Integration of host plant resistance and biological control:

Using *Arabidopsis*-insect interactions as a model system

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CONTENTS

Abstract

Two main methods in sustainable pest control are host plant resistance and biological control. These methods have been developed in isolation. However, host plant characteristics can decisively affect the effectiveness of biological control agents, and therefore when altering plant characteristics in a breeding programme, the implications for biological control should be studied as well. Moreover, this may also provide the opportunity to breed for plants that are optimally compatible with biological control agents.

Breeding for host plant resistance has a long history. However, in the past decades a new development was to use transgenes to generate plants resistant to pests and diseases. In more recent years also Plant genetic engineering makes it possible to transfer a foreign gene to a host plant to introduce resistance against insects or to produce a volatile which can attract the natural enemy of the pest after the host plant is wounded by insect herbivores. In this thesis I have modified direct and indirect defence to herbivores in *Arabidopsis* plants through a transgenic approach. In order to evaluate transgenic *Arabidopsis* with different genes that influence direct and indirect defence to the herbivore *Plutella xylostella (*diamondback moth, DBM).

I investigated the effect of a protease inhibitor from mustard plants (Mustard Trypsin Inhibitor 2, MTI2). MTI-2 transgenic *Arabidopsis* did not affect the performance of a Dutch and a Chinese strain of DBM. My data show that the gut enzymes of DBM are insensitive to MTI2, which can be explained by the specific inactivation of MTI2. DBM has apparently developed MTI2 inactivation as a way to protect itself against this protease inhibitor. This makes ecological sense as MTI2 is part of the defense of its brassicaceous host plants.

Terpenoids are among the plant volatiles involved in indirect defence of many plant species. I exploited the availability of a linalool synthase (LIS) gene to develop transgenic herbivore-inducible linalool-producing *Arabidopsis* plants and developed Arabidopsis plants with this linalool synthase gene under the protease inhibitor 2 (PI2) promoter from potato. As a result the linalool synthase was inducible by methyl jasmonate application, but not by *P. xylostella* infestation.

To investigate a transgenic approach to combining direct and indirect defence, a gene encoding a Bt toxin and a gene encoding linalool synthase were integrated into *Arabidopsis* as a model. The data show that hybrid lines carrying Bt and LIS genes in Arabidopsis combine toxicity to the DBM larvae (due to *Bt*) and repellence to the adults of DBM (due to linalool). Moreover, the linalool emission also affected parasitoid behaviour when the plants were damaged. DBM-infested dual transgenic plants were more attractive to the parasitoid *D. semiclausum* than undamaged dual transgenic plants, but less attractive than DBM-infested untransformed plants. The percentage parasitization of DBM larvae on transgenic plants was not affected by the transgenes.

In conclusion, my data provide interesting options for the development of transgenic crops that interfere with the biology of pests and enhance the effectiveness of the pest's natural enemies. In this way transgenic crops that integrate host plant resistance with biological control may be developed.

Chapter 1

General Introduction

Yang, L.M.

1. Background

The biological control of pests in agricultural systems can be traced back to the third century AD, when Chinese farmers released ants in their crops to reduce pest numbers. However, since the introduction of synthetic pesticides in the 1940s, carnivorous arthropods and beneficial microorganisms have usually been eliminated from crop systems where chemical pest control was practiced (Dicke 1999). Major disadvantages of pesticide use are its negative side-effects on beneficial organisms and other non-target species, the persistence of such synthetic organic insecticides in the environment and a rapid increase of resistance in herbivorous species. To produce cultivars resistant to pests is still the major approach to crop protection against insect pests (Smith, 2005). However, just like insects can develop resistance to pesticides, they can overcome plant resistance too. With the development of the genetic engineering technology, a new possibility has been made available to develop pest-resistant plants. The genes involved in transgenic host-plant resistance programs mainly encode Bt toxins, proteinase inhibitors and lectins. Most research and utilization efforts focus on *Bacillus thuringiensis* (*Bt*) genes. Bt-crops can be considered as possessing a specific form of host plant resistance. However, during its development, possible effects on natural enemies have largely been ignored (Groot & Dicke, 2001). Some studies showed that Bt-crops may not affect the environment and natural enemies of target or non-target pests (Head et al., 2002; Sisterson et al., 2004; Romeis et al., 2006). In most cases, both host-plant resistance and biological control represent self-renewing processes, insofar as the control itself is built into the seed, in the case of host plant resistance, or is present in the crop's environment, in the case of biological control. In both cases, control extends between pest generations over the cropping season and, potentially indefinitely, as long as resistance persists in the crop line and natural enemies persist in the agroecosystem. It is essential to analyze plant-herbivore-natural enemy interactions for crop protection programs in which both host plant resistance and biological control are important components of pest control (Thomas & Waage, 1996).

2. Host plant resistance (HPR) and biological control (BC)

Plants have two ways of defence against herbivores. One is direct defence, which affects herbivores directly through physical or chemical means, such as thorns, toxins, digestibility reducers. The other is indirect defence, which promotes the effectiveness of carnivores (Dicke, 1999). A direct defence is to employ toxins such as alkaloids, phenolics, terpenoids, which are lethal for herbivores in some cases. While another direct defence is to produce substances such as tannins and phenolics as digestibility reducers to delay herbivore development. A plant's indirect defence can promote the performance of carnivores in several ways. For example plant morphological characteristics that can support the activities of natural enemies,

or plant substances prolonging herbivore development which results in the herbivore remaining in a stage that is susceptible to carnivores for a longer period of time, or induced plant volatiles that help carnivores to find their herbivorous prey (Dicke, 1999; Groot & Dicke, 2001).

It has been recognized that many of the plant traits and processes that negatively affect herbivores change following attack. Karban & Baldwin (1997) refer to changes in plants following damage or stress as induced responses. Those induced responses that decrease the negative fitness consequences of herbivore attack on plants are termed induced defences, including induced direct and indirect defences. Induced direct defence normally acts through preventing herbivores from converting a plant's tissues into their own tissues after damage or stress. In induced indirect defences, two types can be distinguished. One is wound-induced change in the production of extrafloral nectar. The other acts through the emission of induced volatile compounds in response to herbivory that attracts predators and parasitoids of herbivores. Many induced responses to wounding are systemic. In such cases the damaged plant tissue may produce a signal that is transmitted systemically throughout undamaged parts of the plant, causing the induction of new morphological or physiological states, the induced response. Several different signal transduction pathways are involved, including chemical and electrical signaling. Signalling compounds include 1) oligosaccharide fragments of plant cell walls; 2) systemin (an oligopeptide); 3) salicylic acid; 4) ethylene, which mediates induced defences such as induced volatile production; 5) abscisic acid; 6) jasmonic acid and methyl jasmonate; and 7) electrical signals (Karban & Baldwin, 1997; León et al., 2001; Lorenzo et al., 2004; Adie et al., 2007).

The volatile compounds that plants emit when they are damaged typically are mixtures of C6-alcohols, -aldehydes, and -esters produced by the oxidation of membrane-derived fatty acids, and terpenoids and aromatic compounds such as methyl salicylate and indole. These herbivore-induced volatiles released by plants were found to increase the foraging efficiency of carnivores, and carnivores may learn to associate the volatiles with actively feeding herbivores (Vet & Dicke, 1992; Karban & Baldwin, 1997; Dicke & van Loon 2000; Turlings & Ton, 2006).

Plant defences have been exploited by humans to develop two methods of environmentally-benign pest control: plant resistance based on direct defence and biological control based on indirect defence.

Host plant resistance (HPR) can be defined as the inherited property that enables a plant to avoid, tolerate, or recover from injury by insect populations. For crop production, host plant resistance represents the inherent ability of crop plants to restrict, retard or overcome pest infestations and thereby to improve yield or quality of the harvestable product. Three mechanisms of plant resistance to insects are commonly recognized: antixenosis (non-preference), antibiosis, and tolerance. Antixenosis is defined as a relatively low acceptability of a plant as a host to an insect herbivore. Plants that exhibit antixenotic resistance would be expected to have reduced initial infestation or a higher emigration rate of the insect than susceptible plants. The basis of this resistance mechanism can be morphological or chemical. Antibiosis is defined as the mechanism leading to negative effects of a resistant plant on the biology of an insect which has colonised the plant. Both chemical and morphological plant traits can have antibiotic effects. The consequences of antibiotic

resistance may vary from mild effects that influence fecundity, development times and body size, through to acute direct toxic effects resulting in increased insect mortality. Tolerance is the degree to which a plant can support an insect population that under similar conditions would severely damage a susceptible plant. That is, when two cultivars are equally infested the less tolerant one has a smaller yield (Thomas & Waage, 1996).

Biological control is the suppression of a pest population using predators, parasitoids and pathogens. There are three main forms of biological control: 1) conservation of natural enemies already present in the environment; 2) augmentation and dissemination of natural enemies of pests, such as microorganisms, nematodes, and arthropods. 3) classical biological control—to suppress a pest population permanently through a single introduction of a natural enemy (Thomas & Waage, 1996). A parasitoid is an insect that as a juvenile, lives at the expense of another one (host) and it usually does not kill its host immediately but at the end of juvenile development. Parasitoids may parasitize eggs, larvae, pupae or adult hosts and each species is usually highly specific in this. A predator is an insect that eats more than one other organism during its life and kills its prey immediately. Predators are normally larger than their prey and are generally much more generalistic than parasitoids. In a successful biological control programme the pest kill rate of effective natural enemies should be always higher than the potential maximum rate of population increase of the pest species (Dicke, 1996; Hawkins & Cornell, 1999).

The natural enemies of herbivores can be influenced by plant characteristics independently of the herbivore or mediated through herbivore activities. The relevant plant characteristics include the following aspects: i) plant tissues and products that are used as a source of nutrition by natural enemies; ii) plant morphology; iii) visual and vibrational cues; iv) secondary plant chemicals; v) plant volatiles (Thomas & Waage, 1996).

Many pest management practitioners have found that host plant resistance is fundamentally compatible with biological control (Verkerk et al, 1998). Positive interactions result where natural enemies use herbivore-induced synomones emitted by plants as cues to find prey (Vet & Dicke, 1992; Dicke et al., 2003; Arimura et al., 2005; D'Alessandro et al., 2006). Partial plant resistance may also provide benefits for the third trophic level by reducing the growth rate of prey which in turn increases the duration of their availability to natural enemies (Feeny, 1976; Price et al., 1980). However, in some cases, negative interactions between plant resistance and biological control are caused by toxic secondary plant compounds which can be passed on through herbivores to their natural enemies (Hare, 1992; Harvey et al. 2003). They may also be caused by plant physical factors which can impede natural enemy effectiveness (e.g. leaf toughness, cuticle thickness, trichomes (Price, 1986). Therefore it is important to integrate HPR and BC dexterously.

In the future, fundamental and applied pest control research will have to be more closely integrated and coordinated to increase efficiency. In this project, the integration of host plant resistance and biological control will be studied.

3. Herbivore-induced plant volatiles as an important connection in tritrophic systems

Plants, herbivores, and natural enemies of herbivores in food webs of living plants are strongly dependent on each other. Traits of individual plants may modify interactions between herbivores and their enemies. When plant defensive traits result in rapid death of

herbivores, the opportunity to influence interactions between herbivores and enemies will be minor. Herbivores may either evolve resistance or avoidance responses. In this case, enemies could not play a role. Digestibility-reducers differ in their effects on herbivores from toxins through exerting sublethal effects such as impairing growth, reducing resistance to disease, and reducing fecundity. Digestibility reducers usually delay development of larvae, resulting in longer exposure to their enemies, thus increasing mortality. They may also affect herbivore-enemy interactions by causing decreased herbivore body size and hence fecundity. Mustard Trypsin Inhibitor 2 (MTI2), a serine proteinase inhibitor, expected to be detrimental to *Plutella xylostella*, is used in this thesis for the purpose of delaying larval development, and thus to increase the attack opportunities for a parasitoid of *P. xylostella*.

The chemical information from plants can be exploited both by herbivores and carnivores during foraging for food. The volatiles constitutively produced from intact plants generally do not provide relevant information to foraging carnivores while herbivore-induced plant volatiles can present a reliable cue for carnivores. The volatiles released from damaged plant tissue are in many cases systemic and they differ from those released upon herbivore feeding and mechanical damage (Dicke & van Loon, 2000). The biosynthesis of terpenoids can be induced by the application of jasmonic acid or its methyl ester, methyl jasmonate (Xu et al., 1993; Martin et al., 2003). In cucumber and Lima bean many of the volatiles, including the acyclic terpenes (*E, E*)- α -farnesene, (*E*)- β -farnesene, (*E*)- β -ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, are biosynthesized *de novo* following herbivore damage. Other volatile constituents are synthesized from stored intermediates (Paré & Tumlinson, 1997; Bouwmeester et al, 1999). Expressing a (3S)-(*E*)-nerolidol synthase from strawberry in *Arabidopsis* indeed results in the production of (3S)-(*E*)-nerolidol and 4,8-dimethyl-1,3(*E*),7-nonatriene and in attraction of a carnivorous mite (Aharoni et al., 2003; Kappers et al., 2005). Linalool, an acyclic monoterpene alcohol, is one of the volatile compounds released as an infochemical after herbivore attack in some plant species. It may attract predators or parasitoids of the herbivores (Dicke et al., 1990). In the current project this last characteristic of linalool will be exploited.

4. *Arabidopsis* as an important model plant in this project

Arabidopsis thaliana (L.) Heynh., or thale cress, is a small old-world weed in the mustard or crucifer family (Brassicaceae syn. Cruciferae), which has long been a research object for plant genetics. It has a short life cycle and can go from seed to seed in 8—12 weeks. Individuals are usually self-pollinating and tend to be highly homozygous at all genetic loci. However, outcrossing can be performed manually and may occur in nature as *Arabidopsis* flowers emit volatiles, suggesting the involvement of pollinators (Chen et al., 2003). *Arabidopsis* plants are prolific seed producers. Because this plant has been well studied at the molecular level, it can be exploited to understand in molecular terms the evolution and distribution of resistance genes and defence mechanisms in 'wild' species.

Arabidopsis has been adopted as a model plant for many aspects of plant biology, hastening development of tools necessary for its emergence as a model in plant-pathogen interactions (Uknes et al., 1992; Axtell & Staskawicz, 2003). A wealth of information is available regarding many aspects of its biology. Its rapid life cycle accelerates Mendelian genetic analysis. Mutagenesis of seeds or pollen via chemical and physical means is

straightforward. Through traditional mutagenic approaches, saturation screens can be developed for traits of interest, and several hundred mutations leading to visible phenotypes have been used to construct a dense genetic map on five linkage groups. *Arabidopsis* is readily transformed with either *Agrobacterium* or naked DNA. The isolation of *Arabidopsis* genes known only through their mutant phenotype and map position is also highly advanced. The nuclear genome is very small compared to other plant species, approximately 120 megabase pairs with an estimated 20,000 genes, and nearly devoid of repetitive DNA. Physical mapping of cosmid contigs covering 90% of the genome is finished (Dangl, 1993). The recombinant inbred (RI) map illustrates locations of cloned genes and molecular markers based on recombination within a defined mapping population produced through repeated selfing of progeny plants in successive generations. Markers on this map include restriction fragment length polymorphisms (RFLPs), simple sequence length polymorphisms (SSLPs), cleaved amplified polymorphic sequences (CAPSs), and a variety of cloned genes, expressed sequence tags (ESTs), and the ends of bacterial (BAC) and yeast (YAC) artificial chromosomes. More than 790 markers are included on the RI map (Meink et al., 1998). Also, a library of genomic *Arabidopsis* DNA, maintained in plant-transformation competent *Agrobacterium* has recently been made available to the research community. Cloning strategies based on identification and isolation of sequences deleted at a locus of interest have also recently been developed for use with *Arabidopsis*. In 2000 the complete genome of *Arabidopsis* has been sequenced, which provides ample opportunities for functional genomics studies. The intense scrutiny given to the *Arabidopsis* genome will revolutionize our understanding of many basic plant processes.

With respect to plant-pathogen interactions, it is particularly important that over 100 land races or ecotypes of *Arabidopsis* from various locations around the northern hemisphere are available. Moreover, it is not difficult to collect *Arabidopsis*, often infected with pathogens, from appropriate climates. Any local genetic variation can be expected to be rapidly frozen after several generations, due to its high rate of inbreeding. Thus, evolution in the presence or absence of a particular pathogen can be expected to lead to natural variation in resistance-related gene functions.

Only a few studies on insect-plant interactions have used *Arabidopsis* as a host plant and even fewer focussed on indirect defences. Yet *Arabidopsis* is known to be consumed by insects, such as caterpillars, and these caterpillars are hosts of parasitoids. The work of Van Poecke et al. (2001) indicated that *Arabidopsis* is a good candidate for studying signal transduction in direct and indirect defences in plants (Van Poecke & Dicke, 2004). Their studies showed: 1) Adult females of *Cotesia rubecula*, a specialist parasitoid wasp of *Pieris rapae* caterpillars, were attracted to *P. rapae*-infested *Arabidopsis* plants. 2) *Arabidopsis*, both infested by *P. rapae* and wounded mechanically emits volatiles from several major biosynthetic pathways, including terpenoids, methyl salicylate and green leaf volatiles. The blends from both kinds of plants are similar but not identical. Differences can be found with respect to several components of the blend, such as two nitriles and the monoterpene myrcene, that were produced exclusively by caterpillar-infested plants, and methyl salicylate, that was produced in larger amounts by caterpillar-infested plants. 3) Genes from major biosynthetic pathways involved in volatile production are induced by caterpillar feeding. These include *AtTPS10*, encoding a terpene synthase involved in myrcene production,

AtPAL1, encoding phenylalanine ammonialyase involved in methyl salicylate production, and *AtLOX2* and *AtHPL*, encoding lipoxygenase and hydroperoxide lyase respectively, both involved in the production of green leaf volatiles. Another gene *AtAOS*, encoding allene oxide synthase, involved in the production of jasmonic acid, also was induced by herbivory (van Poecke et al., 2001).

Responses of *Arabidopsis* to various pathogens and insects involve several signaling pathways, including SA, JA and ethylene. The potential contribution of these pathways to defence gene expression was examined by Stotz and co-workers (2000). They determined the influence of ethylene signaling on resistance against two lepidopteran insects, i.e. the diamondback moth (*Plutella xylostella*) and the Egyptian cotton worm (*Spodoptera littoralis*) (Stotz et al., 2000). Related studies show similar results for the role of jasmonate in plant defence against other insects (McConn et al., 1997).

5. Herbivores and carnivores used in this project—*Plutella xylostella* and *Diadegma semiclausum*; *Pieris brassicae* and *Cotesia glomerata*

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae), occurs wherever cruciferous crops are grown and is considered the most important insect pest of crucifers worldwide (Talekar & Shelton, 1993). This insect has an ability to survive a wide range of temperatures. DBM is an oligophagous insect that specializes on Brassicaceae. It will feed on plants that contain mustard oil glucosides (Ooi, 1986). Its eggs are whitish yellow, 0.5 mm in size. There are four larval instars and the total larval period extends from 11 to 21 days depending on rearing temperature. The 1st instar larvae mine into the leaf. The larvae generally stay in the mines for about two days. The 3rd instar larvae generally feed on mature leaves for two to three days. The 4th instar larvae consume the largest quantity of leaf tissue and last for 2 to 4 days, excluding the prepupal period, depending on temperature. Pupation takes place in a loose mesh of silken cocoon spun by the caterpillar. Adults are brownish-yellow moths with a wing expansion of 14 mm. Their longevity ranges from 3 to 11 days. Adults were found to emerge during the evening and rarely in the morning hours. The moths mate at dusk on the day of emergence. Mating lasts one to two hours and females mate only once. Most females lay eggs on the day of emergence. Oviposition begins in the evening and proceeds during the scotophase. Typically, eggs are laid in depressions on the leaf along the midrib and larger veins or on concave surfaces near smaller veins. The majority of the eggs are laid on the upper leaf surface. The oviposition period extends up to 10 days with peak oviposition occurring on the day of emergence. The fecundity ranges from 70 to 200 eggs/female and is increased when exposed to increased photoperiod (Chelliah & Srinivasan, 1986; Harcourt, 1986).

The secondary chemistry of the Brassicaceae is characterized by the presence of glucosinolates. Glucosinolates have been shown to adversely affect the growth and survival of many bacteria, fungi and mammals and are generally toxic to insect species which do not usually feed on Brassicaceae (e.g. generalist feeders) and feeding on such plants may cause death or at least drastically reduced fitness. *Plutella xylostella* possesses a sulfatase enzyme as an adaptation to detoxify glucosinolates (Li et al., 2000; Ratzka et al. 2002; Sarfraz et al., 2006; Després et al., 2007). In contrast, specific glucosinolates (or derivatives) are known to be powerful feeding and oviposition stimulants to crucifer-specialist insects (Reed et al.,

1989; van Loon et al., 2002; Renwick et al., 2006).

Several factors contribute to the pest status of this species: its high reproductive potential, wide range of alternative weed hosts, its dispersal ability and its demonstrated capacity to develop resistance to a wide range of insecticides (Schroeder et al., 2000). The increasing failure of insecticides to control *P. xylostella* has stimulated the development of alternative tactics including inoculative release of parasitoids, conservation of natural enemies, mating disruption using pheromones, and various cultural practices (Talekar & Shelton, 1993). A wide range of parasitoids has been associated with *P. xylostella*. However, Lim (1986) asserts that key parasitoids capable of exerting significant levels of control over *P. xylostella* belong only to the hymenopteran genera *Diadegma*, *Cotesia* and *Microplitis*. The genera *Diadegma* (Ichneumonidae) and *Cotesia* (Braconidae) are regarded as the most important primary parasitoids of *P. xylostella*. It was found that the presence of silk on leaves stimulated searching by the parasitoid to a greater extent than clean leaves, but to a lesser extent than leaves with both hosts and silk. Lim (1982) showed that floral nectaries could significantly increase parasitoid female longevity and consequently the duration of potential oviposition. It was found that host plant species could have a significant effect on parasitism success, e.g. parasitism by the solitary *C. plutellae* was greatest with larvae on *B. pekinensis* compared with larvae on *B. oleracea* var. *capitata*, *B. oleracea* var. *botrytis* and *B. oleracea* var. *italica* (Verkerk & Wright,1996). Schuler et al. (1999) using Bt-resistant *P. xylostella* evaluated indirect effects of Bt toxins on the biology of *C. plutellae*. In a choice test that compared Bt leaves damaged by either Bt-resistant hosts or Bt-susceptible hosts, most of the *C. plutellae* flew to the Bt leaves damaged by resistant hosts (*P. xylostella*). The reason for this most likely is that resistant caterpillars feed more than non-resistant caterpillars (Schuler et al., 1999). A recent study demonstrated that *Arabidopsis* is a suitable model plant for studying plant-*Plutella*-parasitoid interactions (Barker et al., 2007). Shiojiri et al. (2000) observed the host-searching behaviour of *C. plutellae* on a cabbage plant infested by *P. xylostella* larvae, and found that the wasps showed their antennation behaviour only on the host-infested site. The searching time of *C. plutellae* on cabbage leaves infested by host larvae was significantly longer than that on leaves infested by nonhost (*Pieris rapae*) larvae and that on artificially damaged leaves (Shiojiri et al., 2000).

Diadegma semiclausum (Hellen), one of the most important natural enemies of *Plutella xylostella*, is a solitary larval endoparasitoid. The size of the adult female varies from 5.0-7.0 mm and that of the male from 4.4-6.4 mm. The egg-to-adult development time ranges from 13-21 days depending on temperature. The wasps prefer to parasitize 2nd and 3rd instar *P. xylostella* larvae. Most of the activities related to reproduction, such as mating and oviposition, are stimulated by daylight (Azidah et al., 2000).

Pieris brassicae, the large cabbage white butterfly, is one of the main pests in *Brassica* crops, such as cabbage (*B. oleracea*), Chinese cabbage (*B. rapa* L.), oilseed rape (*B. napus* L.). The larvae feed on the leaves or flowers of the plants (Smallegange et al., 2007). The final instar larvae can cause severe damage to *Brassica*-crops.

Cotesia glomerata, a gregarious endoparasitoid of *P. brassicae*, is a braconid wasp that develops within the larvae of *P. brassicae*. The adults are black, ca. 5 mm long, and feed on nectar of flowers. Adult female wasps parasitize generally first and second instars, and they lay up to 25-30 eggs inside each host larva. The caterpillar continues its own development

until the wasp larvae emerge from its body to spin their cocoons in a group. The life cycle, from egg to adult, is approximately 22-30 days, depending on the temperature.

The main tritrophic system studied in this thesis is *Arabidopsis-Plutella xylostella – Diadegma semiclausum* (figure 1). For some experiments also *Pieris brassicae* and *Cotesia glomerata* will be used.

