Zebitz, C.P.W. and Heckel, D.G. (2006). A host shift of diamondback moth from crucifers to peas: Life history traits and genetic mechanisms. 5th International Workshop on Management of the Diamondback Moth and Other Crucifer Insect Pests, Beijing, China. Oral presentation

Janssen, K., Reineke, A., Scheirs, J.A., Zebitz, C.P.W. and Heckel, D.G. (2006). A host shift of diamondback moth from crucifers to peas: Life history traits and genetic mechanisms. The seventh International Workshop on Molecular Biology and Genetics of the Lepidoptera, Crete, Greece. Poster