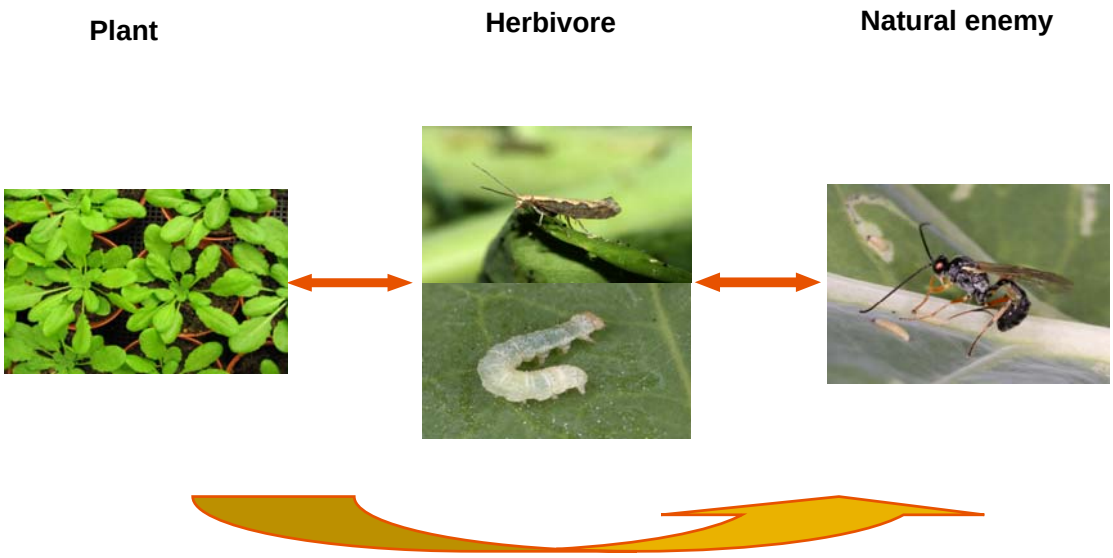

Figure 1. The main tritrophic system of *Arabidopsis -Plutella xylostella-Diadegma semiclausum* used in this project. Pictures of the insects by Tibor Bukovinszky (www.bugsinthepicture.com).

6. Transgenic approaches to combine direct and indirect plant defences

Within a decade after the first discoveries of transgenic "model" plants, gene transfer has become an established and routine technique in numerous laboratories around the world. Gene transfer became a reality in the middle of the 1970s soon after it was discovered that the soil bacterium *Agrobacterium tumefaciens* contained a plasmid, part of it being transferred to competent plant cells. Host range limitations of *Agrobacterium*-mediated gene transfer prompted the search for alternative gene transfer systems, leading soon to the development of "direct gene transfer to protoplasts". Further limitations in both gene transfer systems led to the exploration of a great variety of further approaches such as pollen transformation, pollen tube pathway, electroporation, microlaser, liposome-fusion and liposome-injection, macroinjection, and direct DNA application in numerous variations. However, *Agrobacterium*-mediated gene transfer to tissues has remained the major technique, which has not only allowed efficient and routine production of transgenic "model plants", but which has also opened the route for genetic engineering of all major crop plants. There are additional novel techniques, including tissue-electroporation, silicon carbide fiber-mediated transformation, micro-injection, site-directed recombination (Hennet et al., 1995; Zhang et al., 1996) and microtargeting, which may become important (Potrykus & Spangenberg, 1995). Production of transgenic plants requires reliable gene transfer techniques and also requires the cells which have received and integrated the transgene in

their genome. These cells should have the capacity to regenerate to fertile plants, and they are provided with external conditions which allow them to do so. It finally also requires establishing proof for integrative transformation (Potrykus & Spangenberg, 1995).

The ability to suppress endogenous genes in crop plants and to introduce novel genes from diverse sources, including bacteria, fungi, and exotic plants, opens up many opportunities not available through mutation and conventional breeding techniques. These approaches are largely focused on traits such as disease resistance, herbicide resistance, insect resistance, etc. (Knauf, 1995). Strong et al. (1990) argued that transgenic techniques need to be refined so that toxins are only expressed in a subset of crucial tissues and at specific developmental stages and they should also be integrated into an ecological framework if they are to be effective and contribute to biological control. Van Emden and Wratten (1991) warned that modern gene transfer techniques aimed at creating resistant crop varieties are more likely than traditional plant breeding methods to use an allelochemical mechanism of resistance (antibiosis) which might be damaging to natural enemies. Recent work has shown, however, that the potential also exists for creating genetically engineered plants which emit increased amounts of natural enemy-attracting volatiles (synomones) (Verkerk et al., 1998; Aharoni et al., 2003; Kappers et al., 2005; Schnee et al., 2006;). The latter approach will be attempted in the present project, using genes of plant origin.

There is considerable interest in the use of inducible systems for the expression of genes introduced into plants, not only because they allow expression of genes which may, for example, be developmentally lethal, but also because they allow for controlled experiments to be performed in a true isogenic background. Such systems also find use in the manipulation of levels of expression in order to understand more fully individual gene function, or to provide a means for the overproduction or deletion, by reverse genetics, of a particular gene product (Salter et al., 1998; Li et al., 2005). This is a rapidly developing area of research in plant molecular biology. The need for inducible expression systems is high, not only for their obvious use as research tools, but also for the potential of inducible expression of desired characters in crops. A wide range of promoter systems can be envisaged which could potentially allow inducible control of genes to be introduced into plants. These could be broadly described as falling into three general categories.

Firstly, there are those which rely on plant-based developmental processes. Such promoters could, for example, include those regulated by plant hormones or which are otherwise developmentally regulated. The advantage of such systems is clearly that all components of the necessary signal transduction pathways are already present in the plant. They also provide a means for the coordinated expression of a gene product within a defined stage of plant growth and development.

The second category of promoter systems includes control sequences which respond to particular environmental signals. These potential control systems include heat-shock and senescence-specific promoters, as well as systems which are responsive to nutritional status or wounding by mechanical damage or herbivory. These sorts of promoter systems may be profitable for controlling expression of characters in the field, as opposed to the laboratory situation. This is because no application of specific inducers or defined conditions for growth are necessary, and the desired expression of a gene at a particular growth stage of the plant could be ‘self-regulating’. In this thesis, I adopt the use of the wound and herbivore inducible

promoter of the Proteinase Inhibitor II from potato (An et al., 1989; Palm et al., 1990; Duan et al., 1996).

The third group of promoters are those which are introduced from non-plant backgrounds. This includes animal hormone receptor/activators, antibiotic resistance control mechanisms from bacteria and promoters responsive to chemical inducers. Such systems require the introduction of the appropriate transcription factor systems into the plant background together with the inducible promoter. They have the potential advantage that signal transduction systems are therefore unique to the gene which is being induced and allow timing of expression which is totally independent from the timetable of plant processes and from plant transcription factors (Tomsett et al., 2004; Li et al., 2005).

Genes coding for proteins that are part of plant natural defence systems have been characterized and transferred to plant species in which they do not naturally occur. These proteins e.g. protease inhibitors, lectins, peroxidases, interfere with digestion of plant food. In this project the mustard trypsin inhibitor 2 (MTI2) which belongs to a class of serine protease inhibitors (see paragraph 7) and *Bt* (SN19) which belongs to the Cry-1 proteins of *Bacillus thuringiensis* (see paragraph 8 below), will be used for the research on direct plant defence. Linalool synthase (see paragraph 9) will be used with both constitutive and wound-induced promoters for the research on indirect plant defence. Transgenic approaches followed by crossing of different single transgenic lines may integrate direct and indirect defence of the host plant.

7. Protease inhibitors (PIs)

Most protease inhibitors are not directly toxic to herbivores but reduce their efficiency to digest dietary protein by inhibiting gut proteases. Plant protease inhibitors in leaves are usually induced by herbivore feeding and constitutively expressed in storage organs such as tubers, unripe fruits and seeds all with the apparent aim to prevent unwanted (tubers, seeds) or premature (fruits) herbivory. Insects that feed on plant material possess specialized midguts with micro-environments adapted for digestion of plant compounds. In response to insect attack, plants mount a counter-defence consisting of a number of protease inhibitors that are active against different classes of proteases. Insects may counter-adapt to plant PIs. In several cases larvae of different species were not affected by the PIs in their diet, even though it was shown that the inhibitors had bound to midgut proteases (Jongsma et., 1995; Bolter & Jongsma, 1995; Broadway, 1996; Jongsma and Beekwilder, 2008). It was shown that a wide range of insect species, using different classes of proteases for protein digestion, all adapt to plant PIs by over-expressing PI-insensitive proteases or by proteolytically degrading the inhibitors to different degrees, usually inducing only some delay in larval development. However, this delay may affect the success of natural enemies by increasing the ability and success of natural enemies to parasitize and knock down the herbivore population (Thaler, 2002).

Protease inhibitors expressed in transgenic plants and ingested through normal feeding by an insect will be exposed to a series of complex biochemical and physiological processes in the midgut region of the intestinal tract, the primary interface between phytophagous insects and their trophic environment. The degree to which the ingested inhibitor can interact with its target enzyme within this complex system will affect its usefulness as a pest control agent. In

addition to information obtained from direct *in vitro* and *in vivo* studies of enzyme inhibitor interactions, a detailed knowledge of the overall digestive process itself, in a given pest insect species, will maximize the likelihood of a successful use of these inhibitors in a pest management program. The small size of many insects complicates the analysis of food breakdown and nutrient utilization (Van Loon et al., 2005). Nevertheless, there has been excellent progress in the understanding of the biochemistry and physiology of many aspects of insect digestion (Carozzi & Koziel, 1997).

Inhibitors of serine proteases are the most extensively characterized and they are currently subdivided into at least eight families of structurally different molecules. In seeds of mustard, two different trypsin inhibitors, MTI and MTI2 have been identified. MTI2 was the first member of this protease inhibitor family which was found in the Brassicaceae family. In mustard the gene encoding the MTI2 protein is expressed in seeds towards the end of maturation and in leaves only after wounding (Volpicella et al., 2000). Tobacco and *Arabidopsis* transgenic plants expressing the mustard trypsin inhibitor 2 (MTI2) at different levels resulted in opposite effects on larval development. Plant lines with expression levels within the range previously shown to provide insect resistance in transgenic plants resulted in a reduced developmental rate of the larvae, together with a reduction of leaf damage, thus providing effective pest resistance to the plant. On the contrary, when MTI2 was expressed at lower levels in tobacco plants, the larvae of *Sposoptera littoralis* developed faster, were bigger than on control plants, and caused more damage to the leaves (De Leo et al., 1998). The mechanisms underlying this increased rate of development and leaf consumption are still unknown. The opposite effects observed in larvae fed leaves expressing high or low levels of MTI2 suggest the occurrence of a sensitivity threshold in *S. littoralis* toward MTI2. The design of a pest-defence strategy for a crop using a particular PI may therefore be dependent on the assessment of the sensitivity threshold of the target pest toward the chosen PI (De Leo et al., 1998). In the study of De Leo et al (2001), the effects of mustard trypsin inhibitor MTI2 expressed at different levels in transgenic tobacco, *Arabidopsis* and oilseed rape lines were evaluated against *Mamestra brassicae* (L.), *Spodoptera littoralis* (Boisduval) and *Plutella xylostella* (L.): *P. xylostella* larvae were most sensitive to the ingestion of MTI2. The inhibitor expressed at high levels in *Arabidopsis* plants caused rapid and complete mortality. High mortality was also detectable in oilseed rape expressing MTI2. Based on these and other results the design of a crop protection strategy based on PIs should consider at least the following parameters: 1) expression should be high enough to avoid stimulation of larval development 2) both larval mortality and larval development should preferably be affected by the right choice of inhibitor(s) and a sufficient level of expression; and 3) any adaptation of each insect species to PIs both in terms of induction of insensitive proteases and specific degradation of inhibitors should be assessed (Jongsma et al., 2008) .

8. *Bacillus thuringiensis (Bt)*

Bacillus thuringiensis (*Bt*), existing in many locations, is a gram-positive soil bacterium that forms parasporal crystals during sporulation. The parasporal crystals consist of one or more δ-endotoxins or crystal (Cry) proteins. Following ingestion, the alkaline environment of the insect midgut causes the crystals to dissolve and release their constituent protoxins. The protoxins are subsequently proteolytically converted by gut proteases to an N-terminal, 65-70

kDa truncated form – the activated toxin. The toxin binds to specific receptors on the cell membranes of the midgut epithelial cells, inserts itself into the membrane, and generates pores that disturb osmotic balance and thus kill the epithelial cells (Höfte & Whiteley, 1989; de Maagd et al., 1999).

Bt δ-endotoxins are a big group of homologous proteins and still increasing in number– more than 390 Cry genes have been identified to date (http://epunix.biols.susx.ac.uk/Hom/Neil_Crickmore/Bt/index.html). These genes form a rich source of diversity in insect specificities. This specificity is an important aspect of the *Bt* Cry proteins: each protein is active only in species within one or two insect orders. The members of the Cry gene family have been grouped in subfamilies according to their specificity for members of the insect orders Lepidoptera, Diptera, and Coleoptera (Schnepf et al., 1998; de Maagd et al., 1999).

The use of *Bt* formulations (spore and crystal mixtures) as insecticidal sprays can be traced back to the 1930s, but large scale production started in the late 1950s. Several factors restricted the efficiency of pest control using *Bt* formulations: lack of stability, failure to penetrate plant tissues, and too narrow a specificity. The first two problems have been effectively solved by creating transgenic plants that express the crystal proteins. The problem of narrow specificity may be overcome by expression of pyramiding/stacking Bt genes (Peferoen, 1997; de Maagd et al., 1999). In the early 1980s, when *Bt* crystal protein genes were being cloned and the stable transformation of plants using *Agrobacterium tumefaciens* was demonstrated, efforts were directed towards the engineering of *Bt* genes into plants. At the beginning of transgenic studies, both full-length and truncated *Cry* genes were introduced into tobacco and tomato by *Agrobacterium tumefaciens*-mediated transformation (Vaeck et al., 1987; Fischoff et al., 1987; Barton et al., 1987), but it soon became clear that only truncated versions of *Cry* genes resulted in plants with significant levels of the insecticidal protein and thereby protected from feeding damage by the pest insects. Since then numerous studies focused on Bt transformation. An interesting innovation made use of the *Bt cry*2Aa2 operon to demonstrate operon expression and crystal formation via the chloroplast genome (De Cosa et al., 2001) and a chemically inducible promoter-*Bt cry*1Ab expression cassette was transferred into broccoli plants (Cao et al., 2006).

The first insect-resistant transgenic crops expressing genes from *Bt* are being grown commercially in a number of countries since 1996 (Bates et al., 2005). In the USA, the six biotechnology-derived crops planted in 2003 produced an additional 2.4 million tons of food and fibre and increased farm income by US$1.9 billion. Meanwhile, these crops reduced the use of pesticides by 21000 tons. However, the expanding cultivation of transgenic crops is at risk of losing these advantages to the evolution of resistance in the targeted insect pests (Christou et al., 2006). In the laboratory, fitness costs have been observed with some resistant strains of Indian meal moth (*Plodia interpunctella*), Pink bollworm (*Pectinophora gossypiella*), diamondback moth (*Plutella xylostella*), Colorado potato beetle (*Leptinotarsa decemlineata*), cabbage looper (*Trichoplusia ni*), and cotton bollworm (*Helicoverpa armigera*). These fitness costs will ensure that without selection pressure the wild types will predominate. However, diamondback moth and the corn earworm (*Helicoverpa zea)* have been reported to have evolved resistance to Bt also in open field conditions (Bates et al., 2005; Zhao et al., 2005; Tabashnik et al., 2008). Due to the current commercial significance of *Bt* crops

strategies for resistance management are urgently required. There are at least eight possible types of tactics to slow selection for *Bt*-insensitivity by transgenic plants: 1) express toxin genes only moderately; 2) modify the expression of the genes through tissue-specific, time-specific or inducible promoters; 3) express the toxins to levels as high as is agronomically acceptable; 4) deploy different toxins individually in different varieties simultaneously; 5) deploy different toxins sequentially; 6) deploy plants with a mixture of toxins (pyramid); 7) leave non-transgenic crop as 'refuges' for susceptible insects; and 8) deploy the crops as part of an overall integrated pest management program. From the list above, strategies 6) (pyramid multiple toxins in the same plants) and 7) (refuges) are most popularly used in resistance management (Roush, 1998).

9. Linalool synthase (LIS)

Linalool, 3,7-dimethyl-1,6-octadien-3-ol, is an acyclic monoterpene alcohol with a sweet, pleasant fragrance that occurs widely among diverse monocot and dicot families. It is one of the most frequently encountered floral scent compounds in the world. Because of the chiral properties of its hydroxylated third carbon, linalool occurs in two enantiomeric forms: (R)-linalool and (S)-linalool. In plant-insect interactions linalool plays a role in attraction of pollinators, herbivores, and carnivores (Dicke et al., 1990; Raguso & Pichersky, 1999; Wei & Kang, 2006) and repellence of herbivores (Aharoni et al., 2003). In higher plants, monoterpenoids, such as linalool, are derived from isopentenyl pyrophosphate via the universal isoprenoid intermediate, geranyl pyrophosphate (GPP), through a class of enzymes called monoterpene synthases. Linalool synthase (LIS) converts GPP to (S)-linalool and was first characterized from *Clarkia breweri* floral tissues (Raguso & Pichersky, 1999).

LIS genes have been isolated from strawberry and evening primrose and transformed into different plant species with the production of linalool and its volatile and non-volatile derivatives, such as hydroxylated linalool and linalool glycoside (Lücker et al., 2001; Lewinsohn et al., 2001; Aharoni et al., 2003). The FaNES1 (LIS) gene, isolated from strawberry (*Fragaria ananassae)*, was capable of the biosynthesis of both the monoterpene linalool and its sesquiterpene counterpart nerolidol in transgenic *Arabidopsis*. When the protein is targeted to the plastid or cytosol, linalool-producing transgenic plants were obtained and these showed a repellence to the aphid *Myzus persicae* (Aharoni et al., 2003). When the enzyme was targeted to the mitochondrion it resulted in the production of nerolidol and this transgenic *Arabidopsis* line was attractive to predatory mites (Kappers et al., 2005).

Research aims

The central aim of the present PhD-study is to investigate whether host plant resistance and biological control can be effectively integrated through a transgenic approach. The combination of a pest-resistance gene and volatile emission gene is expected to show a synergism of two methods of environmentally benign pest control. Proteinase inhibitor MTI2 and *Bt* transgenic *Arabidopsis* will be investigated for their impact on *Plutella xylostella*. Linalool synthase transgenic *Arabidopsis* will be investigated for its impact on the plant-feeding insect *P. xylostella*, and its parasitoids *Diadegma semiclausum* and the parasitoid of *P. brassicae*, *Cotesia glomerata*. Plants with a single transgene as well as plants

with a combination of the two transgenes will be investigated. Interactions of the plants with members of two trophic levels (herbivores and their parasitoids) will be investigated. The research will be carried out with the model plant *Arabidopsis* because this plant has been proven to be a valuable model for plant-insect interactions and can be relatively easily transformed.

Thesis outline

Question 1: Is transgenic *Arabidopsis* expressing a Mustard Trypsin Inhibitor 2 (MTI2) resistant to *Plutella xylostella*?

Although former studies showed that MTI2 transgenic plants displayed obvious resistance to larvae of the diamondback moth (De Leo et al., 2001; Ferry et al., 2005), we did not arrive at the same conclusions with transgenic *Arabidopsis* to the same insect species although the transgene was produced by an event homologous with that used in De Leo's study. Therefore, I investigated the biochemical basis of the apparent resistance mechanism of DBM to the inhibitor. This research is presented in **chapter 2** of my thesis.

Question 2: Can the wound-inducible promoter from Potato proteinase inhibitor 2 (PI2) in combination with the linalool synthase (LIS) gene be expressed by the induction of methyl jasmonate and insect feeding?

In **chapter 3** I report on my work to make the P_{PI2}-LIS' expression cassette with the linalool-intron gene under the control of the PI2 promoter-terminator and its transfer to *Arabidopsis*. Gene presence and transcription were investigated and phenotype expression in terms of linalool emission were assessed. Inducible linalool emission was investigated in response to induction by methyl jasmonate and diamondback moth feeding.

Question 3: What is the effect of a *Bt* transgene, constitutive expression of a LIS transgene, and the combination of the *Bt* and LIS transgenes on *Plutella xylostella*?

In **chapter 4** I created homozygous *Bt* and LIS transgenic *Arabidopsis* and made crosses to produce lines that incorporated each of the two transgenes. I investigated constitutive emission of linalool for P_{35S}-LIS transgenic plants with SPME-GC-MS. The effects of *Bt* and LIS single and dual transgene on diamondback moth were investigated by addressing larval mortality, larval weight, and oviposition choice of *Plutella* moths.

Question 4: What are the effects of constitutively expressing LIS as a single transgene and LIS in combination with Bt on the behaviour of two parasitoid species?

In **chapter 5** LIS transgenic *Arabidopsis* plants constitutively emitting linalool only and combined with the Bt-transgene were investigated with respect to behaviour of the parasitoids *D. semiclausum* and *Cotesia glomerata.* This included induction treatments such as mechanical damage, and *P. xylostella* infestation.

In a general discussion (**chapter 6**) the data from this thesis are integratively discussed.

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Chapter 2

The diamondback moth, *Plutella xylostella*, specifically inactivates Mustard Trypsin Inhibitor 2 (MTI2) to overcome host plant defence

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Abstract

The mustard trypsin inhibitor family has so far only been described among cruciferous species which represent the host plants for the specialist diamondback moth (DBM), *P. xylostella*. The performance of a Dutch and Chinese strain of DBM was assessed on transgenic *Arabidopsis* expressing Mustard Trypsin Inhibitor 2 (MTI2) at a level of 84 µg/g fresh weight equivalent to 12 µM. In contrast to earlier reports (De Leo et al., 2001a; Ferry et al., 2005) no significant differences in larval mortality or development were found relative to the control. Trypsin activity in gut extracts from larvae feeding on either control or transgenic plants were titrated with MTI2 and SKTI (Soybean Kunitz Trypsin Inhibitor) to assess the basis of the insensitivity to MTI2. The specific trypsin activity per gut of larvae reared on MTI2 plants was not significantly higher compared to the control, and ca. 80% of trypsin activity could be inhibited by both inhibitors in both treatments, suggesting no specific induction of PI-insensitive activity in response to MTI2 in the diet. On the basis of the apparent equilibrium dissociation constant of *Plutella* trypsins for MTI2 (80 nM), the gut trypsin concentration (4.8 µM), and the MTI2 concentration in the leaves (12 µM) it was calculated that 99% of the gut trypsin activity sensitive to MTI2 should be inhibited *in vivo*, unless MTI2 was degraded. Indeed, we found that a pre-incubation of MTI2 and SKTI with gut proteases for 3 hours resulted in complete loss of inhibitory activity of MTI2, but not of SKTI, at the concentration ratios found *in planta*. Gut extracts of larvae reared on control or MTI2 leaves were equally well capable of this degradation indicating that the inactivating enzymes are constitutively expressed. In conclusion, it appears that the insensitivity of the diamondback moth to MTI2 can be sufficiently explained by the specific degradation of MTI2, thereby protecting itself against this protease inhibitor which is part of the defence of cruciferous plant species.

1. Introduction

The diamondback moth (DBM), *Plutella xylostella* (L.) (*Lepidoptera*: *Yponomeutidae*), occurs wherever cruciferous crops are grown and is considered to be the most important insect pest of crucifers (*Brassicaceae*) worldwide (Talekar and Shelton, 1993). DBM is a specialist herbivore of plants in the *Brassicaceae* family and has adapted to the characteristic secondary chemicals of this family, the glucosinolates, which make the plants toxic to most generalist feeders (Chelliah and Srinivasan, 1986; Harcourt, 1986; Ooi, 1986). The characteristics which explain its pest status are its high reproductive potential, its ability to use a range of alternative weedy hosts, its dispersal ability, and its capacity to develop resistance to a wide range of insecticides (Schroeder et al., 2000). The increasing failure of insecticides to control DBM has stimulated the development of alternative strategies, among which transgenic approaches (Christou et al., 2006) to enhance host plant resistance to this pest using Bt genes

(Schuler et al., 2003; Zhao et al., 2003; Zhao et al., 2000) and protease inhibitors (De Leo et al., 2001a; Ferry et al., 2005).

Protease inhibitors (PIs) in plants are known to be involved in the defence against pathogens and insect pests (Christou et al., 2006; Jongsma and Bolter, 1997). PIs are generally not directly toxic to herbivores, but reduce the digestive efficiency to utilize plant protein for growth and development. Different insects depend on different classes of proteases for their digestion depending on the pH of the gut. The lepidopterans are known to have guts with pH levels varying from 8-11 and employ mostly serine endopeptidases for digestion. It is well established that specific serine PIs can thus reduce the growth rate and survival of lepidopteran insects (Abdeen et al., 2005; De Leo et al., 2001a; Ranjekar et al., 2003; Zavala et al., 2004). The mustard trypsin inhibitor-2 (MTI2), which is classified as a serine PI, was the first member of this family to be characterized. It is a potent inhibitor of trypsin with no activity towards chymotrypsin and reported to be toxic for lepidopteran and other insects (Ceci et al., 2003; De Leo et al., 1998; De Leo et al., 2001b; De Leo and Gallerani, 2002; Volpicella et al., 2000). In mustard and *Arabidopsis* expression of the gene is upregulated in response to wounding and jasmonic acid (Ceci et al., 1995; Clauss and Mitchell-Olds, 2004).

To investigate its functional role in plant defence MTI2 cDNA was previously transformed to *A. thaliana* under the control of the CaMV 35S promoter. Plant lines showing a high expression level of the MTI2 gene were used for feeding DBM larvae on detached leaves and this was reported to lead to rapid and complete mortality (De Leo et al., 2001a). In the case of oilseed rape expressing MTI2 at low levels an increase in mortality of DBM larvae and lower mean weights of the surviving larvae were observed (Ferry et al., 2005). Here, we report on experiments that were aimed at precise quantification of the level of resistance of transgenic *A. thaliana* MTI2 plants to DBM larvae, using plants originating from the same transformation event as reported by (De Leo et al., 2001a). However, in contrast to data reported by De Leo we did not observe any sign of resistance of MTI2 plants. This lead us instead to investigate the biochemical basis of the apparent resistance mechanism of DBM to the inhibitor.

Essentially two types of resistance or adaptation to protease inhibitors have been described. The first one depends on the presence of alternative proteases which are insensitive to the action of the inhibitors. These can be both constitutively expressed and induced in response to inhibition of other enzymes to compensate for the loss of activity (Bayes et al., 2005; Bayes et al., 2006; Bolter and Jongsma, 1995; Jongsma et al., 1995; Lopes et al., 2004). The second resistance mechanism depends on the presence of alternative proteases which can degrade the inhibitor so that its inhibitory action will not last long enough in the gut to reduce the availability of protein (Girard et al., 1998; Giri et al., 1998; Ishimoto and Chrispeels, 1996; Michaud et al., 1995; Zhu-Salzman et al., 2003)..

In this report DBM larvae reared on control and transgenic MTI2 plants were used to investigate the fate of MTI2 in diamondback moth larvae. Titrations of trypsin activity were done with MTI2 and Soybean Kunitz Trypsin Inhibitor (SKTI) to investigate the type of adaptation that DBM evolved to overcome the effects of this host plant inhibitor.

2. Materials and methods

2.1 Insect strains

Two strains of DBM were used for the bioassays. A Dutch strain of DBM was reared on greenhouse-grown Brussels sprouts plants, *Brassica oleracea* L. var. *gemmifera* cv Icarus (Sluis & Groot, Enkhuizen, The Netherlands) under a L16:D8 photoperiod, at 23-25°C and 60-70% r.h. This colony has been established in the laboratory for over 200 generations and was started with material collected in the Netherlands and since then reared continuously in the Laboratory of Entomology, Wageningen University. A Chinese strain originated from the Institute of Vegetables & Flowers (IVF), Chinese Academy of Agricultural Sciences, Beijing, China. It was reared in a climate room at 24-26°C and 60-70% r.h, under a L16:D8 photoperiod. The colony of DBM was fed on greenhouse-grown white cabbage (*B. oleracea* L. var. *capitata*, cv JingFeng No.1). This colony has been established at IVF in 1996, originating from Wuhan, Hubei province, China. Pupae of the Chinese strain were shipped to Wageningen and reared for two generations in the Laboratory of Entomology, well separated from the Dutch strain under the conditions given above.

2.2 Plant material

Arabidopsis thaliana ecotype Wassilewskija (Ws) lines 3A5 (Ws transformed with empty vector pKYLX35S2), and 7B3 (homozygous MTI2 T4 line in Ws background) were a gift obtained from Lise Jouanin (INRA, France) (De Leo et al., 2001a). The plants were grown in 8x8 cm plastic pots in standard sterilized, humus-rich potting soil (Lentse potgrond), in a climatised greenhouse at 18-22 °C and a L8:D16 photoperiod.

2.3 Detection of MTI2 expression in transgenic *Arabidopsis*

Leaf samples (a single young leaf of around 10 mg) were weighed and extracts were prepared on ice by adding three volumes of water relative to the leaf weight and crushing the leaf using a plastic potter fitting the tube. The extracts were centrifuged for 10 minutes at maximum speed and the supernatant was stored for later use at -80 °C. MTI2 expression was quantified by a radial diffusion assay as described previously (Jongsma et al., 1993) on agar plates containing 42 nM fully active bovine trypsin using a dilution series of purified MTI2 (gift from M. Volpicella, Bari, Italy) of known titrated concentration for the reference curve.

2.4 Insect bioassays

Insect bioassays were carried out in a climate chamber at 25±1 °C, L8:D16 illuminated by HF fluorescent tubes (Philips, 84 °, high frequency). The irradiance level was 16.6 W/m^2. Plants were inoculated with neonate larvae of *P. xylostella* that had no previous experience with plant material. This was achieved by having adult female moths oviposit on Parafilm® that was offered as the only oviposition substrate. Three replicate experiments were carried out. Experiment 1 was carried out with the Dutch strain, and experiments 2 and 3 were carried out with both strains simultaneously to ensure identical circumstances for both. Surviving larvae were counted every two days in experiment 1 and every day in experiments 2 and 3. For each experiment 10-20 *A. thaliana* plants were used. In experiment 1 two larvae were introduced onto each plant but reduced to one larva on day 3 as one plant would not provide sufficient food. Experiments 2 and 3 were done with one larva per plant. Mortality was assessed on day 2 and day 7 in experiment 1, and on day 7 in experiments 2 and 3. Larval body fresh weights were measured individually at day 3 and day 7 in experiments 2 and 3.

2.5 Proteinase assays

The final instar larvae of DBM fed on lines WS (wt), 3A5 (transformed control) or 7B3 (MTI2 line) were dissected on day 7 (Markwick et al., 1996). The guts of cold-anaesthesized larvae were transferred individually to preweighed 1.5 ml Eppendorf tubes, weighed (1.2-3.5 mg), frozen in liquid nitrogen, and stored at -80 °C. Guts were subsequently homogenized individually in 9 gut volumes of ice-cold extraction buffer (0.9% NaCl; 5% polyvinylpolypyrrolidone; 0.5% sodium diethyldithiocarbamate) using a plastic potter. Homogenates were clarified by centrifugation (10 min, 12.000 x g, 4°C). The supernatant was transferred to a clean tube and clarified again by the same procedure. Clear green/yellow supernatants were subsequently stored at -80 °C for use in activity assays to determine the statistical average. For the purpose of performing titrations with different protease inhibitors a larger quantity of uniform gut extract was needed and twelve guts (from insects on control or transgenic plants) were pooled and 10 times diluted with 9 volumes of Tris-buffer (100mM TrisHCl, pH 8, 0.1mg/ml BSA fraction V).

The assay of trypsin activity was done by adding 5 µl of 10-fold diluted gut enzyme to 95 µl Tris-buffer in a microtiterplate, and then adding and mixing 50 µl protease inhibitor solutions of different concentrations (stock solutions of 41 µM concentration of MTI2 obtained as a gift from M. Volpicella (Bari, Italy) and SKTI (Sigma-Aldrich)). After 30 (normal) or 180 (inactivation test) minutes on a microtiterplate shaker (room temperature) 50 µl BApNA substrate solution (15mg BApNA dissolved in 1ml DMSO, 60% diluted with DMSO, and then 60% diluted with Tris-buffer) was added to each well. Plates were measured immediately at 405 nm at approximate time intervals of 20 seconds (over a period of 20 minutes) and 20 minutes over a period of 50 minutes. Proteases were finally measured at a concentration which is 400-fold less concentrated compared to the situation in the gut.

2.6 Statistical analysis

Survival data were analysed by the Chi-square test using the raw scores. Larval weight was analysed using one-way analysis of variance (ANOVA, α=0.05).

3. Results and discussion

3.1 MTI2 expression level in *Arabidopsis*

The expression of the protease inhibitor MTI2 in *Arabidopsis* was determined by a radial diffusion assay using purified and titrated MTI2 for the reference curve. The concentration in young leaves of MTI2 line 7B3 was determined to be 84$\pm$2 µg MTI2/g of fresh tissue equaling a molar concentration of ca. 12 µM, assuming the leaf weight to largely represent water (Table 1). The concentration as percentage of total soluble protein was not measured but was probably around 0.8% of total soluble protein considering that the protein concentration in leaves is mostly around 10 mg/g fresh weight. This expression level would then be similar to the levels reported for the lines 7A and 8A which were reported to be toxic for DBM by (De Leo et al., 2001a). The latter paper, however, reports two conflicting values: 0.6-0.8% of total soluble protein and 21-28 ng/g tissue. The latter figure would be 4000-fold lower than our data and very unlikely to exhibit any effect on the larvae. However, 21-28 microgram MTI2 /gram fresh

weight would represent 0.6-0.8% of total soluble protein if we assume a very low total soluble protein content in *Arabidopsis* leaves of 3.5 mg/g fresh weight (normally around 10 mg/g). So we assume this to be a typographic error and the MTI2 levels to be in the same range. In *Arabidopsis* there is no detectable constitutive expression of trypsin inhibitors as demonstrated using the radial diffusion assay (Table 1), although it has been demonstrated that some members of the MTI2 gene family can be induced by jasmonate and herbivore feeding and are expressed systemically also in non-wounded leaves of a wounded plant (Clauss and Mitchell-Olds, 2004).

Table 1. Estimation of MTI2 activity in different *Arabidopsis* lines using a radial diffusion assay

Arabidopsis line	Average µg trypsin inhibitor/g of fresh tissue	SEM	n
WS (wildtype)	0	-	20
3A5 (empty vector)	0	-	20
7B3 (MTI2 line)	84	2	19

3.2 Effect of MTI2 on larval mortality and development

To re-assess the reported toxicity of MTI2 to DBM larvae several experiments were done with first instar larvae inoculated on whole plants. To our surprise we did not find any statistically significant difference in larval mortality of insects reared on line 7B3 compared to the controls using either of the two insect strains from mainland Europe or China (Table 2 & Table 3). We replicated our results in three separate experiments with two different *P. xylostella* populations. In each experiment new plants from a new batch of seeds were used. As argued above the plants used in our study probably had a similar or slightly higher MTI2 expression level to the plants used in the study by De Leo (De Leo et al., 2001a), and much higher levels compared to the 0.05% TSP oilseed rape plants of Ferry (Ferry et al., 2005). Usually higher expression levels result in stronger inhibition of insect growth although De Leo et al. (1998) reported that above a certain threshold level *Spodoptera littoralis* larvae were able to adapt better to MTI2 expressed in transgenic tobacco by producing new PI-insensitive proteinases. A difference with the experiments of De Leo et al. (1998) is the use of intact plants rather than detached leaves. We consider the method we used as biologically more relevant, because in nature caterpillars will be exposed to intact plants instead of to detached leaves. To confirm that our results were not specific to our lab strain we used 2 different, unrelated, DBM populations from the Netherlands and China. The population used in the experiments of De Leo et al. (1998) was from the Pacific island La Réunion and in the experiments of Ferry from the Atlantic Island of Great Britain. Unfortunately, the La Réunion-strain was no longer available for testing when we requested it. For us the question remained how the Eurasian continental DBM strains managed to remain unaffected by the presence of MTI2 in their diet.

3.3 How does DBM overcome the inhibitory effect of MTI2?

Protease inhibitors expressed in transgenic plants and ingested through normal feeding by an insect will be exposed to a series of complex biochemical and physiological processes in the midgut region of the intestinal tract, the primary interface between phytophagous insects and their trophic environment. The degree to which the ingested inhibitor can interact with its

target enzyme within this complex system will affect its usefulness as a pest control agent. Several insect species have been shown to adapt to plant protease inhibitors either by overexpressing PI-insensitive proteases, thus circumventing the action of the inhibitors (Bolter and Jongsma, 1995; Bown et al., 2004; Broadway, 1997; Jongsma et al., 1995; Jongsma and Bolter, 1997), or by degrading/inactivating the inhibitor with specific proteases (Bolter and Jongsma, 1995; Giri et al., 1998; Ishimoto and Chrispeels, 1996; Jongsma et al., 1995; Jongsma and Bolter, 1997; Michaud et al., 1995; Zhu-Salzman et al., 2003).

Table 2. Mortality (%) of diamondback moth larvae of 2 different populations (Dutch and Chinese) on three *Arabidopsis* lines in different replicate experiments.

Plutella population - time of assessing mortality (number of larvae)	*Arabidopsis* lines			P-value
	WS wildtype	3A5 empty vector	7B3 MTI2	
Exp 1 Dutch-2 days (n=38-40)	5.0 a	7.5 a	2.6 a	0.62
Exp 2 Dutch-7 days (n=19-20)	10 a	10 a	21 a	0.51
Exp 2 Dutch-7 days (n=12)	50 a	67 a	42 a	0.46
Exp 2 Chinese-7 days (n=12)	33 a	67 a	33 a	0.17
Exp 3 Dutch-7 days (n=10)	40 a	30 a	40 a	0.87
Exp 3 Chinese-7 days (n=10)	10 a	20 a	30 a	0.54

Table 3. Analysis of variance (ANOVA) of larval fresh weights of *P. xylostella* of two populations, after feeding on wildtype *A. thaliana*, on MTI2 transgenic line 7B3 or on a line transformed with an empty vector (3A5).

Insects	Day	weight			F value	Pr<F
		WS	3A5	7B3		
Dutch *Plutella*	Day 3	0.324 a	0.348 a	0.368 a	0.89	0.4191
	Day 7	6.623 a	6.420 a	6.407 a	0.05	0.9510
Chinese *Plutella*	Day 3	0.288 a	0.387 a	0.311 a	2.78	0.072
	Day 7	5.495 a	5.253 a	5.219 a	0.07	0.9335

The lack of sensitivity of our DBM populations to MTI2 led us to investigate by which mechanism DBM had become insensitive in the course of its association with Brassicaceae. We asked the following questions: 1) Is MTI2 capable of inhibiting a significant portion of the gut trypsin activity of DBM *in vivo*; 2) Is the MTI2 insensitive fraction of DBM trypsin activity induced in response to the presence of MTI2 in the diet; and if not 3) is MTI2 inactivated by DBM gut proteases after ingestion?

3.4 MTI2 inhibits 80 % of *Plutella* trypsin protease activity both *in vitro* and *in vivo*

To demonstrate that *Plutella* trypsin activity is sensitive to MTI2 prior to exposure to this inhibitor, control gut extracts were titrated against two inhibitors: MTI2 and SKTI using the trypsin substrate BApNA. We suspected that the insensitivity to MTI2 might be a specific phenomenon, and, therefore, we included the comparison to an inhibitor which was never part

of the diet of DBM. SKTI is isolated from soybean and this specific protein is not expressed in *Brassica* species although the gene family does occur in *Arabidopsis* (http://merops.sanger.ac.uk/).

First MTI2 and SKTI stock solutions were both titrated against 42 nM active bovine trypsin to determine the molar concentrations of active inhibitors (Figure 1). Then, gut trypsin activity derived from DBM larvae reared on control plants was titrated against both MTI2 and SKTI (Fig. 2A). Figure 2A shows that the maximum inhibition which could be achieved with either MTI2 or SKTI is approximately 80% of total trypsin activity. This result implies that MTI2 and SKTI must at least partially overlap in their specificity for *Plutella* trypsins and that 20% of the total trypsin activity in the control guts represents MTI2 insensitive trypsin activity.

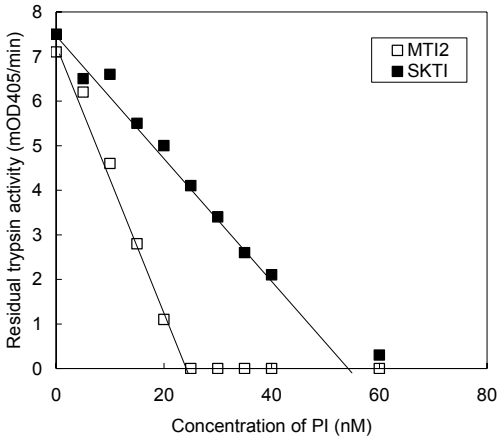

Figure 1. Titration of 42 nM bovine trypsin with MTI2 and SKTI to determine the molar concentration of active MTI2 and SKTI in stock solutions.

Figure 2A also shows the concentration of SKTI and MTI2 required to yield 50% inhibition of the PI-sensitive fraction of *Plutella* trypsin. SKTI is shown to be a much better inhibitor of these trypsins than MTI2, as SKTI is inhibiting the same amount of gut trypsin at 6 nM instead of approximately 80 nM for MTI2. The DBM gut extract in all likelihood represents a mixture of trypsin enzymes with different dissociation constants for the inhibitors used. This means that on the basis of these titrations the concentration of trypsin cannot be reliably determined. A rough estimation of the maximum trypsin concentration present in the gut and the maximum average K_d can be made, however. The *Plutella* trypsin activity could be titrated 50% using 6 nM of SKTI. If we assume that we are dealing with a tight inhibitor which is nearly fully complexing all available sensitive trypsin then we can estimate the titration assay-mix to contain at maximum ca. 12 nM of *Plutella* trypsin. With the estimated protease concentration based on titration with SKTI we can estimate the dissociation constant of *Plutella* trypsins for MTI2 using the data for 50% inhibition where the complexed (Y) and free enzyme (12-Y in this case) are balanced:

K_d = [free protease] x [free inhibitor] / [protease-inhibitor complex]

=[12-Y] x [80-Y]/Y

= [6 nM] x [80 nM – 6 nM] / 6 nM = 74 nM for MTI2

If the actual trypsin concentration would be half the estimated maximum value, the K_d would only change from 74 to 77 nM, so that the use of this maximum concentration value (12 nM) is safe to calculate an estimated minimum K_d value considering its usefulness in the calculation of the *in vivo* situation.

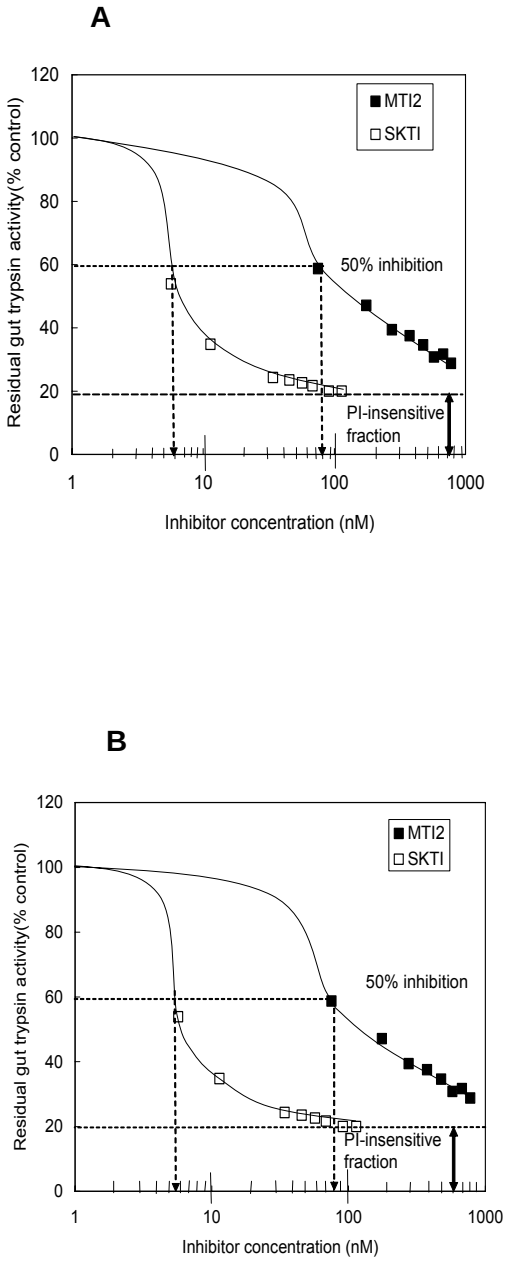

Figure 2. Sensitivity of larval *Plutella* gut trypsin activity for MTI2 and SKTI

A: Titration of trypsin activity (BApNA) with MTI2 and SKTI in extracts derived from larvae reared on control plants. For SKTI 6 nM and for MTI2 80 nM inhibitor was required to achieve 50% inhibition (13 fold difference)

B: Same titration but using larvae reared on MTI2 plants. For SKTI 5.6 nM and for MTI2 80 nM inhibitor was required to achieve 50% inhibition (14 fold difference).

For the *in vivo* situation we should take account of the fact that in the assay-mix the gut extract was diluted 400-fold. This implies that the actual trypsin concentration in the gut is at maximum 400 x 12 nM = 4.8 µM. This maximum amount of MTI2-sensitive *Plutella* trypsin (4.8 µM) is complexed with approximately 12 µM MTI2 in the *Arabidopsis* line 7B3 when the leaf material is ingested. At these *in vivo* concentrations most trypsin would be bound in the complex as the formula for the dissociation constant would predict:

K_d = [free protease] x [free inhibitor] / [protease-inhibitor complex]
In vivo: 0.074 µM = [4.8-Y] x [12-Y] / [Y]
Complexed protease Y = 4.75 µM

This implies that, if MTI2 would remain fully active in the gut and if no protease activity would be specifically induced in response to MTI2, 99 % of the total trypsin activity would be complexed under *in vivo* conditions.

3.5 PI-insensitive activity is not specifically induced

The average total trypsin activity in gut extracts from larvae reared on MTI2 plants was 30% higher compared to the control (10.6 vs 8.1 units per mg gut), but the difference with 12 different guts was not significant due to the high individual variation between guts. This could indicate in principle three different processes, in combination or alone: (i) induction of MTI2-insensitive activity (if less activity could be inhibited in the MTI2-extract); (ii) overproduction of MTI2-sensitive trypsin to overcome the effect of inhibition, or (iii) the inactivation of MTI2 once it enters the gut.

Figure 2 shows that the trypsin activity from both control (2A) - and MTI2 (2B) - reared larvae could be inhibited *in vitro* to virtually the same extent (ca. 80%) although more MTI2 than SKTI was needed to achieve this level of inhibition in both extracts. This demonstrated that the ratio between MTI2- and SKTI-insensitive fractions to the MTI2-insensitive proteases did not change significantly due to the exposure to MTI2 in the *Arabidopsis* leaves.

Caution needs to be exercised when analyzing enzyme data of complexes with inhibitors when they are carried out under strongly diluted circumstances, however. As explained in the previous paragraph at in vivo concentrations 99% of gut MTI2 sensitive trypsin would be bound in the complex. At the 400-fold dilution of the assay, however, most of the Plutella MTI2-sensitive trypsin would be expected to be dissociated from the inhibitor. The previous formula can also be used to calculate this amount of dissociated protease under in vitro (400-fold diluted) conditions:

Kd = [free protease] x [free inhibitor] / [protease-inhibitor complex]
In vitro: 0.074 µM = [0.012-Y] x [0.03-Y] / [Y]
Complexed protease Y = 0.0030 µM (25%)

This implies that, if MTI2 would be fully active and if no protease activity would be induced only 25% instead of 99% of the activity would be inhibited under our *in vitro* conditions before adding MTI2. However, we did not observe such a decrease in the total trypsin concentration from 0.012 to 0.009µM, nor did we observe a change in the fraction of the activity which is

sensitive to MTI2. In fact, if anything, we observed a small - non-significant – increase in total trypsin activity of 30%, which under *in vivo* conditions would be 80% inhibited (the sensitive fraction) unless MTI2 would be degraded.

If no MTI2-insensitive activity is induced relative to the sensitive fraction two possible explanations remain for the lack of a biological effect of MTI2 on the development of the larvae: either (i) *Plutella* larvae are overproducing MTI2 sensitive proteases to overcome any inhibition or (ii) MTI2 is effectively inactivated, presumably by other proteases. In the first case, we should again consider that the gut extracts are assayed at a 400-fold lower concentration than *in vivo*. Under *in vivo* circumstances it is necessary that DBM raises the expression of sensitive trypsin significantly from 4.8 to 16.6 µM in order to overcome the inhibitor levels in the plant:

K_d = [free protease] x [free inhibitor] / [protease-inhibitor complex]
In vivo: 0.074 µM = [16.6-Y] x [12-Y] / [Y]
Complexed protease Y = 11.8 µM (71%)
Free protease 16.6 – Y = 4.8 µM and identical to the control

Under the dilute (400x) *in vitro* conditions this situation would be assayed as a 173% higher concentration compared to the control:

In vitro: 0.074 µM = [0.042-Y] x [0.03-Y] / [Y]
Complexed protease Y = 0.0092 µM
Free protease 0.042-0.0092 = 0.0328 µM (78%) and 173% higher than the control with a free protease concentration of 0.012 µM

In reality we observe only a minor – non-significant - increase of 30% in the level of activity which would be insufficient to overcome the effect of the inhibitor. The possibility that MTI2 is actively degraded in the gut is, therefore, more likely.

3.6 *Plutella xylostella* extract specifically inactivates MTI2

To assess whether MTI2 specifically inactivates MTI2 we pre-incubated the extracts containing the equivalent of 12 nM *Plutella* trypsin with 10-80 fold excess MTI2 and SKTI for both 3 hours (6-fold longer) and 30 minutes (the usual pre-incubation time). The period of three hours more or less corresponds to the time an ingested meal spends in a larval gut. We found that in 3 hours MTI2 was inactivated completely at excess concentrations 10-20 fold to the protease, while even at 80-fold excess half of MTI2 was inactivated. This process did not differ between extracts derived from MTI2 or control reared larvae (Fig 3AC) indicating that the inactivating enzymes are constitutively expressed in the guts of the larvae. In our transgenic *Arabidopsis* line, MTI2 was present in 2-3 fold excess relative to the trypsin concentration in the gut, so that nearly complete inactivation is likely to take place in less than an hour. The possible (non-significant in our experiments) increase of 30% in total trypsin activity, however, may represent an adaptation to partially inhibited enzymes because in the steady state situation with continuous influx of fresh MTI2 not all of it will be immediately degraded under *in vivo* conditions. After extraction and during our assay preincubation also that part of MTI2 is degraded and may raise the observed total trypsin activity per gut.

In contrast, SKTI was not inactivated at all and yielded an identical titration independent of the pre-incubation time in both extracts of MTI2 and control reared larvae (Fig 3BD). This demonstrates that the inactivation of MTI2 is a specific phenomenon which possibly evolved due to the common presence of this inhibitor in the cruciferous host plants of DBM.

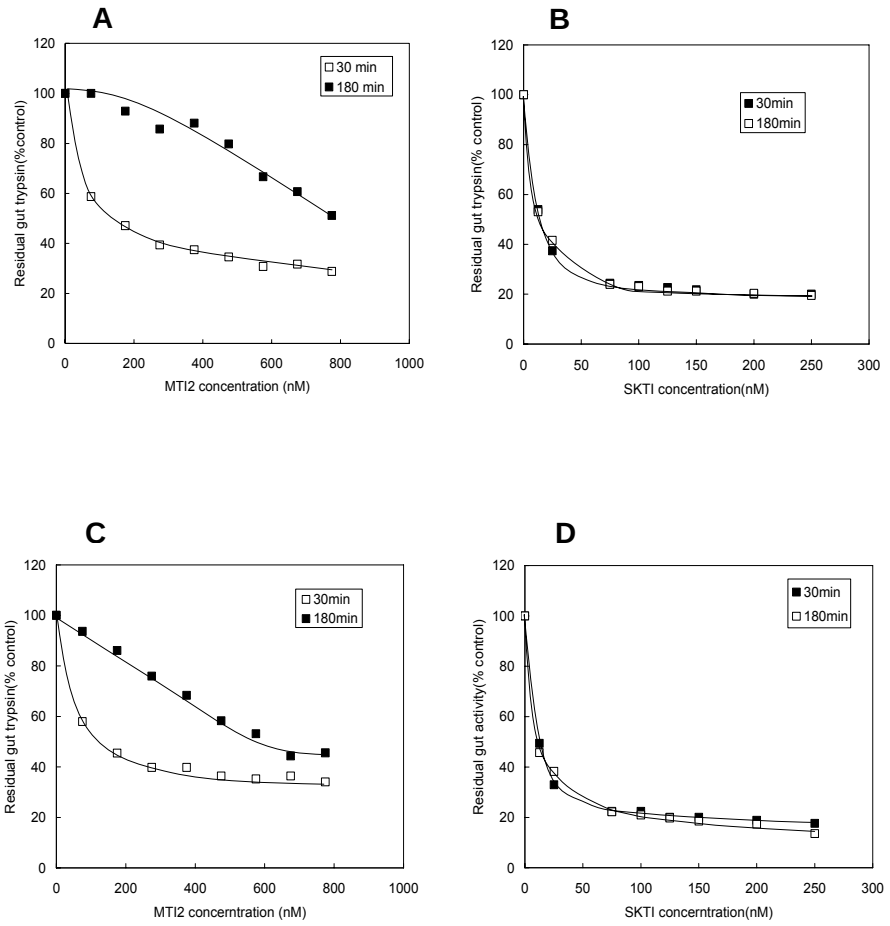

Figure 3. Sensitivity of MTI2 and SKTI for degradation by larval *Plutella* gut proteases

3A: Comparison of residual larval gut trypsin activity from larvae reared on MTI2 plants 30 min and 180 min after addition of MTI2 to 12 nM gut trypsin in the assay

3B: the same as A but after addition of SKTI

3C: the same as A but using gut extracts from larvae reared on control plants

3D: the same C but after addition of SKTI

4. Conclusion

This paper presents a novel perspective on the analysis of protease activity in insect guts. It has been pointed out before that to understand the effects of inhibitors in insects it is crucial to calculate dissociation constants for inhibitors and to convert the *in vitro* assay results back to the *in vivo* situation (Jongsma and Bolter, 1997). We demonstrate that an enzyme activity which would be completely inhibited *in vivo* could be largely active under dilute *in vitro* assay conditions. In the *in vivo* case one would conclude that the insect must be suffering from the direct inhibition of the enzymes whereas in the latter case the opposite conclusion would be

that the insect is suffering from hyperproduction of proteases. Many papers base their conclusions, however, purely on the *in vitro* observations without calculating the actual *in vivo* situation leading to potentially false conclusions about the consequences for the insects.

As shown here, using an approach based on elementary enzymological principles, a more confident assessment can be made of what happens in the gut. It seems that DBM's ability to inactivate MTI2, possibly in combination with a minor increase in trypsin activity, represents the mechanism that the larvae employ to overcome inhibition of trypsin proteases and counteract the antinutritional effects of this inhibitor. The dissociation constant of 74 nM and the MTI2 levels expressed in *Arabidopsis* are sufficient to block most activity at *in vivo* concentrations, but we demonstrated that due to the rapid inactivation an effective MTI2-trypsin complex is most likely hardly formed. This also explains why we observe no induction of MTI2-insensitive trypsin activity relative to sensitive activity in response to exposure to the inhibitor that would compensate the inhibition. The apparent small raise in total trypsin activity of both MTI2-sensitive and insensitive trypsins is insufficient to counteract high concentrations of MTI2, but it could very well represent a compensatory trypsin secretion to counter MTI2 which is intact when it enters the gut. The inactivation was assessed under dilute *in vitro* conditions, but we would expect that under *in vivo* conditions, if anything, the process would be the same or even more rapid. Interestingly this process is specific for MTI2 and not observed for SKTI suggesting that DBM has evolved this resistance specifically against the inhibitor MTI2 in a way similar to other plant-insect combinations (Girard et al., 1998; Giri et al., 1998; Ishimoto and Chrispeels, 1996; Michaud et al., 1996; Zhu-Salzman et al., 2003).

Acknowledgements

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Chapter 3

Expression in *Arabidopsis* of a strawberry linalool synthase gene under the control of the inducible potato PI2 promoter

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Abstract

To investigate the role of inducible linalool in *Arabidopsis*-insect interactions, the FaNES1 linalool synthase (LIS) cDNA from strawberry with plastid targeting and a synthetic intron (LIS') was placed under the control of the wound inducible Proteinase Inhibitor 2 (PI2) promoter from potato. The construct pBin-P_{PI2}-LIS' was transformed to *Arabidopsis thaliana* ecotype Columbia 0. Kanamycin resistant T_0 seedlings were confirmed for the presence and transcription of the LIS' gene by PCR analysis on genomic DNA and by RT-PCR analysis on RNA. Genomic and RT-PCR products were sequenced to confirm correct splicing of the synthetic intron. The expression of active linalool synthase by the P_{PI2}-LIS' gene construct in the transgenic lines was assessed by measuring linalool emission using solid phase micro-extraction (SPME) GC-MS measurements after induction with methyl jasmonate. Among 30 tested independent T_2 transgenic lines, 10 exhibited linalool production. Linalool expression could be induced by methyl jasmonate treatment, but not by Diamondback moth larvae.

1. Introduction

Terpenoids comprise one of the largest families of compounds produced by plants. They have many functions, and for example act as hormones, mediators of polysaccharide synthesis, photosynthetic pigments (chlorophyll and carotenoids), and membrane components (Chappell, 1995). They also mediate interactions between plants and their environment: they are involved in interactions between plants and microorganisms, between plants and herbivorous arthropods or between plants and carnivorous arthropods (Cseke et al., 1998; Aharoni et al., 2003; 2006; Kappers et al., 2005). Moreover, plants may emit terpenoids that attract insect pollinators (Dobson et al., 1993; Knudsen and Tollsten, 1993).

Linalool (3,7-dimethyl-1,6-octadien-3-ol), a C10 monoterpene volatile, is present in the floral fragrance of diverse plant families. Due to its sweet, pleasant fragrance it is used extensively by the flavour and fragrance industry. Linalool mediates interactions among plants and other organisms, e.g. it has been shown to act either as an attractant or a repellent/deterrent to a broad spectrum of pollinators, herbivores and parasitoids (Dicke et al., 1990; Raguso & Pichersky, 1999; Wei & Kang, 2006).

For the purpose of our research, the latter aspect is of special interest. Attraction was documented for insects such as the tarnished plant bug (Chinta et al., 1994), and repellence/deterrence of e.g. the peach aphid and tobacco hornworm moth (Aharoni et al., 2003; Kessler and Baldwin, 2001). In addition, linalool released from bean plants attracted a carnivorous enemy of an herbivore and this showed a role of linalool as infochemical in indirect defence against herbivores in tritrophic interactions (Dicke et al., 1990).

Because of terpenoid diversity and the distinct roles of these compounds in plants, many efforts have been made to identify and isolate the compounds and corresponding biosynthetic genes by overexpression and knock outs/downs. This approach is proving to be highly helpful to understand both the metabolism and the physiological and ecological effects of terpenoids. A model plant such as *Arabidopsis thaliana* is ideally suited for determining the natural roles of secondary metabolites due to the available information on the tissue-specific and environmentally induced expression patterns and the availability of T-DNA knock outs for virtually each gene. Furthermore, it has been shown to be excellently suited for evaluating metabolic engineering strategies (Aharoni et al., 2003). Flowers of *Arabidopsis* produce a range of mono- and sesquiterpenes including linalool, but its leaves produce only trace amounts of the monoterpenes limonene, β-myrcene, and the homoterpene (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (van Poecke et al., 2001; Aharoni et al., 2003; Chen et al., 2003; 2004). Also under conditions of stress leaves of *Arabidopsis* ecotype Columbia 0 (Col0) plants do not emit linalool (van Poecke et al., 2001), but the introduction of a foreign gene coding for linalool biosynthesis led to very high volatile emissions (Aharoni et al., 2003).

Several studies focused on transgenic plants expressing a linalool synthase gene from strawberry or evening primrose resulting in the biosynthesis of linalool and its volatile and non-volatile derivatives, such as hydroxylated linalool and linalool glycoside (Lücker et al., 2001; Lewinsohn et al., 2001; Aharoni et al., 2003). The FaNES1 (LIS) gene was isolated from strawberry and recombinant FaNES1 catalyzes the biosynthesis of the monoterpene linalool and its sesquiterpene counterpart nerolidol from the precursors geranyl diphosphate (GDP) and farnesyl diphosphate (FDP), respectively. When the protein is targeted to the plastid, linalool is observed as the exclusive product. Nerolidol is only produced in minute quantities even if the gene is targeted to the cytosol (Aharoni et al., 2003). When the enzyme was targeted to the mitochondrion it resulted in the production of nerolidol and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) (Kappers et al. 2005). In all these studies the expression of the transgene was regulated by a constitutive or tissue-specific promoter (Lücker et al., 2001; Lewinsohn et al., 2001; Lavy et al., 2002; Aharoni et al., 2003). Inducible promoters allow the on-demand regulation of the transgenic traits at times when their expression is needed, thus avoiding energetically wasteful and ecologically or physiologically undesirable constitutive expression. Among inducible promoters, wound-inducible promoters are the most interesting in the context of plant defence against herbivores.

The PI2 promoter from the potato proteinase inhibitor II gene is constitutively expressed in potato tubers and inducible in leaves (Palm et al., 1990; Duan et al., 1996; An et al., 1998). In potato and tomato PI2 gene expression can be induced by mechanical injury, herbivore damage, microorganism invasion, phytohormones such as abscisic acid (ABA), jasmonic acid (JA) or its methylester (Me-JA), the peptide hormone systemin, heat treatment, and electrical current application (Pena-Cortes et al., 1995). Methyl jasmonate is a known mediator of herbivory-induced defences and has been shown to induce terpene synthase gene expression in various plants (Martin & Tholl, 2002; Martin et al., 2003; Fäldt et al., 2004; Mercke et al., 2004).

In the present study the linalool synthase gene was for the first time engineered under the control of a wound-inducible promoter with the aim of enhancing the attraction of natural

enemies to *Arabidopsis* experiencing feeding damage by herbivorous insects. The effectiveness of methyl jasmonate treatment and herbivory by Diamondback moth larvae to induce linalool release from leaves was investigated.

2. Materials and methods

2.1 Insect rearing

Plutella xylostella was reared on greenhouse-grown Brussels sprouts plants, *Brassica oleracea* L. var. *gemmifera* cv Icarus (Sluis & Groot, Enkhuizen, The Netherlands) under a L16:D8 photoperiod, at 23-25 °C and 60-70% r.h. This colony has been established in the laboratory for over 200 generations and was started with material collected in The Netherlands and has since then been reared continuously in the Laboratory of Entomology, Wageningen University.

2.2 Vector construction

The FaNES 1 linalool synthase gene (LIS) (Aharoni et al., 2003) from strawberry was used to construct the expression cassette. LIS cDNA (without synthetic intron) and pUC 19 plasmid with the linalool synthase (iMuT1) gene (with synthetic intron but without chloroplast transit peptide) were obtained from G. Rouwendal (Plant Research International (PRI), The Netherlands). The introduction of the LIS gene was found to cause toxicity to *E. coli* due to leaky promoter activity of the PI2 promoter and functional gene expression in *E. coli* was prevented by introducing an intron. For this purpose, an 89 bp intron present in the iMuT1 cDNA was used to construct the final expression cassette: The LIS gene with the intron (iMuT1) was excised from its vector with the restriction enzymes *Nco*I and *Bam*HI, and the PI2 promoter-terminator multiple cloning site in vector pUC19 (from N. Outchkourov, PRI) was cut with the compatible enzymes *Nco*I and *Bgl*II. The purified insert and vector fragments were ligated to form PI2/iMuT1 (3' part) in pUC19. Next, cDNA of LIS (PCR amplified with a forward primer introducing an NcoI site at the beginning of the gene using the primers 5'-TAATACCATGGCATCGTCTTCTTGGG-3' and 5'-GCGGAGATCTGGATCCTTACATTGATA -3') was cut with NcoI, and the vector PI2/iMuT1 was cut with the same enzyme. The purified fragments were ligated to form the P_{PI2}-LIS' expression cassette with the linalool-intron gene under the control of the PI2 promoter-terminator (Fig 1A). Finally the cassette was digested with AscI and BamHI and inserted into binary vector plant transformation vector pBINPLUS (van Engelen et al., 1995) digested with the same enzymes resulting in the vector pBin-P_{PI2}-LIS' (Fig. 1B)

2.3 *Arabidopsis* transformation and selection

Plants of *Arabidopsis thaliana* ecotype Columbia 0 were used for floral dip transformation. Nine seeds were sown per pot (12 x 12 cm) filled with well-wetted soil. In total 36 plants were used for transformation. The pots were covered with plastic for ca. one week until the first pair of true leaves was well developed. The pots were placed in a climate chamber (22±1 ºC, L:D 16:8). The plants were used for transformation 4-5 weeks after sowing. One week before transformation the main branch of the plant was clipped to stimulate the emergence of more bolts. Plants were transformed with *Agrobacterium tumefaciens* containing pBin-P_{PI2}-LIS'

according to the flower dip method of Clough & Bent (1998). Plants were subsequently allowed to mature for seed production. The seeds were sterilized with 4% bleach, including 3 drops Tween/30ml, for 20 minutes, and rinsed 4 times with sterile MQ water. Sterile seeds were plated on agar-1/2 Murashige and Skoog (1/2 MS) medium with 50 mg/l kanamycin and 50 mg/l cefotaxime. The plates were incubated in a cold room (4 ºC) for 3 days and then transferred to a growth chamber with long day conditions (16 hrs light, 24±1 ºC). After 1 or 2 weeks antibiotic resistant plants were transferred to soil and placed in the greenhouse (24±4 ºC, 16 hours light). After two generations of selfing, followed by kanamycin selection (methods as mentioned above), T_2 transgenic seeds were obtained, which no longer segregated for kanamycin resistance and were thus classified as homozygous.

2.4 Isolation of *Arabidopsis* DNA

One leaf disc from a well-developed leaf was harvested from each plant, flash frozen (-196 ºC) and ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was added to 500 µl of extraction buffer (25 ml buffer 1 (0.6 M NaCl, 100 mM Tris pH = 7.5, 40 mM EDTA, 4% sarkosyl), 20 ml 12 M Urea, 2.5 ml phenol, 2.5 ml water), vortexed until the entire green material was in slurry, and put on ice till all samples were treated. A volume of 400 µl phenol/chloroform/IAA (24:24:1) was added to the homogenate, mixed by gently inverting 20 times, and centrifuged at 3500 rpm for 15 minutes. The upper phase was carefully taken and transferred to a new tube, 300 µl isopropanol were added, mixed by hand for 10 minutes at room temperature. The supernatant was removed without touching the pellet, and 500 µl 70% ethanol was added to wash it. The pellet was dried in air at room temperature for 10 minutes, dissolved in 50 µl TE buffer with 10 µg/ml Rnase, and stored at –20 ºC.

2.5 Detection of LIS' gene integration and expression

Following kanamycin selection, for the generations T_0 to T_2 PCR analyses of the LIS and LIS' gene were done on genomic DNA to confirm the presence of the gene. The upper primer fwD (5'-TCATGGCCAGGAACTTTTTC-3') starting at position 731 of the linalool synthase gene and the lower primer RevD (5'-TGAATGATGCTGGAAATGGA-3') starting at position 1631 were used for PCR detection. The same primers were used in RT-PCR and sequencing to confirm transcription and correct splicing of the synthetic intron. Total RNA was extracted by the TriPure-small sample method (Roche Applied Science). cDNA synthesis was done by following the Invitrogen protocol of Superscript II and was carried out by adding 2 µl oligo-dT (0.25 ug/µl) and 1 µl dNTP mix (10 mM each) to 1 µg total RNA sample, up to 12 µl MQ water, keeping at 65 ºC for 5 minutes, transferring directly on ice, adding 4 µl first strand buffer, 2 µl 0.1 M DTT, 1 µl Rnasin, and 1 µl Superscript II RT, keeping at 42 ºC for 50 minutes and then 70 ºC for 15 minutes. A volume of 1 µl of this cDNA was used for PCR, which was performed at the following conditions: 95 ºC, 3 min; 30 cycles of 94 ºC, 45 sec; 56 ºC, 30 sec; 72 ºC, 1 min; followed by 72 ºC, 4.5 min. The sequencing samples of DNA and cDNA were prepared by adding 3 µl DETT buffer, 1 µl DETT dye, and 1 µl 1 µM primer to 500 ng template DNA, making up with water until 10 µl, followed by PCR (94 ºC, 20 sec; 50 ºC, 15 sec; 60 ºC, 1 min; for 30 cycles). The samples were sequenced by Greenomics (PRI).

2.6 Induction of linalool expression and GC-MS measurements

To detect the expression of linalool under the control of a wound-inducible promoter leaves needed to be pretreated with methyl jasmonate or insect herbivory. Water agar (1%) was poured into 12×12 cm square Petri dishes. After cooling down of the agar, the detached leaves were placed on the agar layer. We took one leaf from each plant and 5-8 detached leaves were located in each Petri dish. A drop (ca. 20 µl) of methyl jasmonate (Duchefa, The Netherlands) on a small piece of filter paper was placed in the Petri dish. After sealing the Petri dishes with Parafilm, they were placed in a climate room (25±1 ºC, L:D 8:16) for 24 hours to induce linalool production. Induction was also done by insect wounding for a different set of plants from the same batch. Five or ten 2nd instar larvae of *Plutella xylostella* were placed on each *Arabidopsis* plant and 24 or 48 hours later the larvae were removed. P_{35S}-LIS transgenic *Arabidopsis* plants with the transgene under the control of the constitutive CaMV35S promoter were used as a positive control and the wild type (ecotype Col0) as a negative control.

Intact leaves of untreated P_{35S}-LIS and wild type plants were placed in a 1.5-ml crimp cap vial with 10 µl MQ water and capped. Leaf samples of P_{PI2}-LIS' and wild type plants, both induced and non-induced, were also analyzed using the calcium chloride method. Leaves were ground to a fine powder in liquid nitrogen using a mortar and pestle. Around 30 mg fresh weight of leaf material was weighed into a 1.5-ml screw-cap vial, closed, and incubated at 30 ºC for 10 min. Subsequently, 5.5 M $CaCl_2$ was immediately added to give a final concentration of 5 M. The closed vials were then sonicated for 5 min. Subsequently 80 µl of the pulp was transferred into the 1.5-ml crimp cap vial and capped. Both intact samples and $CaCl_2$-extracted samples were finally analyzed by SPME-GC-MS in the following way. A 50 ppm solution of linalool was used as the reference sample. Each day the SPME fiber was conditioned by inserting it into the machine under 270 ºC for 10 min before the first analysis. Headspace volatiles in the vials were collected by exposing a 65-mm polydimethylsiloxane-divinyl-benzene SPME fiber (Supelco) into the vial headspace for 20 min. The $CaCl_2$-extracted samples were in addition held under continuous agitation and heating at 50 ºC. The collected samples were injected into the GC-MS via a Combi PAL autosampler (CTC Analytics AG). In this procedure the fiber was inserted into a GC8000 (Fisons Instruments) injection port and volatiles were desorbed for 1 min at 250 ºC. Gas chromatography was performed on a HP-5 (50 m×0.32 mm×1.05 µm) column with helium as carrier gas (37 KPa). The GC temperature program began at 45 ºC (2 min), was then raised to 250 ºC at a rate of 5 ºC /min, and finally held at 250 ºC for 5 min. The total run time, including oven cooling, was 60 min. The GC interface and MS source temperatures were 260 ºC and 250ºC, respectively. Mass spectra in the 35 to 400 m/z range were recorded by an MD800 electron impact MS (Fisons Instruments) at a scanning speed of 2.8 scans/s and an ionization energy of 70 eV. The chromatographic and spectral data were evaluated using Xcalibur software (Tikunov et al., 2005).

3. Results

3.1 Acquisition of primary transformants

An 894-bp 3'-fragment of the LIS' gene containing a synthetic intron was cut with *Nco*I and *Bam*HI from the iMuT1 vector and ligated into the vector pUC19-P_{PI2}. Subsequently a 1764-bp PCR product was amplified from the LIS cDNA and cut with *Nco*I. The 939-bp fragment was isolated and ligated into the *Nco*I-digested pUC19-P_{PI2} vector with part of iMuT1. The LIS' gene

(with intron) expression cassette which now also possessed plastid targeting under the control of the PI2 promoter was cut from the vector using *Asc*I and *Bam*HI restriction enzymes and ligated into the binary vector pBINPLUS (Fig 1). After mobilization of the genes to *A. tumefaciens* and infection of *Arabidopsis* flowers, more than 20,000 seeds were obtained from 36 transformed plants. Ten to 14 days later seedlings resistant to kanamycin were well developed with a normal green color and susceptible individuals had not germinated, not rooted or had turned whitish in color. In total 0.2-2% of the seeds were resistant to kanamycin and in total 56 healthy seedlings were obtained (Fig. 2). In contrast, no green seedlings were regenerated in the presence of kanamycin from wild type *Arabidopsis*.

AscI ***HindIII***

*GGCGCGCCAAGCTT*CCCCAATTCAAAGAACCTTGTTCATTAATTATTATTTTTCCAGCAAAATGAAAAT
TTTTAGTTAGGATGATAAAATTTATCCAAATTTCTACCACCACCCAAAACTAAATCTTCCAATTCAGAA
AAAATCATTCATTACAACTCTTGGATTTGTTTGTATGATGTGTTAGAAGCAGAAATGGGTTTAAAAGGT
GAATTAAATGGTATTTTTAACTAAAGAAATGAATGCGGGATATATTTAAGTTTTTTCAGATAATTCATG
AATATTTTTATCCTCTTTCCGTGTGCAATAATAGAGTTCCAACTTAATTATCACGTGGACTTATAAGAA
ACCGATGCTGGATGATAATTATTTAAAAAAACAAGCAAGTCGGGGTCAGTACAGCTTGCTGAATAGGAG
AAATTAAAGATAGCAACCAGGAAAAGTTAAGAGCTTTTGGCTCCTCCGTCCAATTATAACCGTCCACTA
TATATATTTGAGACTCGTATTGAGAAACAATAAATAATAAGAATGATATTACTATATTACCTTTTGAAT
ATATTAAATTTAATGCTTTGAAAAATATATTTGATAATGAATAATATCTAATAGCAGCAAGAATAAAAT
AGACACAAACAAGTAAAATTACTCATTGATCTTTCAAATTAGACAAGTATTATCGGACATCTACTTTTA
GTATAGTAAACAAGTAAAGATCGGATAAAGAGAGTAATAAAGAAGAAGCAAGCGTAAGTACCTTGCCAA
AATAATTAACTAACAAGCACATCTTTTTTTTTTTATCAAATATTAATAAAAATAATTTATATTAATATG
AAGAAAAAAAAGGTTTTAGTTTGCTATCTTTTTGATCACTCGTTTGCTATAAATAGGTGGAGGAGGACA
GACACTCTTCACCCCAAAATTAAAAGAAAAAGAGGCAGTACTAATTAATTATCCA*CC*ATGGCATCGTCT

NcoI

TCTTGGGCCTTCTTTAAAGTATTCAATCCCCAAATTGCTCCAAAAAGTATCTCACATATTGGCCAGTCT
GACCTCATGCAGCTTACACATAAGAAGCAGCTGCCTACTTTTCAAAGACGGGGCATTGCCGAAGATAGC
TTGCTTCCCAGTTCTACTACTCCCATAAAGCCGATGAACGTTGAAACCAAGCATACTAGAACTATGGGT
GACATTTTTGTCCAACATTCTCAGAAGTTGGAACTATTGAAAACTGTCTTGAGGAATGTAGCAGAGCTA
GATGCCCTTGAAGGTTTGAATATGATCGATGCTGTTCAAAGGCTAGGCATCGATTACAACTTTCAACGA
GAAATCGACGAAATCCTGCACAAGCAAATGAGTATTGTGTCTGCCCGTGATGATCTTCATGAGGTTGCA
CTTCGCTTTCGACTACTGAGACAACATGGTTACTTCGTGCCTGAAGATGTGTTTAACAACTTCAAGGAC
AGCAAAGGAACGTTCAAGCAAGTTCTGGGTGAAGACATCAAGGGATTGATGAGCTTATACGAAGCTTCG
CAGCTAGGTACAGAAGGAGAAGATATACTTGTTGAAGCTGAAAAGTTTAGCGGCCATCTGCTAAAGACT
TCTCTGTCACATCTTGATCATCATCGAGTCAGAATTGTTGCAAATACATTGAGGAATCCTCATCACAAA
AGCTTGGCCCCATTCATGGCCAGGAACTTTTTCGTTACTTCTCAAGCCACCAATTCATGGTTAAATTTG
CTAAAAGAAGTAGCAAAAACAGATTTCAATATGGTCCGGTCTCTGCACCAGAATGAAATAGTTCAAATG
TCCAAATGGTGGAAGGAGCTTGGATTGGCTAAGGAACTGAAGTTTGCAAGAGATCAACCACTGAAATGG
TACATTTGGT*CCATGG*CATGCCTGACAGATCCAAAGTTATCAGAGGAGAGGGTTGAGCTCACAAAACCC

NcoI

ATCTCTTTTGTCTATTTGATAGATGACATTTTCGATGTTTATGGAACCCTTGATGACCTCATTCTCTTC
ACAGAAGCTGTTAATCG*gtatatatgaattatatgcgtcagtgatgaaatataatcagacttgttacca*
*atttatgattgatcaacaacctattgcatacatacag***ATGGGAAATTACTGCTATAGACCACTTACCAG**
ACTATATGAAGATATGCTTCAAGGCTCTCTATGATATGACTAATGAATTCAGCAGCAAGGTCTATCTGA
AGCATGGATGGAACCCCTTACAATCTTTGAAAATTTCGTGGGCGAGTCTTTGCAATGCATTTTTGGTGG
AAGCAAAATGGTTCGCCTCTGGGAAGCTGCCGAAGTCAGAAGAGTACTTGAAGAATGGCATCGTTTCTT
CTGGGGTAAATGTGGTTCTAGTCCACATGTTTTTTCTCTTGGGTCAGAACATAACCAGAAAGAGTGTGG
AGTTGTTGAATGAAACTCCAGCCATTATATCGTCCTCAGCAGCAATTCTTCGACTCTGGGACGATTTAG
GAAGTGCAAAGGATGAGAACCAGGATGGGAACGATGGGTCGTATGTAAGGTGCTACTTAGAGGAACATG
AAGGCTGTTCCATTGAGGAGGCACGAGAAAAGACGATTAATATGATTTCAGATGAATGGAAGAAACTGA
ACAGAGAACTGCTCTCTCCAAATCCATTTCCAGCATCATTCACATTGGCTTCTCTTAATCTCGCAAGAA
TGATCCCCTTGATGTATAGCTACGATGGCAACCAATGCCTTCCATCTCTTAAAGAGTATATGAAACTGA
TGTTGTATGAGACTGTATCAATGTAA*GGATCT*TAATGTATGAAATAAAAGGATGCACACATAGTGACAT

Fusion ***BamHI/BglII***

GCTAATCACTATAATGTGGGCATCAAAGTTGTGTGTTATGTGTAATTACTAGTTATCTGAATAAAAGAG
AAAGAGGTCATCCATATTTCTTTTCCTAAAGAAATGTCACGTGTCTTTATAATTCTTTGATGAACCAGA
TGCATTTTATTAACCAAATCCATATACATATAAATATTAATCATATATAATTAATATCAATTGGGTTAG
CAAAACAAATCTAGTCTAGGTGTGTTTTGCTAATTATTGGGGGATAGTGCAGAAAGAAATCTACGTTCT
CAATAATTCAGATAGAAAACTTAATAAAGTGAGATAATTTACA*AAGCTT*GCATGCCTGCAGGTCGACTC
TAGA*GGATCC*

HindIII

BamHI

Figure 1A. The P_{PI2}-LIS' expression cassette. The sequence of the PI2 promoter and terminator fused to the LIS' cDNA with a synthetic intron is shown. The bold part is the LIS' sequence; the lower case sequence in italics represents the intron; the frames indicate the start and end of translation; the underlined italics indicate the restriction sites of enzymes, and the underlined part shows the PCR fragment that was amplified using the fwD and revD primers.

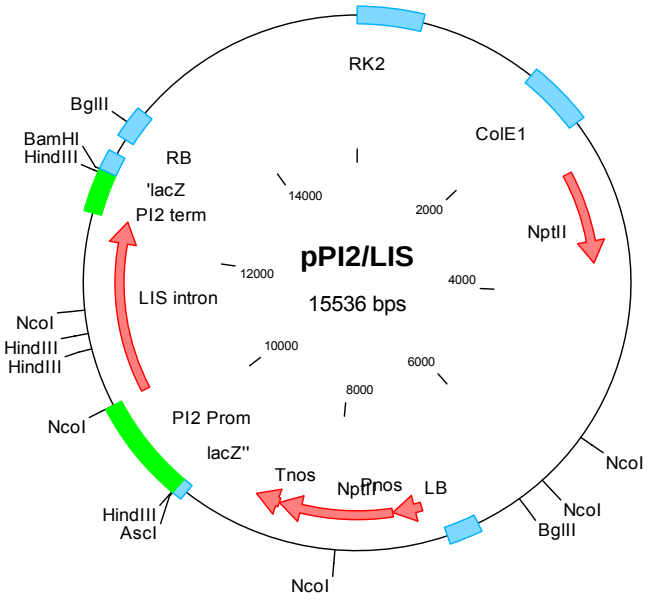


Figure 1B. The physical map of the pBinplus binary vector carrying the P_{PI2}-LIS' gene used for transformation of *Arabidopsis*.

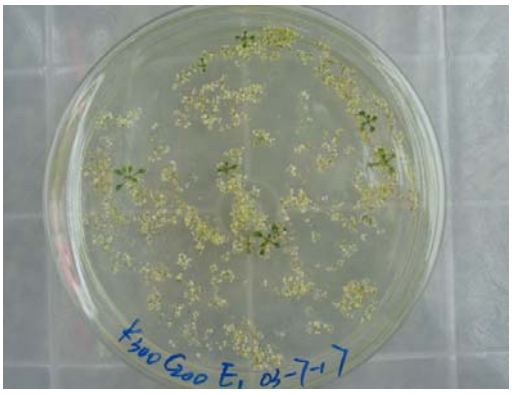


Figure 2. Kanamycin selection of first generation (T_0) of transgenic *Arabidopsis thaliana* lines. The green, well developed seedlings are kanamycin resistant.

3.2 Linalool synthase transformation and expression analysis

In order to test for the presence of the LIS' gene in kanamycin-resistant plants, DNA was isolated, followed by PCR analysis. In this way the numbers of plants analyzed were 56, 33, and 65 in the T_0, T_1, and T_2 generations respectively. More than 80% of the selected, kanamycin-resistant, plants were PCR-positive for a part (994 bp) of the linalool synthase gene. Furthermore, no amplification was observed in untransformed seedlings from wild type plants (Fig. 3). In the T_2 generation, progenies from 8 T_1 plants showed no segregation upon

germina s.

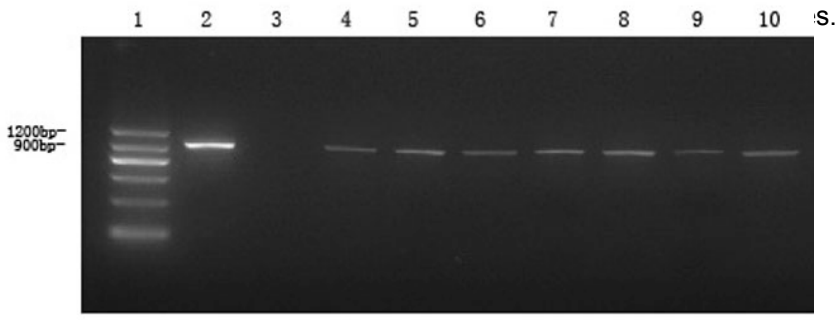


Figure 3. PCR detection for T_0, T_1, and T_2 generation of plants. Lane 2: positive control using plasmid DNA as a template; lane 3: negative control using wild type *Arabidopsis* genomic DNA as template; lanes 4-10: P_{PI2}-LIS' transgenic plants yielding a 994 bp band.

Two single homozygous plants of P_{35S}-LIS and P_{PI2}-LIS' (T_2 generation) were selected respectively for PCR on genomic DNA and RT-PCR on cDNA using identical primer pairs. After induction of P_{PI2}-LIS' with methyl jasmonate both types of plants turned out to transcribe the LIS gene as can be deduced from the presence of a 905 bp band in the RT-PCR experiment (Fig. 4). Furthermore, P_{PI2}-LIS' plants efficiently spliced out the intron from the primary transcript if we compare the PCR product from genomic DNA (994 bp) and cDNA (905 bp) (Fig. 4). To demonstrate correct splicing the RT-PCR product was sequenced. Transcript sequencing revealed that the intron was present in the PCR product of genomic DNA of the P_{PI2}-LIS' plant 23 and that the 89 bp intron was correctly removed during mRNA maturation.

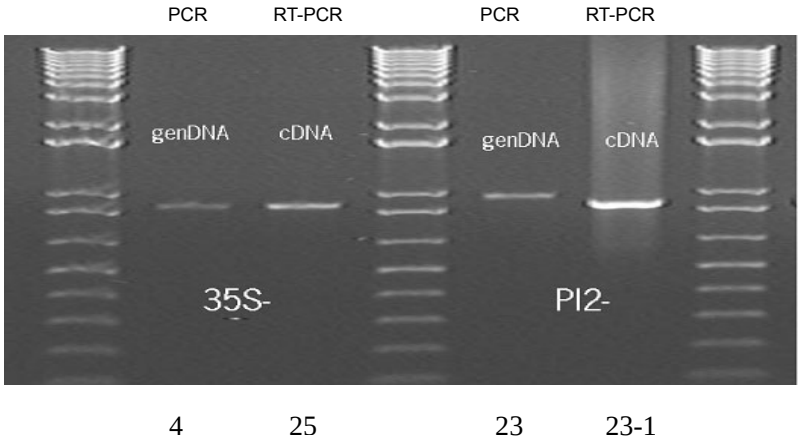


Figure 4. Comparison of PCR and RT-PCR on genomic DNA and total RNA isolated from a P_{35S}-LIS (without intron) and P_{PI2}-LIS' (with intron) plants after induction with methyl jasmonate. Lanes 1, 4 and 7: DNA size marker; Lanes 2 and 5: PCR fragments using P_{35S}-LIS and P_{PI2}-LIS' plant genomic DNA templates; Lanes 3 and 6: RT-PCR fragments using P_{35S}-LIS and P_{PI2}-LIS' cDNA . The fragment size in lanes 2, 3 and 6 is 905 bp, and the size in lane 5 is 994 bp including the intron of 89 bp.

3.3 Linalool production induced with methyl jasmonate induction

Thirty T_2 generation transgenic homozygous plants were screened for linalool emission by SPME-GC-MS analysis of $CaCl_2$ extracts obtained from P_{PI2}-LIS' leaves. As expected, the control leaf samples of uninduced and methyl jasmonate induced wild-type *Arabidopsis* did not produce any detectable linalool (Fig. 5B, D). Only the transgenic plants showed varying levels

of linalool emission after induction with methyl jasmonate (among 30 transgenic plants, 10 showed linalool emission; Fig. 5C). Surprisingly, there was no detectable linalool production in P_{PI2}-LIS' transgenic plants after 24-48 hours of herbivory by second instar larvae of *Plutella xylostella* (Fig. 6).

One year later, when aiming for combining P_{PI2}-LIS' by crossing with other genes in *Arabidopsis* individuals, SPME-GC-MS analyses were carried out for plants grown from seeds of the same batch of P_{PI2}-LIS' plants as used here as well for their progeny using identical protocols, but linalool emission could not be detected from any of them.

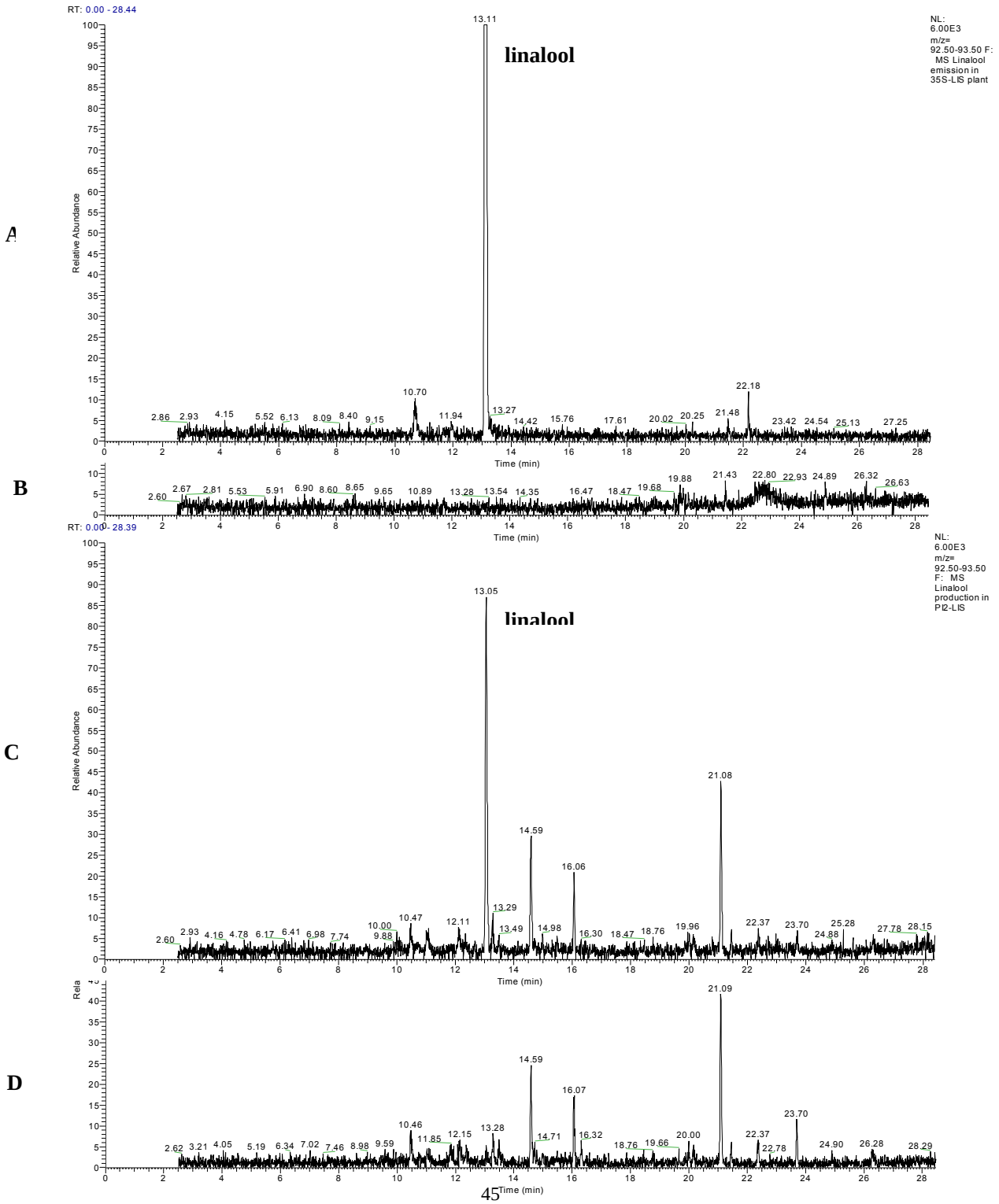
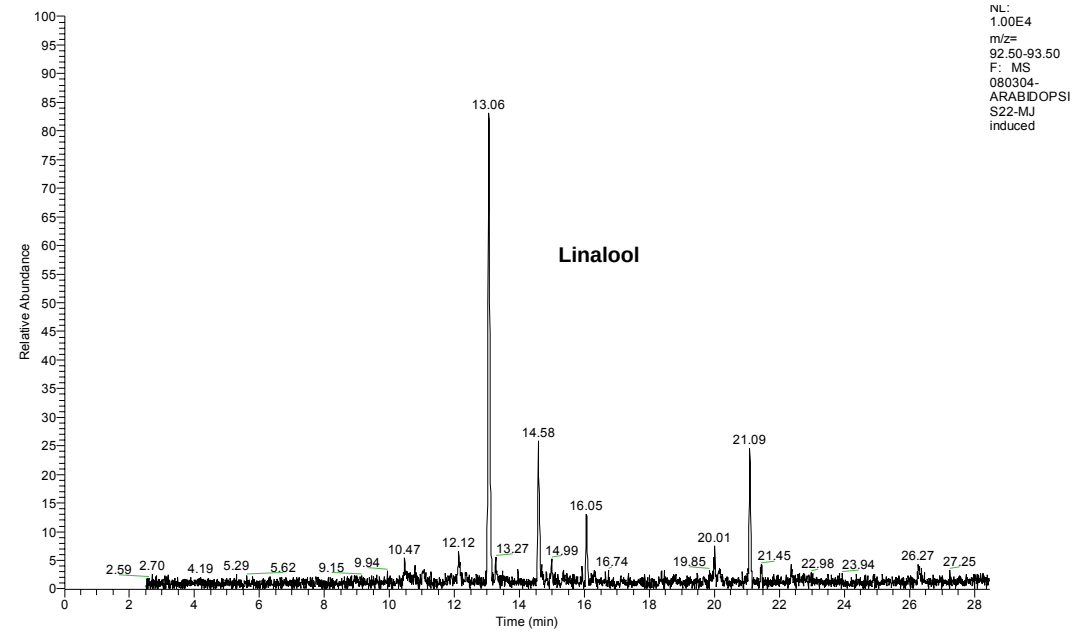


Figure 5. Headspace profile from $CaCl_2$-extracted and live leaves to detect linalool emission from P_{35S}-LIS and P_{PI2}-LIS' transgenic *Arabidopsis* plants respectively by SPME-GC-MS. Panel A: Linalool emission from P_{35S}-LIS Leaves; Panel B: emission spectrum from wild type leaves; Panel C: Linalool emission from P_{PI2}-LIS' $CaCl_2$-extracted leaves induced with methyl jasmonate; Panel D: emission spectrum from wild type $CaCl_2$-extracted leaves induced with methyl jasmonate. Shown are abundances of the masses in the range of 92.5-93.5 specific for monoterpenes on the absolute scale of panel A.

A



B

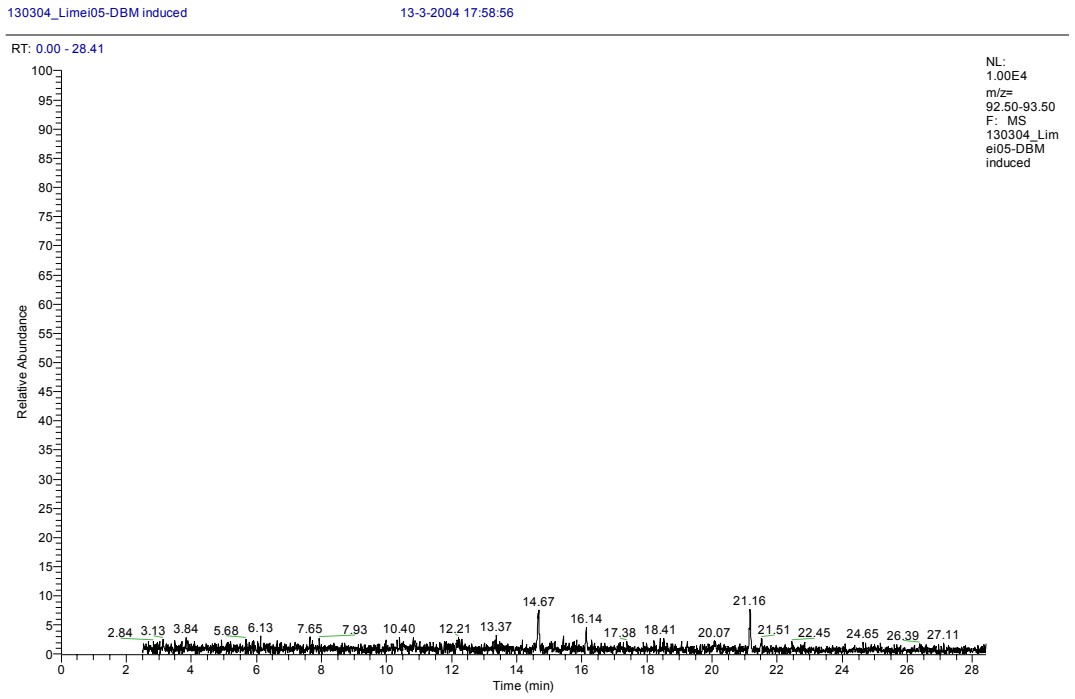


Figure 6. Comparison of linalool expression in P_{PI2}-LIS' transgenic *Arabidopsis* plants induced by either methyl jasmonate (panel A) or the L2 larvae of *P. xylostella* (panel B). Shown are abundances of the masses in the range of 92.5-93.5 specific for monoterpenes on the absolute scale of panel A.

3.4 Growth comparison between P_{35S}-LIS and P_{PI2}-LIS' transgenic *Arabidopsis*

P_{35S}-LIS homozygous transgenic *Arabidopsis* (from A. Aharoni, PRI) were used as the positive control for linalool emission. These plants exhibited a developmental delay of approximately two weeks compared with Col0 control plants. This phenomenon was not observed in P_{PI2}-LIS' homozygote plants (Fig. 7). Due to the delayed development and very high linalool expression of P_{35S}-LIS transgenic plants, they had an obvious phenotype, of lighter, younger looking green leaves with some small yellow spots (Fig. 7). However, eventually they produced flowering stems and seeds.

Figure 7. Growth retardation by constitutive expression of linalool in transgenic *Arabidopsis*. Homozygous P_{35S}-LIS, Columbia 0 ecotype control, and P_{PI2}-LIS' plants sown on the same day. All plants shown are 6 weeks old since sowing.

4. Discussion

In recent years more and more researchers focused on metabolic engineering of terpenoids due to their potential implication in various aspects of plant-insect interactions. Some studied direct plant defence (Bohlmann et al., 1998; Zhang et al., 2002; Aharoni et al., 2003; Beale et al. 2006), and others worked on the effect of new components on indirect plant defence (Aharoni et al., 2005; Kappers et al., 2005; Schnee et al., 2006; Beale et al. 2006). So far, in all reported examples constitutive or tissue-specific promoters have been used so that the presence of the compound is not correlated to the invading/attacking pest. However, regulated transgene expression, whereby a promoter is specifically activated in response to pathogen invasion or pest attack, may have distinct advantages for genetic engineering disease/pest resistant traits in plants, by reducing the cost involved in producing the metabolites when they are not yet required. Furthermore, coupling of a herbivore-inducible promoter to a terpenoid synthase gene will allow biological control agents to detect the induced terpenoid only after damage inflicted by attacking herbivores (Kappers et al., 2005). Different gene expression patterns can be created using inducible promoters responsive to mechanical wounding, herbivore damage, or pathogen invasion (Keil et al., 1989; Siebertz et al., 1989; Hansen et al., 1996; Yevtushenko et al., 2004). Jasmonate family members including OPDA, dnOPDA, and

jasmonic acid can be induced in *Arabidopsis* by *Pieris rapae* feeding (Reymond et al., 2004). Up to now, there have been no reports on genetic engineering of monoterpene synthases with wound-inducible promoters. Our results show that the use of the PI2 promoter from potato resulted in methyl-jasmonate inducible linalool expression in *Arabidopsis,* although it failed to yield a response to herbivory by *Plutella xylostella* larvae.

Several studies demonstrated that gene expression can be manipulated with wound-inducible promoters. Most of them focused on β-glucuronidase (GUS) reporter gene expression (Keil et al., 1989; Hansen et al., 1996; Yevtushenko et al., 2004). The promoter of proteinase inhibitor II in potato was proven to be both wound- and herbivore-inducible in different plant species (Keil et al., 1989; Palm et al., 1990; Xu et al., 1993; Pena-Cortes et al., 1995; Duan et al., 1996). Among them, Duan's group reported that wound-inducible expression of the pin2 gene driven by its own promoter resulted in high-level accumulation of the PINII protein in transgenic rice with increased resistance to a lepidopteran, the pink stem borer (*Sesamia nonagrioides* Lef). The observations reported here for *Arabidopsis* that linalool expression is only observed in response to methyl jasmonate and not to herbivore damage is therefore unexpected.

Jasmonic acid and its methyl ester, methyl jasmonate, play important roles in plant defence as intra- and intercellular signaling molecules. They mediate the activation of gene expression in response to wounding, elicitor treatment, and pathogen infection. Application of exogenous methyl jasmonate can induce a pattern of gene expression very similar to mechanical wounding, pathogen infection, and herbivore damage (Hildmann et al., 1992; Ellard-Ivey et al., 1996; McConn et al., 1997; Dicke et al., 1999; Van Dam et al., 2001; Fäldt et al., 2003). Plant defences against pathogens and insects are regulated by signaling pathways in which salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play key roles. Comparison of transcript profiles revealed that consistent changes induced by pathogens and insects with very different modes of attack can show considerable overlap. The majority of the changes in JA-responsive gene expression were attacker-specific. JA plays a dominant role in the transcriptional reprogramming of the response of *Arabidopsis* to an array of attackers (De Vos et al., 2005). MeJA can induce transcription by the PI2 promoter and its response mechanism is different from the induction by sucrose which controls its tissue-specific expression in potato tubers (Kim et al., 1992). In our case, we successfully induced linalool production though application of MeJA, but failed when insect feeding was applied. Possible reasons for this are: 1) MeJA is a more powerful inducer than insect damage or the amount of JA applied was high relative to the strength of natural induction by feeding; Both MeJA and mechanical wounding can induce the expression of *pin2-Gus* systemically in transgenic rice, however (Xu et al., 1993). 2) the damage duration and the sampling time for linalool detection were not optimal because insect damage will be low at first and increases over time compared to one large boost of MeJA at the start of the experiment; The induction by lepidopteran larvae of genes in both *Solanaceae* and *Arabidopsis* occurs within 24 hours however (Jongsma et al., 1995; Sarosh et al 2007). 3) Some unknown components in saliva of *Plutella* larvae might directly or indirectly suppress a step in the JA-induced signal transduction pathway normally leading to the activation of the PI2-promoter considering that there is little overlap in MeJA- and *Plutella*-inducible genes in some Brassicaceae (Sarosh et al 2007). A further example of this is the study of Kahl et al. (2000) in which *Manduca sexta* saliva-induced ethylene suppresses JA-responsive nicotine

accumulation, but enhances JA-responsive volatile emission. They showed that nicotine accumulation was proportional to the amount of leaf wounding and the resulting increases in jasmonic acid (JA) concentrations in tobacco plants. In contrast, feeding by the nicotine-tolerant larvae of *M. sexta* led to large increases in volatile terpenoids, ethylene, and accumulations of endogenous JA pools, but to similar or decreased nicotine accumulation, which can be strongly induced by JA. The ethylene release was sufficient to explain the attenuated nicotine response (Kahl et al., 2000). A similar situation might exist in our case.

The retardation of plant growth, observed in P_{35S}-LIS plants, was not observed in P_{PI2}-LIS' plants relative to the wild type control. Previously, growth retardation in *Arabidopsis* was reported to be the result of the high constitutive expression of linalool (Aharoni et al., 2003; Kappers et al., 2005). A possible explanation for this retarded and light green phenotype in the transgenic lines is the depletion of the isopentenyl diphosphate (IPP) pool in the plastids, which may lead to reductions in the levels of essential isoprenoids (Aharoni et al., 2003) or part of the metabolic energy goes into the biosynthesis of linalool and can not be used for growth (Herms and Mattson, 1992). Alternatively, it is possible that the levels of linalool or its derivatives produced in these high level expressors are phytotoxic to some degree. In any case our results show that the use of an inducible promoter can in principle overcome these negative effects of constitutive promoters on plant phenotype.

Arabidopsis plants emit a number of different volatile organic compounds (VOCs), including monoterpenes and sesquiterpenes from different organs (e.g. leaf, flower, stem, silique, root) and depending on developmental or stress conditions (Van Poecke et al., 2001; Chen et al., 2003). Several *Arabidopsis* terpene synthase (TPS) genes are expressed exclusively or almost exclusively in the flowers. Among these, At1g61680 encodes a linalool synthase which is petal- and anther-specific (Chen et al., 2003; www.arabidopsis.org). In the vegetative stage of *Arabidopsis* the terpenoids myrcene, β-ionone and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene can be emitted in response to infestation by *P. rapae* caterpillars so that future experiments aimed at inducible linalool production may profit from focusing on promoters of genes responsible for these emissions (van Poecke et al., 2001). The introduction of wound-inducible linalool production in *Arabidopsis* leaves will increase our understanding of the biological role of inducible plant terpenoids, e.g. the effects of induced and inducible linalool emission on herbivores and their parasitoids.

Further research on induced linalool emission in response to insect attack will be relevant for the engineering of indirect plant defence. Compared with constitutive production, the use of herbivore-inducible terpenoid expression will result in a more reliable cue to affect the behaviour of the natural enemies of herbivores, will save metabolic resources for plant development and reduce any negative phenotypes associated with the constititutive expression of the gene.

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Chapter 4

Combining linalool synthase gene with a *Bacillus thuringiensis* toxin gene in *Arabidopsis thaliana* reduces oviposition by *Plutella xylostella* adults and may thus reduce selection of *Bt*-resistant larvae

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Abstract

Homozygous transgenic *Arabidopsis* plants expressing the *Bt* toxin gene SN19 and the strawberry linalool synthase gene FaNES1 (LIS plants) were crossed to wildtypes and each other to obtain hybrid lines for these traits in different combinations. Larval feeding bioassays showed that the expressed level of SN19 was toxic to the larvae of the diamondback moth (DBM), *Plutella xylostella*, in both homozygous and hybrid plant lines. The emission of high levels of linalool by hybrid LIS transgenic *Arabidopsis* did not affect the survival and development of larvae of DBM. However, oviposition choice experiments showed that LIS plants and LIS plants with *Bt* toxin were both repellent to DBM female moths to the extent that significantly fewer eggs were laid on LIS transgenic plants compared to control plants or *Bt* toxin plants without linalool. This repellence may increase the probability that potentially resistant insects from *Bt* toxin plants will mate with susceptible insects colonising non-transgenic plants that constitute refuges. Whether the combined effects of both *Bt* toxin and linalool synthase on DBM will increase the efficiency of a high-dose / refuge strategy should be further investigated.

1. Introduction

The Diamondback moth (DBM), *Plutella xylostella* (L.) is one of the most destructive pests of *Brassica* and other crucifer crops throughout the world. DBM can live under a wide range of climatic conditions and is cosmopolitic. Several of its biological traits contribute to problems in controlling DBM: its short life cycle, its high fecundity, a wide range of alternative weedy hosts, and its capacity to develop resistance to multiple insecticides (Schroeder et al., 2000). The cost of DBM control worldwide has been estimated at approximately one billion US$ annually (Talekar & Shelton, 1993).

Insecticide resistance in DBM is a major factor causing its pest status on cruciferous crops. In addition, synthetic pesticides have long-term detrimental effects, leading to environmental pollution and elimination of natural enemies such as parasitoids and predators. The use of pesticides of biological origin has been gaining increased acceptance. A major microbial pesticide is a toxic protein derived from the soil bacterium *Bacillus thuringiensis (Bt).* Advantages of *Bt* toxins are their specific toxicity against target insects, lack of polluting residues and safety to non-target organisms (Kaur, 2006). Its use is limited because of negative attributes such as short persistence in free form and the need of the target insect to consume *Bt* spores or crystals. These disadvantages have been overcome by incorporation of toxic-protein-producing genes of *B. thuringiensis* into crop plants, which results in the

expression of the toxin throughout the plant. Since the first commercial transgenic *Bt*-cotton was released in 1996, many favourable accounts of the economic, environmental and health benefits of insect resistant transgenic crops have been published (Ferry et al., 2006). Several studies showed that *Bt*-transgenic *Brassica* plants are resistant to DBM (Ramachandran et al., 1998; Jin et al., 2000; Cao et al., 2006). *Bt*-plants were shown to be more effective for managing DBM than *Bt*-foliar sprays, because *Bt*-toxin concentrations can be regulated more effectively in transgenic plants than with sprays, and *Bt*-Cry toxins can be programmed for expression only in specific plant stages or structures (Sarfraz et al., 2006). However, the sustainability and durability of pest resistance to *Bt*-plants continues to be discussed (Christou et al., 2006). As adoption rates of insect-resistant crops rise globally, selection pressure, leading to the development of insect pest resistance to transgenic insecticidal gene products, will increase. Some laboratory studies have shown that DBM has the ability to develop resistance to *Bt*-plants expressing high levels of Cry1Ac (Metz et al., 1995) and Cry1C (Zhao et al., 2000).

Several alternative strategies have been proposed to delay the evolution and selection of insect resistance to transgenic crops: 1) Moderate toxin dosage to ensure survival of a fraction of susceptible insects; 2) High toxin dosage to kill all insects heterozygous for a resistance allele; 3) Provision of a non-toxic plant refuge (Bates et al., 2005). Among these the refuge or the high-dose/refuge strategy is currently the most popular (Roush, 1997; 1998; Could, 1998; Shelton et al., 2000; Onstad et al., 2002; Bourguet et al., 2005; Sisterson et al., 2005). The principle of the refuge strategy is that any resistant insects emerging from *Bt* toxin crops are more likely to mate with members of the much larger number of susceptible adults emerging from the neighbouring refuges than with each other, thereby decreasing the selection for *Bt* resistance alleles. Recently, additional strategies for integrated resistance management comprising pyramiding/stacking of genes encoding different *Bt* toxins have been developed as a method for preventing development of pest resistance and for conferring higher levels of pest control (Zhao et al., 2003). Examples include expressing insecticidal proteins from sources other than *Bt*, e.g. *Photorhabdus* and *Xenorhabdus* protease inhibitors (PIs), lectins (Ferry et al., 2006), and using novel proteins from insect biological control agents and insect hormones to generate transgenic crops (Tortiglione et al., 2003). The next generation of insect-resistant transgenic plants must be designed to delay or prevent the development of resistance and thus provide more durable levels of crop protection (Ferry et al., 2006).

Plants respond to insect herbivory by synthesizing and releasing complex blends of volatiles that are attractive to natural enemies of the herbivores (Dicke et al., 1990; Turlings et al., 1990; Takabayashi et al., 1991; Arimura et al. 2005). Terpenes, which represent a major fraction of plant volatiles, play a remarkable role also in insect repellence (De Moraes et al., 2001; Wang et al., 2002; Vallat et al., 2005; Delphia et al., 2007).

Linalool (3,7-dimethyl-1,6-octadien-3-ol), a C10 monoterpene volatile, is present in the floral fragrance of various plant families (Raguso & Pichersky, 1999) and in the blend emitted by several plant species in response to insect herbivory (e.g. Dicke et al. 1990; Arimura et al., 2004; Wei & Kang, 2007). As an important component of plant odors and insect pheromones, linalool can be both attractive and repellent to insect herbivores. Attraction was documented for insects such as tarnished plant bugs (Chinta et al., 1994), but repellence for *e.g.* the green peach aphid (*Myzus persicae)* (Aharoni et al., 2003), thrips (Visser et al., 2007), and tobacco

hornworm (*Manduca sexta*) moths (Kessler and Baldwin 2001). Several studies focused on transgenic plants expressing linalool-synthase genes from strawberry or evening primrose during the past years (Lücker et al., 2001; Lewinsohn et al., 2001; Lavy et al., 2002; Aharoni et al., 2003). Previously, transgenic plants with the FaNES1 (LIS) gene from strawberry resulted in the expression of linalool or its sesquiterpene counterpart nerolidol depending on targeting the protein to the plastids or the cytosol/mitochondria (Aharoni et al., 2003; Kappers et al., 2005). Transgenic LIS *Arabidopsis* plants expressing linalool were repellent/deterrent to *M. persicae* aphids (Aharoni et al., 2003) while those expressing (3S)-(*E*)-nerolidol and (*E*)-DMNT (4,8-dimethyl-1,3,7-nonatriene) were attractive to predatory mites, the natural enemies of spider mites (Kappers et al., 2005).

In the current study, the *Bt* toxin gene SN19 and the strawberry linalool gene FaNES1 were integrated into one plant genotype and the effect of the dual transgene on DBM larvae and adults was investigated.

2. Materials and methods

2.1 Plant material

*Arabidopsis thaliana e*cotypes Columbia (Col-0, named CcCc) and Wassilewskija (WS, named CwCw) were used as wild type control plants. Transgenic WS *Arabidopsis* lines expressing the *Bt* gene pTC12 (modified SN19) (called BwBw) were donated by Dr. T. Chaidamsari (PRI, The Netherlands) and transgenic Col-0 *Arabidopsis* lines (T2 generation) expressing the linalool synthase gene FaNES1 (LIS, called LcLc) were obtained from Dr. A. Aharoni (PRI, the Netherlands) and have previously been described (Chaidamsari et al., 2005; Aharoni et al., 2003). The above-mentioned *Arabidopsis* plants were grown in a climate chamber under a L8:D16 photoperiod, at 20-22°C and 60-70% r.h. until the vegetative plants were well developed.

Brussels sprouts plants, *Brassica oleracea* L. var. *gemmifera* cv Icarus (Sluis & Groot, Enkhuizen, The Netherlands) were grown in a greenhouse under a L16:D8 photoperiod, at 23-25°C and 60-70% r.h. until the seedlings were well developed.

2.2 Insects used

Plutella xylostella was reared on Brussels sprouts plants. This colony has been established in the laboratory for over 200 generations and was started with material collected in the Netherlands and since then reared continuously in the Laboratory of Entomology, Wageningen University. Newly molted third instar larvae were used for larval feeding bioassays. Adult moths younger than 24 hours after eclosion were kept in a cage for overnight mating, and then, without prior oviposition experience on cabbage, were used for oviposition bioassays.

2.3 Kanamycin selection for homozygous transgenic lines

Seeds of *A. thaliana* were sterilized with 4% bleach, including 3 drops Tween/30ml, for 20 minutes. The seeds were rinsed 4 times with sterile MQ water and plated on agar-1/2 Murashige and Skoog (1/2 MS) medium with 300 mg/L kanamycin (HuaMei Ltd, Beijing, China). The plates were incubated in a cold room (4 ºC) for 3 days and then transferred to a growth chamber with long-day conditions (16 h light, 24±1 ºC). After 1 or 2 weeks, antibiotic

resistant green seedlings were transferred to soil and placed in the same growth chamber. After two generations of selfing, followed by kanamycin selection (methods as mentioned above), T_3 transgenic seeds were obtained, from which the individual plants without segregation under kanamycin selection were selected as the homozygous T_3 plants.

2.4 Crosses between *A. thaliana* lines

Because the *Bt* transgene was present in the WS background and the LIS transgene in the Col-0 background, the following hybrid crosses were made between WS and Col-0, LIS and WS, and *Bt* and Col to eliminate the genotypic effects of the different ecotypes: WS×Col→CwCc, *Bt*×LIS→BwLc, LIS×WS→LcCw, *Bt*×Col→BwCc. (Abbreviations: Cw: wild type Wassilewskija *Arabidopsis*, Cc: wild type Columbia *Arabidopsis*, Bw: pTC12 (modified SN19) in WS, Lc: FaNES1 in Col-0). Seventy-two T_3 plants from each homozygous line were used for making different cross combinations. Four to five T_3 plants were used for each hybridization and 20-30 flowers from each plant were used for making a manual cross by artificial pollination with a fine tweezer between Col-0, WS, *Bt*, and LIS respectively. Seeds used for the experiments were collected from at least 15 plants for each crossed line and mixed in order to have sufficient seeds from one batch for all subsequent experiments.

2.5 Detection of linalool emission with SPME-GC-MS

The headspace of 5-6-weeks-old LcLc, BwLc and LcCw transgenic *Arabidopsis* plants were analysed with SPME-GC-MS. An *Arabidopsis* leaf (ca. 2 cm long) was collected and placed in a 1.5-ml screw-cap vial with 10 µl MQ water, capped and used for SPME-GC-MS analysis. A 50 ppm solution of linalool was used as the reference sample. Each day the SPME fiber was conditioned by exposure at 270 ºC for 30 min before the first analysis. The samples were automatically extracted and injected into the GC-MS via a Combi PAL autosampler (CTC Analytics AG). Headspace volatiles were extracted by exposing a 65 µm polydimethylsiloxane-divinyl-benzene SPME fiber (Supelco) to the vial headspace for 20 min. The fiber was inserted into a GC8000 (Fisons Instruments) injection port and volatiles were desorbed for 1 min at 250 ºC. Chromatography was performed on an HP-5 (50 m×0.32 mm×1.05 µm) column with helium as carrier gas (37 KPa). The GC temperature program began at 45 ºC (2 min), was then raised to 250 ºC at a rate of 5 ºC/min, and finally held at 250 ºC for 5 min. The total run time, including oven cooling, was 60 min. The GC interface and MS source temperatures were 260 ºC and 250 ºC, respectively. Mass spectra in the 35 to 400 m/z range were recorded by an MD800 electron impact MS (Fisons Instruments) at a scanning speed of 2.8 scans/s and an ionization energy of 70 eV. The chromatographic and spectral data were evaluated using Xcalibur software (Tikunov et al., 2005).

2.6 Larval feeding bioassay

Newly hatched larvae of *P. xylostella* were fed on cabbage plants until moulting to the third instar (L3). Into Petri dishes with 1% water agar leaves from a single test plant and 5 freshly moulted L3 larvae were introduced. The Petri dishes were sealed with Parafilm and incubated in a climate cabinet at 25±1 ºC, L16: D8. New leaves were added daily and live and dead larvae were counted every day until day 3. The dead larvae were removed from the Petri dish. After 5 days of feeding live larvae were weighed individually.

2.7 Adult oviposition bioassay

A transgenic and a control plant (ca. 6 weeks old) of similar size were paired in a plastic cylinder with a ventilated cover (Fig. 1). Hemizygote Lc plants initially developed slower but were finally similar in size and appearance compared to wildtypes that were around one week younger. Three to five mated female adults of *P. xylostella* or 5 newly eclosed adult females plus 1 adult male were released into each cylinder. The adults were kept in cylinders for one day and two nights (ca. 40 hours) after which the eggs laid on leaves of each plant were counted under a stereo-microscope at 60 × magnification.

Figure 1. Photograph showing the oviposition bioassay setup, with paired plants in a transparent plastic cylinder. Sizes of paired plants were matched to be similar to eliminate any bias from leaf surface area.

2.8 Statistical analysis

Larval mortality data were analysed using a contingency table test based on X^2 distribution. Larval weight was analysed with ANOVA. Adult oviposition data were analysed with a paired t-test.

3. Results

3.1 Development of homozygous and crossing lines

After two generations of selfing accompanied with kanamycin selection, homozygous *Bt* (BwBw) and LIS (LcLc) transgenic seeds were obtained. T_3-plants were used for hybrid crosses to each other and to the wild types CwCw and CcCc which yielded more than 2,000 seeds per cross of CwCc, BwLc, LcCw, and BwCc respectively.

3.2 Linalool emission by transgenic linalool synthase lines

Linalool emission could clearly be detected in the different Lc transgenic *A. thaliana* lines (LcCw and BwLc); no linalool emission was recorded from the hybrid wild type *A. thaliana* (CwCc) (Fig. 2).

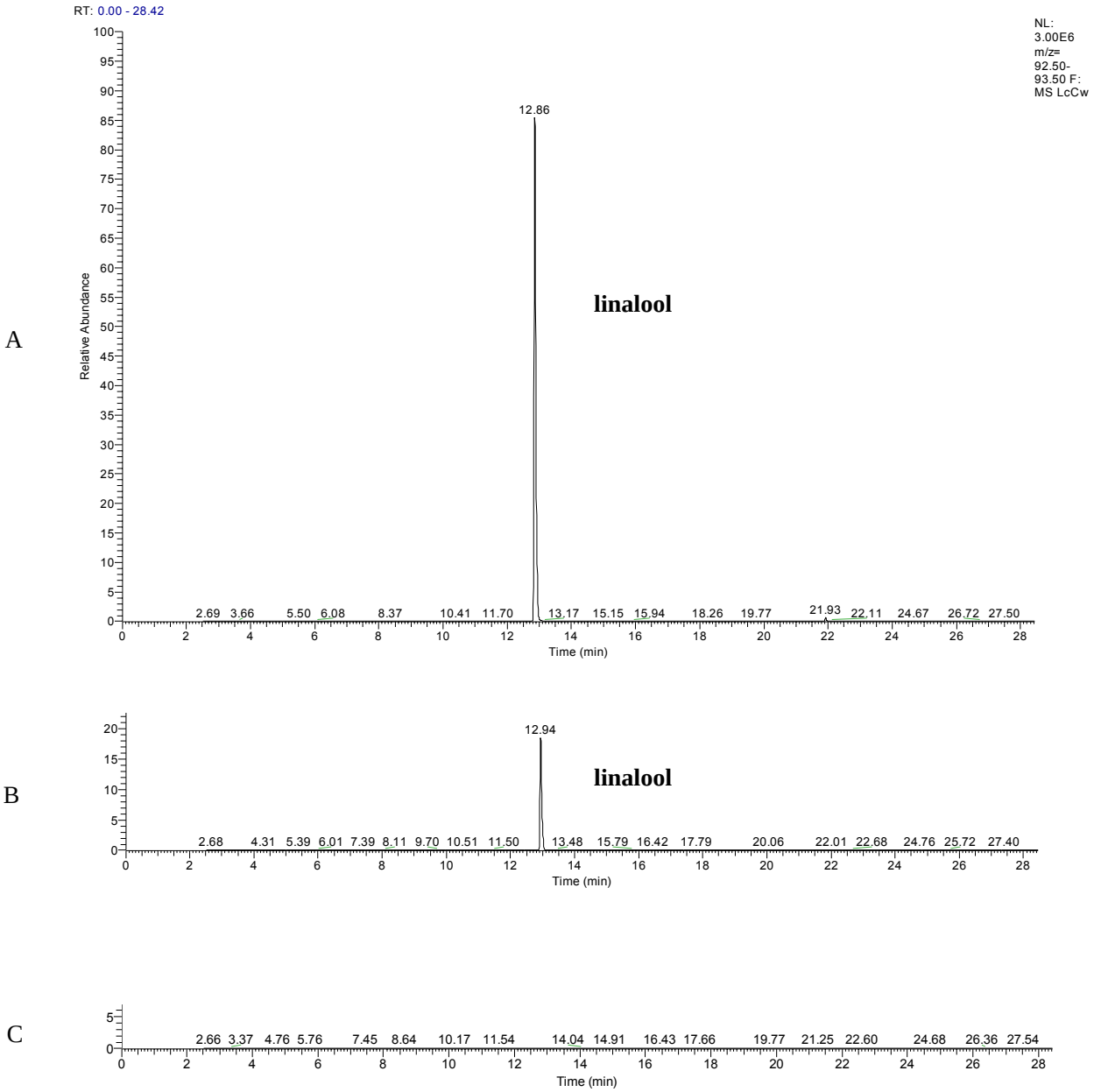

Figure 2. Linalool emission by undamaged plants of the hybrids LcCw (A), BwLc (B), and CwCc (C). The results show the relative abundance of ion masses in the range 92.5-93.5 which is a mass range specific for monoterpenes on the scale of 1A.

The homozygous LcLc line was growing at a different rate compared to the crossed lines as shown in Fig. 3; this was probably due to the expression of the LIS gene. Plants of control hybrid line CcCw (plant D; Fig. 3) and hybrid LcBw (plant C, Fig. 3) were similar in size and

appearance after 5 weeks since sowing. The hybrid LcCw was a slightly smaller (plant B, Fig. 3), while the homozygous LcLc line was the smallest (plant A, Fig. 3). These homozygous LcLc plants were 2-3 weeks retarded in growth and were not used for bioassays. The hybrid LcCw plants were 3-6 days delayed in growth compared to the CwCc or CcCw line and LcBw transgenic plants and in the bioassays one week older plants were used to compensate for the developmental delay.

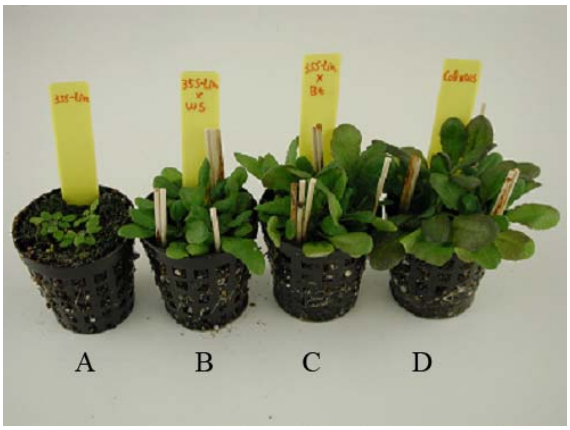

Figure 3. Plant phenotype of different *Arabidopsis thaliana* lines sown on the same day. Homozygous linalool synthase LcLc (A); Hybrid linalool synthase LcCw (B); Hybrid linalool syntase x *Bt*, LcBw (C), and hybrid ecotypes Columbia and Wassilewskija CcCw (D).

3.3 Effect of the expression of *Bt* and LIS genes on larval mortality of *P. xylostella*

Two feeding bioassay experiments were conducted to assess the larval mortality in relation to the expression of the *Bt* and LIS genes. In experiment 1, genotypes CwCc, BwLc, BwCc, and BwBw were used. Six plants were selected from each plant line and five L3 larvae of *P. xylostella* were fed with each plant. In experiment 2, besides the four genotypes used in experiment 1 also LcCw was tested.

In experiment 1 there was no significant difference in larval mortality between BwBw, and BwCc. The mortality of larvae feeding on BwLc, however, was somewhat lower than for larvae feeding on the BwCc and BwBw lines (Fig. 4A) In experiment 2 the larval mortality on BwCc was significantly higher than on BwBw and BwLc, while mortality levels on BwBw and BwLc were significantly higher than on LcCw and CwCc (Fig. 4B). In general, the small differences in acute toxicity of *Bt*-expressing lines were not consistent across duplicate experiments and the difference between *Bt*-expressing lines and the lines not expressing *Bt* was much larger.

After 5 days of feeding all larvae on the *Bt* homozygous line, and *Bt* hybrid lines (BwCc and BwLc) were dead. On the control line CwCc and the hybrid LIS line LcCw most larvae reached the 4th instar. There was no significant weight difference between larvae feeding on either the control line or the LIS hemizygous transgenic lines (Fig. 4C).

In conclusion, the plants carrying the *Bt* SN19 gene were highly toxic to the larvae of *P. xylostella* in both homozygous and hybrid (with half gene dosage) lines in both experiments. The linalool synthase transgenic line LcCw was not toxic to the larvae of *P. xylostella* (Fig. 4A,B).

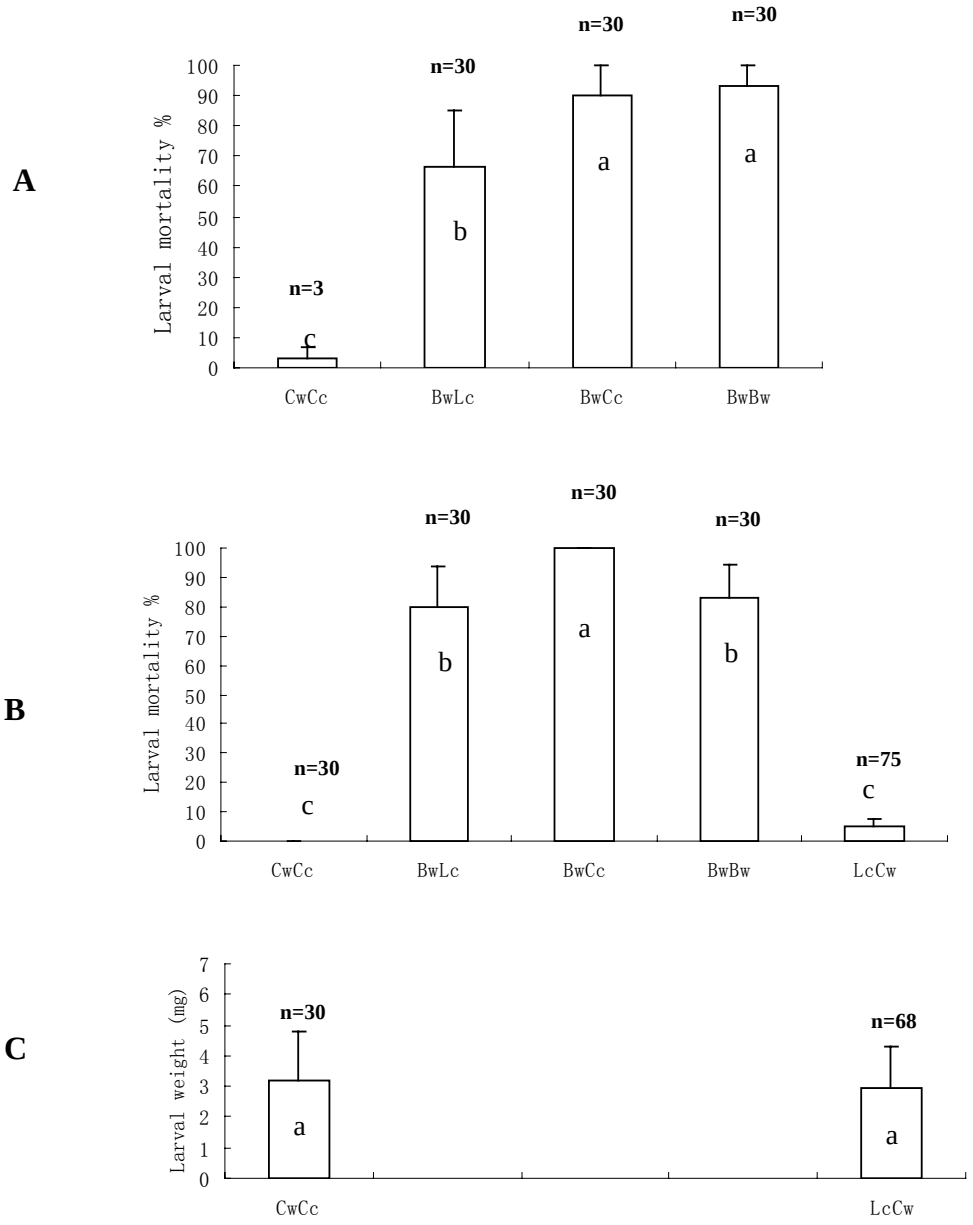

Figure 4.

A: Larval mortality (mean ± SEM) of *P. xylostella* after 3 days in experiment 1 on the control line (CwCc), on the hybrid line *Bt* x LIS (BwLc), on the hybrid *Bt* x control line (BwCc), and on the homozygous *Bt* line (BwBw) (contingency table test based on X^2 distribution , P<<0.001).

B: Larval mortality (mean ± SEM) of *P. xylostella* after 3 days in experiment 2 on the hybrid control line (CwCc), on the hybrid of *Bt* x LIS (BwLc), on the hybrid *Bt* x control line (BwCc), on the homozygous *Bt* line (BwBw), and on the hybrid LIS x control line (LcCw) (contingency table test based on X^2 distribution, P<<0.001).

C: Larval weight (mean ± SEM) of *P. xylostella* feeding for 5 days on the control line (CwCc) and on the hybrid LIS x control (LcCw) line. On day 5 there were no survivors on the other 3 lines. There is no significant difference in larval weight between control line (CwCc) and hybrid of LIS and control line (LcCw) (ANOVA, P=0.393).

Bars within a panel that are marked with different letters are significantly different (contingency table test; α=0.05).

3.4 Effect of the expression of LIS and *Bt* genes on *P. xylostella* oviposition

An experiment was set up to assess the effect of linalool emission on *P. xylostella* oviposition preference in relation to linalool emission (Fig. 5). In the dual choice oviposition experiments fewer eggs were laid on the LIS hybrid LcCw with 63 (37%) eggs versus 105 (63%) eggs on the control hybrid CwCc. Also the hybrid transgenic plants of *Bt* and LIS were significantly less attractive for oviposition with 37 (46%) eggs on BwLc versus 44 (54%) eggs on control plants (CwCc). By contrast, no significant differences were found for the *Bt* hybrid transgenic plants compared to non-transgenic control hybrids with 78 (49%) eggs (BwCc) versus 82 (51%) eggs (CwCc), and on *Bt* homozygous transgenic plants (BwBw) the number of eggs was 98 (49%) versus 101 (51%) on the control plants (CcCc) (Fig. 5). Linalool, resulting from expression of the linalool gene, therefore seems to be detected by adult female *P. xylostella* moths and to act as a repellent, thereby reducing their oviposition on transgenic plants in a choice situation, whereas the expression of the *Bt* toxin gene did not affect their choice.

4. Discussion

The aim of our experiments was to detect whether and how the expression of the *Bt* gene SN19 and the linalool synthase FaNES1 gene affected behaviour and performance of the larvae and adults of the Diamondback moth *P. xylostella*. For this purpose, hybrid *Arabidopsis* plants combining these traits were generated from homozygous parent lines and used for larval feeding bioassays and oviposition preference bioassays. The larval feeding bioassay showed that the expressed *Bt* gene SN19 was toxic to the larvae of DBM in both homozygous and hemizygous dose whereas linalool emitting hybrid LIS transgenic *Arabidopsis* (LsCw) did not impact the development of DBM larvae. The oviposition experiments showed for the first time that linalool emission induced by the LIS gene was repellent to the DBM adults resulting in significantly fewer eggs laid on LcCw and BwLc hybrid plants compared to non-transgenic hybrid control CwCc plants in ventilated plastic cylinders. Interestingly, moth oviposition behaviour was not affected by the presence of the *Bt* toxin so that *Bt* hybrid plants were not deterrent relative to control hybrid plants. In conclusion, the experiments demonstrate that hybrid lines carrying *Bt* and LIS genes in *Arabidopsis* combine toxicity to the larvae and repellence to the adults of DBM.

Numerous studies have shown that plant volatiles play a role in attracting predators and parasitoids to herbivores (Geervliet et al., 1994; Röse et al., 1996; Dicke et al., 1999; Baldwin et al., 1999; Van Poecke et al., 2004; Kappers et al., 2005). Other studies demonstrated that plant volatiles, in contrast, can be repellent to herbivores when emission was constitutive (Aharoni et al., 2003; Jongsma, 2004; Visser et al., 2007) and when emission was induced (Bernasconi et al., 1998; De Moraes et al., 2001; Kessler et al., 2004). Aharoni et al (2003) reported that transgenic *Arabidopsis* lines producing linalool significantly repelled/deterred *M. persicae* aphids, and transgenic chrysanthemum producing linalool repelled/deterred western flower thrips (Jongsma, 2004; Visser et al., 2007). Herbivore-induced emissions of maize volatiles were found to repel the corn leaf aphid (Bernasconi et al., 1998), and several volatile compounds released exclusively at night were highly repellent to female moths of *Heliothis virescens* (De Moraes et al., 2001).

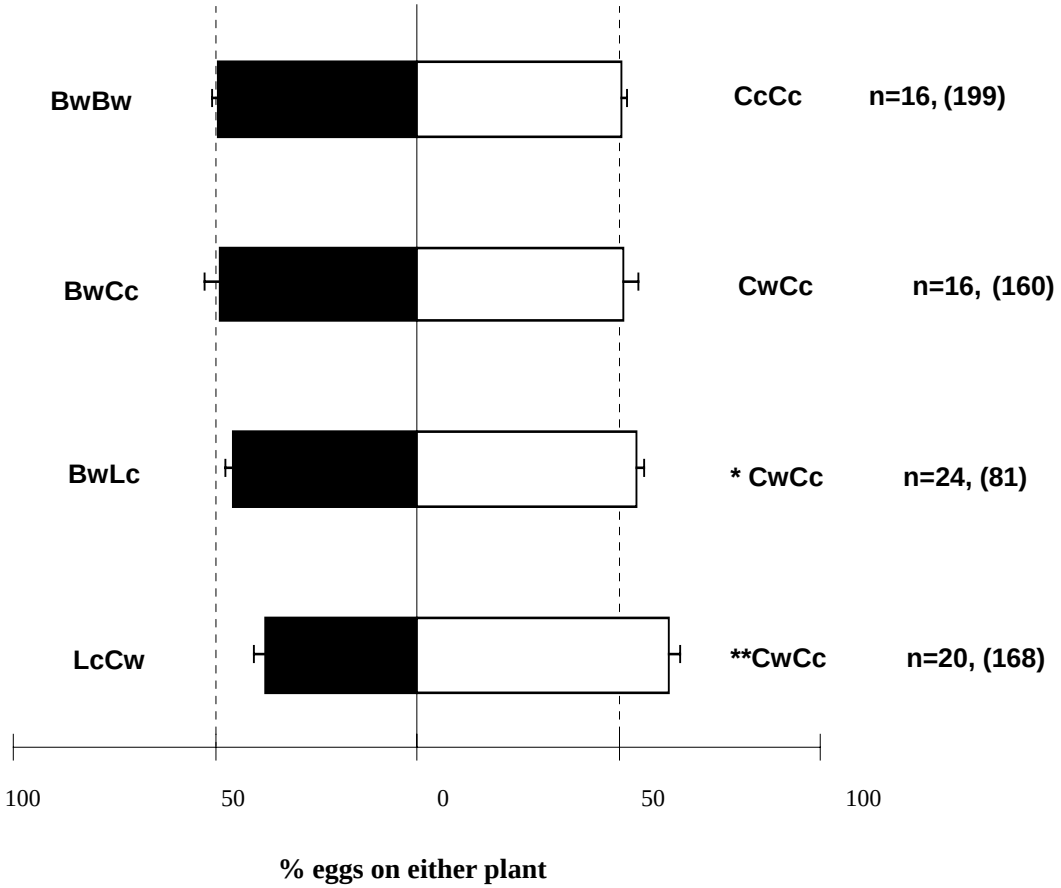

Figure 5. The distribution of the number of eggs deposited by female *P. xylostella* moths on transgenic and control *A. thaliana* plants in two-choice setups. The average total egg numbers counted in each experiment are given between brackets. In the experiments the average absolute total egg number produced on hybrid transgenic LIS x control plants (LcCw) was 63 (37%) and on wildtype control plants (CwCc) 105 (63%) (P<<0.001), on hybrid transgenic *Bt* and LIS plants (BwLc) was 37 (46%) vs control plants (CwCc) was 44 (54%) (P=0.0395), on *Bt* hybrid transgenic and control plants (BwCc) was 78 (49%) vs control plants (CwCc) was 82 (51%) (P=0.766), and on *Bt* homozygous transgenic plants (BwBw) was 98 (49%) vs control plants (CcCc) was 101 (51%) (P=0.654). Asterisks indicate a significant difference within a paired t-test: * P<0.05, ** P<0.01.

Aqueous leaf extracts from the syringa tree, *Melia azedarach,* induced the release of larger quantities of volatiles from cabbage plants and resulted in attraction of *Cotesia plutellae*, a specialist parasitoid of *P. xylostella* (Charleston et al., 2006). Interestingly, the application of the syringa extract resulted in reduced oviposition by *P. xylostella* moths and in feeding deterrence by their larvae (Charleston et al. 2005). Also, plant volatile compounds from extracts of *Chrysanthemum morifolium* were demonstrated to reduce oviposition by DBM and led to increased attraction of natural enemies to DBM (Liu et al., 2006). We show for the first time that linalool is a specific oviposition repellent for DBM moths. Flowers of *Arabidopsis* produce the monoterpenes β-myrcene, limonene, and linalool and the sesquiterpenes

(-)-(*E*)-β-caryophyllene and (+)-thujopsene (Chen et al., 2003). By contrast, the leaves of *Arabidopsis* produce only trace amounts of the monoterpenes limonene, and β-myrcene, and the homoterpene (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (van Poecke et al., 2001; Aharoni et al., 2003).

Linalool emission in *Arabidopsis* was shown to be governed by a diurnal rhythm with high emission rates during day time and low emission rates several hours after dusk until sunrise (Aharoni et al., 2003). This is the case despite control of the LIS gene by the constitutive 35S promoter. It is caused by the fact that the precursors of linalool fluctuate this way in the plastids. Potentially, the low nocturnal emissions of linalool may affect the effectiveness of linalool to repel *P. xylostella* moths. However, our data show that the lower nocturnal emissions did not annihilate the effect on DBM oviposition, that supposedly takes place predominantly during the scotophase but to some extent also during the light phase. Our experiments lasted for 40 h and included a full diurnal cycle of 24 h.

Since the first *Bt* transgenic crops were commercialized in 1996, a number of pest insects were reported to evolve resistance to *Bt* crops (Tabashinik et al., 1997; 2003; Heckel et al., 1999; Zhao et al., 2005). DBM was the first insect to develop resistance to *Bt* toxins in open-field populations (Tabashnik et al., 1997; Heckel et al., 1999; Mehlo et al., 2005). Among the options to delay the evolution of insect resistance, the high-dose refuge strategy is one of the strategies which seems most promising (Zhao et al., 2003; Bates et al., 2005). This strategy assumes that resistance is recessive and is conferred by a single locus with two alleles in three insect genotypes (RR, SS, RS), that resistance alleles are initially rare and that there will be random mating between resistant and susceptible adults (Roush, 1994; Gould, 1998). The success of the high-dose refuge strategy is based on the optimal random mating between susceptible moths from the refuge and any resistant survivors from the *Bt* crop fields. Information regarding insect flight and oviposition behaviour is critical to establish the separation distance between the *Bt* crop planting and refuge. The size of the refuge is based on understanding the dose level expressed by the transgenic crop, the predicted genetics of resistance inheritance, population genetics and ecology of a given target pest, and estimation of the initial resistance allele frequency (Glaser & Matten, 2003). The size of the refuge should be big enough to reduce mortality of less susceptible heterozygous insects but should have no economic trade-offs (Bates et al., 2005).

Bt toxin and linalool synthase genes were integrated into *Arabidopsis* as a model to investigate new methods to improve DBM control. Transgenic plants carrying both traits were found to be toxic to the larvae and repellent to ovipositing adult female DBM. This repellence is expected to increase the probability that potentially resistant insects from the *Bt* crop will mate with susceptible insects from a refuge. This would increase the efficiency of the high-dose refuge, thereby raising the efficiency in controlling DBM, as it would not only reduce the population of the pest but also reduce selection pressure for resistant alleles on *Bt* transgenic plants, thus finally delaying the selection of pest resistance to *Bt* toxins. In fact this strategy to label *Bt* toxin-expressing plants with an odor flag which repels the DBM females could lead to the selection of moths which respond to the flag more and more strongly because of the fitness gain associated with it. If that would happen the proportion of moths that would end up in the refuge would even increase with time. The oviposition preference of adult female herbivores is generally associated with the relative performance of their offspring but they are often not

identical. The diet breadth of the larvae is often wider than the range of plants acceptable as oviposition substrate to the adult female. Natural selection will prevent the development of too great a discrepancy between the preferences of ovipositing females and their offspring (Schoonhoven et al., 2005). Adding a repellent to a crop which remains of good quality for the herbivore will select for the herbivore to neglect the repellent. In contrast, the signal value of a repellent when it represents a low quality food source, will increase selection to respond to the repellent in avoiding this suboptimal food source. The repellence that was recorded in our study (i) may reduce the incidence of resistant insects, when avoiding linalool expressing plants which co-express *Bt* toxins is also observed in the field; (ii) may increase the probability that potentially resistant insects from *Bt* toxin plants will mate with susceptible insects colonising non-transgenic plants that constitute refuges; and (iii) may favour susceptible insects, when avoiding linalool expressing plants provides a selective advantage, as it will when these plants express *Bt* toxins. Thus, the co-expression of linalool may provide a novel option to reduce selection of *Bt*-resistant larvae. This deserves further investigation that also includes the potential effects of experience on the behaviour of the diamondback moth (Liu et al., 2005; 2007; Zhang et al., 2007)..

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Chapter 5

Effect of constitutive emission of linalool by transgenic plants on parasitoid attraction and survival

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Abstract

Transgenic *Arabidopsis* plants expressing a *Bt* gene and a high emission level of linalool were used in a Y-tube olfactometer and greenhouse flight-bioassay to study their effect on the behaviour of the parasitoids *Diadegma semiclausum* and *Cotesia glomerata*. Undamaged Bt x linalool synthase (LIS) transgenic plants were neither attractive nor repellent to the female wasps of *D. semiclausum* compared with undamaged non-transgenic control plants. When LIS plants were damaged by the larvae of *Plutella xylostella* or damaged mechanically they showed a repellent effect on *D. semiclausum* compared with similarly-treated non-transgenic control plants. Yet, when LIS plants were damaged by *P. xylostella* larvae, the plants were more attractive to the parasitoids than undamaged LIS plants. LIS plants that were damaged by *Pieris brassicae* lavae were more attractive to adult female *Cotesia glomerata* wasps when compared with *P. brassicae*-damaged non-transgenic control plants. Experiments addressing the actual parasitization rate of *P. xylostella* by *D. semiclausum* showed that there is no significant difference between the parasitization rates on the transgenic plants and the non-transgenic control plants.

1. Introduction

Plants can defend themselves against herbivores with direct and indirect defence mechanisms. The direct defence affects herbivores directly through physical or chemical means, such as thorns, toxins, and digestibility reducers. Indirect defence includes the attraction of natural enemies of herbivores by emitting specific volatile compounds in response to herbivory.

The production of toxins is one of the most important mechanisms of chemical defence in plants (Schoonhoven et al., 2005). Moreover, in addition to toxins produced by a certain plant species, genes from other organisms coding for toxins can be engineered into plants. Genetic engineering of plants in insect pest control has involved insertion of genes coding for toxins and may be characterized as the incorporation of biopesticides into classical plant breeding. The proteinaceous δ-endotoxins produced by *Bacillus thuringiensis* (*Bt*) were already long known to possess insecticidal activity, but in 1987 it was demonstrated for the first time to be effective when expressed in transgenic tobacco. Since then many studies using *Bt* gene transformation were successful in generating resistance to diverse insects in many crops (Schuler et al., 1998). Transgenic plants expressing *Bt* toxins were first commercialized in 1996 (Bates et al., 2005) and up to now are still the only transgenic insect-resistant crops commercially released worldwide. Among *Bt* genes, the crystal protein endotoxins (*cry*) are utilized most commonly in different crops (Roush, 1998). SN19, a synthetic and truncated

cry1Ba gene with the insertion of the domain II-encoding part of *cry1Ia*, expressed in potato plants, was shown to yield resistance against Colorado potato beetle larvae and adults, potato tuber moth larvae, and European corn borer larvae (Naimov et al., 2003). In order to improve expression in transgenic dicotyledonous plants, a number of single nucleotide changes in fragments encoding the domain III of the hybrid *cry* gene SN19 have been made. Transgenic *Arabidopsis* plants expressing modified SN19 were resistant to *Plutella xylostella* and *Pieris rapae* (Chaidamsari, 2005).

Transgenic insect-resistant crops that express toxins from *Bt* offer significant advantages to pest management, but are at risk of losing these advantages due to the possible evolution of resistance in the targeted insect pests (Shelton et al., 2000; Bates et al., 2005; Zhao et al., 2005; Tabashnik et al., 2008). Moreover, the development and behaviour of non-target beneficial insects may be affected by the planting of *Bt* crops (Schuler et al., 1999). Sustainable pest management will only be possible when negative effects on non-target beneficial arthropods are minimized (Groot & Dicke, 2002).

Indirect plant defence is in many cases mediated by plant volatiles, which guide the searching behaviour of predators and parasitoids to the herbivores. Emitted volatiles may consist of preformed volatiles released immediately after damage and of volatiles that are synthesized *de novo* in response to herbivory (Paré & Tumlinson, 1997; Baldwin & Preston, 1999; Bouwmeester et al., 1999; Dicke et al., 2003; D'Alessandro et al., 2006). These herbivore-induced plant volatiles (HIPVs) can be released specifically in response to herbivore attack, and, therefore, they are reliable cues for parasitoids and predators during searching for herbivorous hosts or prey. However, HIPVs can affect predators or parasitoids also in negative ways. Volatile toxic chemicals, sequestered unaltered from plants, may render herbivorous insects toxic to predators and parasitoids (Price et al., 1980). Some plant species respond with non-specific volatiles that are produced in response to herbivory as well as in response to mechanical damage. Both in the case of specific and non-specific volatiles, the composition of the blend and the amounts emitted can be used by carnivores to discriminate between herbivore-damaged and mechanically damaged plants. Moreover, damage by different herbivore species, or even different herbivore instars, can be distinguished by parasitoids on the basis of herbivore-induced plant volatiles. The information content of the volatile blend emitted by herbivore-infested plants is the most specific when production of novel compounds occurs in response to herbivory (Dicke, 1999; van Poecke et al., 2001).

Terpenoids are known as compounds which can mediate interactions between plants and their environment: they are involved in interactions between plants and microorganisms, between plants and herbivorous arthropods or between plants and carnivorous arthropods (Cseke et al., 1998; Aharoni et al., 2003; 2006; Kappers et al., 2005). Some terpenoids were demonstrated to act as toxins, growth inhibitors, or deterrents to both microorganisms and animals (Gershenzon & Dudareva, 2007). The terpenoid volatiles released from flowers and fruits seem to serve as advertisements to attract pollinators and dispersal agents. Furthermore, in many plant species terpenoid volatiles are components of the odoriferous 'call for help', attracting predators and parasitoids that attack herbivores (Gershenzon & Dudareva, 2007). Thus, terpenoids appear to affect the physiology and behaviour of a large range of organisms. Linalool, a C10 monoterpene volatile, was proven to be either attractive (Dicke et al., 1990a; Raguso & Pichersky, 1999; Takabayashi et al., 1995; Turlings et al., 1995; Takabayashi &

Dicke, 1996; De Moraes et al., 1998; Baldwin & Preston, 1999; Paré & Tumlinson, 1999; Kahl et al., 2000; Dicke & van Loon, 2000; Colazza et al., 2004; Yan & Wang, 2006; Wei & Kang, 2006) or repellent / deterrent (De Moraes et al., 2001; Koschier et al., 2002; Jongsma, 2004; van Tol et al., 2007) to a broad spectrum of pollinators, herbivores and parasitoids.

Progress in research on the molecular mechanisms that are involved in herbivore-induced volatile emissions has provided reason for optimism that we will soon be able to produce crop varieties that have improved abilities to repel herbivores and to attract natural enemies of herbivores (Aharoni et al., 2003; Kappers et al., 2005, Beale et al., 2006; Schnee et al., 2006; Turlings & Ton, 2006). Plant genetic engineering makes it possible to transfer a foreign gene to a crop plant to produce a volatile that can attract the natural enemy of the pest after the host plant is wounded by insect herbivores. Several studies focused on transgenic plants expressing linalool synthase genes from strawberry and evening primrose resulting in the expression of linalool and its volatile and non-volatile derivatives, such as hydroxylated linalool and linalool glycoside (Lücker et al., 2001; Lewinsohn et al., 2001; Aharoni et al., 2003; Kappers et al., 2005). The FaNES1 (LIS) gene was isolated from strawberry (*Fragraria ananassae)* and transferred to *Arabidopsis thaliana* and high linalool emitting transgenic plants were obtained, whereas the leaves of wild type *Arabidopsis* (Col0) plants do not emit linalool (Aharoni et al., 2003). *Arabidopsis* is known to produce herbivore-induced terpenoids and other volatiles in response to the damage of caterpillars of *Pieris rapae* and to attract the parasitic wasp *Cotesia rubecula* as a consequence (van Poecke et al., 2001). The availability of the LIS-transgenic *Arabidopsis* plants provides an excellent opportunity to investigate the effects of enhanced linalool emission on natural enemies of herbivores, such as parasitoids. This can be used to investigate the potential of enhanced linalool emission for the improvement of biological control.

In order to integrate plant direct and indirect defences and to explore the effects of *Bt* and LIS dual transgenes on herbivores and their natural enemies, *Bt* toxin and linalool synthase genes were combined in transgenic *Arabidopsis* plants. In the current study, LIS-transgenic *Arabidopsis* plants (crossed with *Bt* plants or non-transgenic control plants) are used for studying their effect on attraction of *Diadegma semiclausum,* a specialist parasitoid of *Plutella xylostella* and *Cotesia glomerata*, a parasitoid of *Pieris brassicae.* We investigated the effects of volatiles from undamaged, mechanically damaged and caterpillar-damaged LIS-transgenic *Arabidopsis* plants on attraction of the parasitoids. In addition, possible effects on the actual parasitization of *P. xylostella* larvae by the parasitoid *D. semiclausum* on LIS transgenic plants was also addressed.

2. Materials and methods

2.1 Plants

Arabidopsis thaliana ecotypes Columbia-0 (Col-0) and Wassilewskija (WS) were used as wild type control plants. *Bacillus thuringiensis* (pTC12: modified SN19 (Naimov et al., 2003)) transgenic *A. thaliana* WS (donated by Dr. T. Chaidamsari, Plant Research International (PRI), Wageningen, The Netherlands) and T_2 generation plants of linalool synthase (LIS)-transgenic *A. thaliana* Col-0 (obtained from Dr. A. Aharoni, PRI, Wageningen, The Netherlands) were used as the original transgenic plants. Because homozygous LIS plants had a strongly

reduced growth rate compared to control plants, the homozygous LIS line was crossed with *Bt*-WS and with wildtype WS lines: *Bt*×LIS (heterozygous line) and LIS×WS (hemizygous line). To avoid the possible influence of interactions between the two ecotypes, the cross WS×Col-0 was made to serve as the control line. For the method used to produce the cross for *Arabidopsis* plants and for the analysis of the headspace see paragraphs 2.4 and 2.5 in Chapter 4. The notation of the plant genotypes will follow that applied in chapter 4 consistently, i.e. WS × Col → CwCc, *Bt* × LIS → BwLc, LIS × WS → LcCw. Moreover, the hemizygous LIS plants used in the greenhouse flight-choice bioassay for *Cotesia glomerata* were not from an artificial cross but originated from the segregation of the original transgenic event while the control plants used were Col-0.

The above-mentioned *A. thaliana* lines (transgenic lines: BwLc, LcCw; hemizygous LIS; control lines: CwCc and Col-0) were grown from seed in a climate chamber (20 ºC, 60-70% r.h., L8:D16). Vegetative plants of 6-7 weeks old were used for bioassays. The plant sizes of the control hybrid line (CwCc) and the hybrid line between *Bt* and LIS (BwLc) were similar, but the hybrid line between homozygous LIS and WS wildtype plants (LcCw) was smaller. The LcCw plants were 3-6 days delayed in growth compared to the CwCc and BwLc plants and in the bioassays one week older plants were used to compensate for the developmental delay (See Fig. 1 in chapter 4).

2.2 Insects

Plutella xylostella was reared on greenhouse-grown Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Icarus) in a climate room (21±1 ºC, 50-70% r.h., L16:D8).

The parasitoid *Diadegma semiclausum* was reared on *P. xylostella* larvae feeding on Brussels sprouts plants in a climate room (21±1 ºC, 50-70% r.h., L16:D8). For bioassays, *D. semiclausum* cocoons were collected and transferred to an empty cage in the same climate room. Eclosed wasps were collected and transferred to a clean cage to mate; female : male ratio introduced was 10:1 to 10:2. The wasps were provided with water and honey. Mated female wasps, 5-10 days since eclosion, were used for bioassays.

Pieris brassicae was reared on Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Icarus) in a climate room (21±2 ºC, 50-70% r.h., L16:D8).

The parasitoid *Cotesia glomerata* was reared on *P. brassicae* larvae feeding on Brussels sprouts plants, under greenhouse conditions (25±5 ºC, 50-70% r.h., L16:D8). For bioassays, *C. glomerata* cocoons were collected and transferred to a gauze cage in a climate room (23±1 ºC, 50-70% r.h., L16:D8). Eclosed wasps were collected and transferred to an empty gauze cage to allow random mating. The wasps were provided with water and honey. Mated female wasps, 5-8 days since eclosion, were used for the bioassay.

Adult wasps of *D. semiclausum* and *C. glomerata* had not been given oviposition experience and are therefore referred to as ‘naïve’ wasps.

2.3 Plant treatments

Mechanically damaged plants were obtained by making holes with a plastic tubule of 5 mm diameter. The tubule was put on the leaf surface supported by one finger on the other side of the leaf and then turned gently and quickly to produce a hole. Three, six, or nine holes per plant were made respectively, 30 min before the start of the experiment. Four plants per

treatment were used.

Plutella-damaged plants were obtained by placing different numbers (see Results section) of *P. xylostella* larvae on each plant. For LcCw and CwCc plants ten 2nd instar larvae were allowed to feed for 24 hours; ten larvae were placed on the upper surface of different leaves. For BwLc plants twenty 3rd instar larvae were allowed to feed for 24 hours because the product of the *Bt* (SN19) gene is highly toxic to the larvae of *P. xylostella,* especially to the younger larval instars. Four plants per treatment were used.

Pieris-damaged hemizygous LIS plants were obtained by placing two 1st instar larvae on each plant for 24 h of feeding. Twelve plants per odor source were used for a greenhouse flight-choice bioassay.

2.4 Y-tube olfactometer bioassay

The olfactory response of *D. semiclausum* was observed in a Y-tube olfactometer (Takabayashi & Dicke, 1992) (Fig. 1). This setup has previously successfully been used to investigate the attraction of *D. semiclausum* (Bukovinszky et al., 2005). The setup was located in a constant environment (22±1º C) and illuminated by 4 fluorescent tube lights (FTD 32 W/84 HF, Pope, The Netherlands) suspended 90 cm above the Y-tube olfactometer. The airflow through the two jars connected to the B- and C- arms (Fig. 1) was 4 l/min, so 8 l/min through the A-arm (Fig. 1; diameter 40 mm). Four plants served as odour sources in each jar. After a single female wasp was introduced at the release point, the behaviour of the parasitoid was observed. When the wasp crossed the “decision line” (Fig. 1) in either of the two arms and remained beyond that line (in area 3) for at least 15 seconds, this was recorded as a response. If the wasp remained in area 1 for 5 minutes or longer, or moved in area 2 but did not reach the “decision line” within 10 minutes, this was recorded as “no response”. We repeated this bioassay until observations had been made on at least 20 responding wasps, switching treatments between arms of the Y-tube after every five trials to control for possible asymmetry effects. We compared the numbers of wasps responding to the two odour sources with a two-sided binomial test.

2.5 Flight choice bioassay

Cotesia glomerata two-choice flight experiments were conducted with a flight choice bioassay setup (Steinberg et al., 1992) in a greenhouse compartment (23±5 ºC, r.h. 50-60%). The setup consisted of a table in a nylon gauze tent (length 3 m, width 1 m, height 1 m), a wooden block (length 1 m, width 1.5 m) which was put on the table, two fans which were located at one end of the table, a gauze screen which was used to prevent that wasps landed on the plants and a glass cylinder from which the wasps were released (Fig. 2). The distance between the two fans was 50 cm, that between the fans and the plants was 120 cm and the distance between the two plant odour sources was 40 cm. The distance between the midpoint of the cylinder and the screen (standing just in front of plants) was 50 cm.

Twelve plants were used and placed in three rows to act as odour sources (12 *P. brassicae*-infested hemizygous LIS transgenic plants vs 12 *P. brassicae*-infested non-transgenic control (Col-0) plants). Each plant was inoculated with two newly hatched larvae of *P. brassicae.* The wind speed was adjusted to 0.3 m/s in front of the screen and 0.1 m/s at the parasitoid release point, using an anemometer. The experiment was conducted

between 11:00 - 15:00 h.

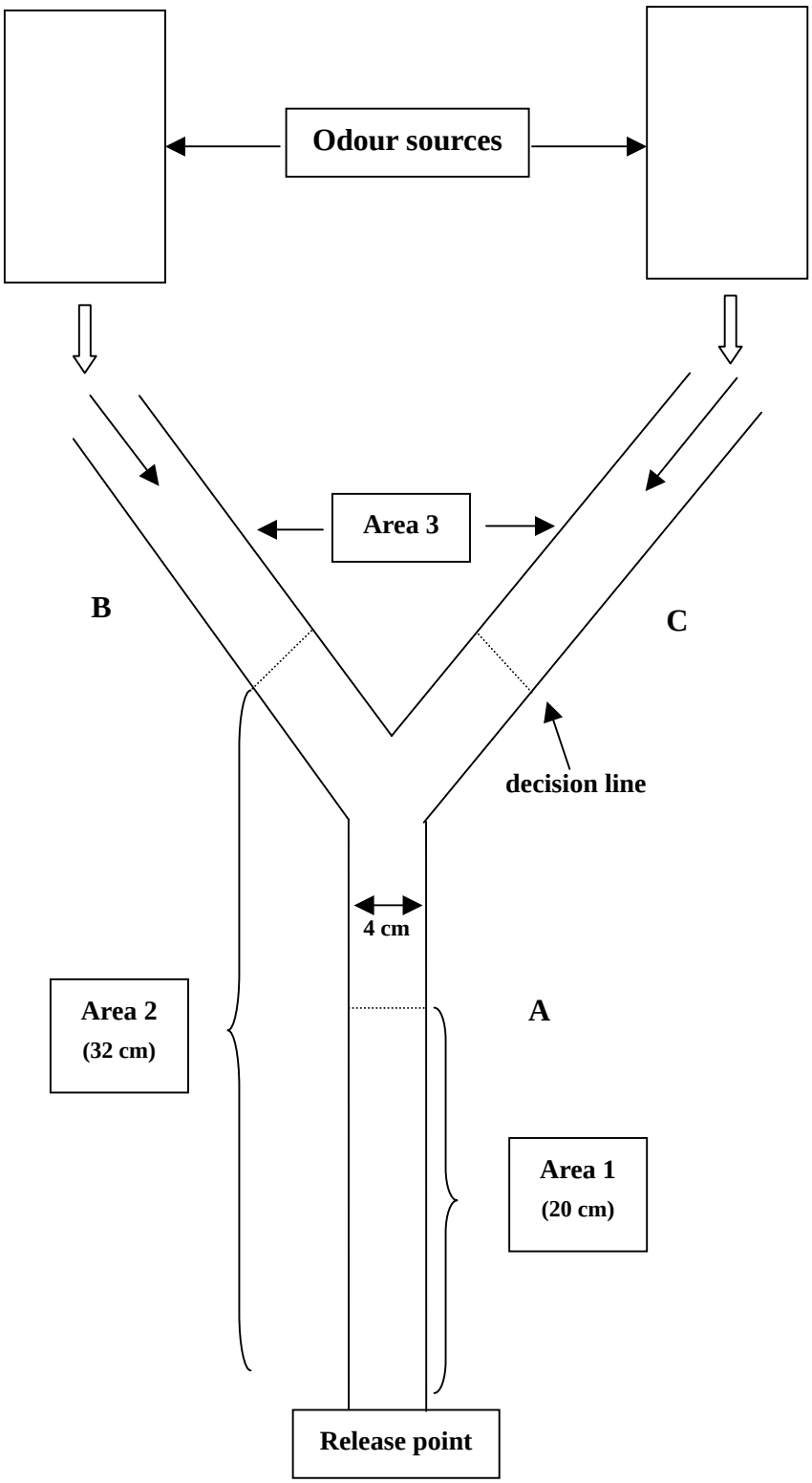

Figure 1. Schematic drawing of Y-tube olfactometer. The wasps were released from the downwind end of the A-arm. The odour sources were connected to the B- and C-arms. Three areas were distinguished.

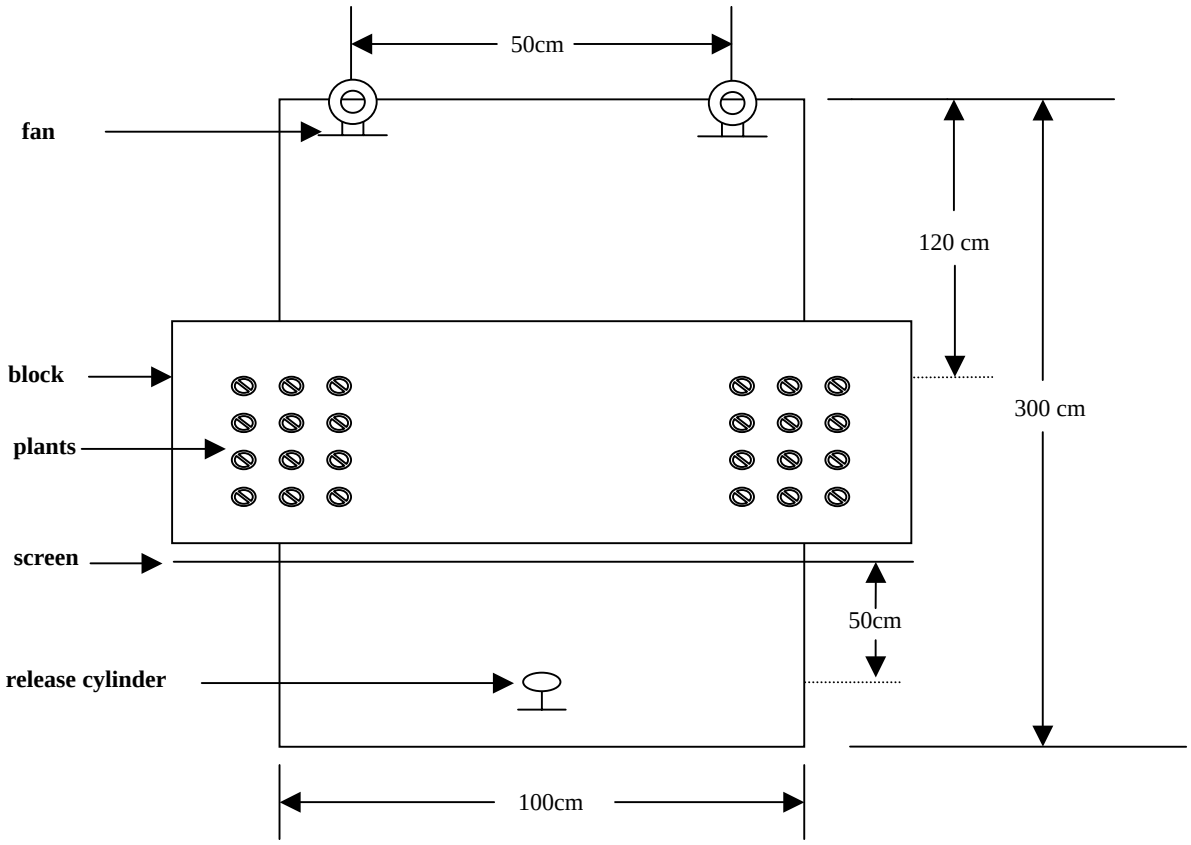

Figure 2. Schematic drawing of flight behaviour assay in the greenhouse.

Five-to-eight-days old naïve female *C. glomerata* wasps were used for the bioassay. When the wasps landed on the screen in front of either odour source within 6 min, this response was recorded as a response to that odour source, otherwise (i.e. landing elsewhere or taking longer than 6 min) a ”no response” was recorded. The wasps´ choice behaviour was statistically analysed with a two-sided binomial test.

2.6 Parasitisation success of *D. semiclausum*

Parasitisation experiments were conducted in a plastic cylinder (15 cm diameter and 25cm high) with a ventilated cover in a climate room (21±1ºC, 50-70% r.h., L16:D8). One LcCw plant (hybrid line between LIS and control plants) or one CwCc plant (non-transgenic control line) were placed in each cylinder. Ten 2nd instar larvae of *P. xylostella* were placed on each plant one day before the experiment. A total of 16 replicate plants were used per treatment. One 8-to-10-days old, mated female *D. semiclausum* wasp was released into each cylinder. After the first larva was parasitised, the parasitoid was recorded for another 10 min, after which it was removed. The cover of the cylinders was sealed with Parafilm to avoid the escape of larvae. The plants were watered every two days until wasp larvae egressed from larvae of *P. xylostella* and spun a cocoon. The number of cocoons obtained on each transgenic or control plant were statistically analysed using a Mann-Whitney U-test with correction for continuity.

3. Results

3.1 Response of *Diadegma semiclausum* in a choice situation between undamaged *Bt*×LIS (BwLc transgenic line) *versus* undamaged WS×Col (CwCc control line)

No significant preference was found for the volatiles emitted by undamaged *Bt*-and-LIS-transgenic plants (BwLc) and undamaged control plants (CwCc) in the Y-tube olfactometer bioassay (binomial test, two-sided; P=0.42). This means that, at the emission level produced by intact transgenic plants, linalool is neither an attractant nor a repellent for naive females of *Diadegma semiclausum* (Fig. 3).

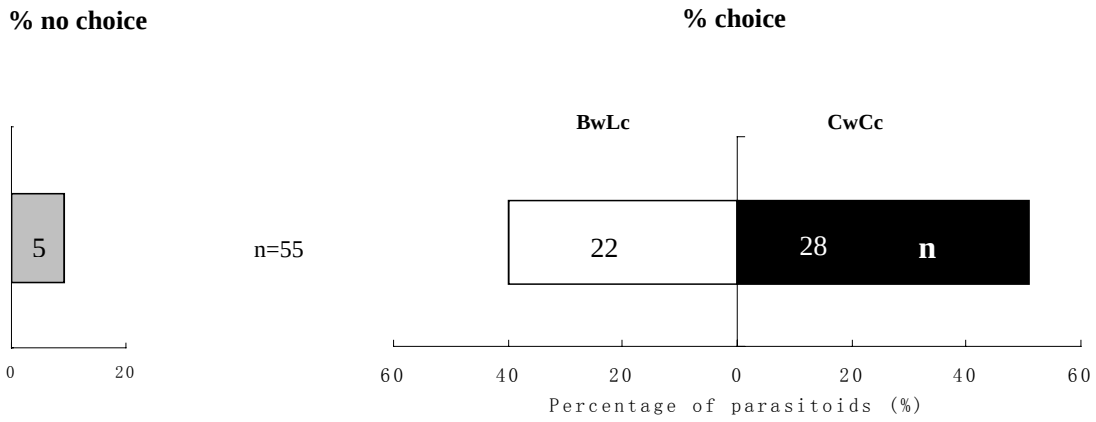

Figure 3. Response of *D. semiclausum* to undamaged LIS transgenic and non-transgenic *Arabidopsis* in a Y-tube olfactometer. Naive *D. semiclausum* females were given a choice between transgenic linalool emitting plants and control plants not producing linalool. The left panel of the figure shows the percentage of wasps that did not make a choice for one of the two odour sources within 5 min in area 1 or within 10 min in area 2 (Fig. 1). The white bar (left hand side) represents the percentage of wasps choosing for the volatile blend emitted by BwLc plants. The black bar (right hand side) represents the percentage of wasps choosing for the volatile blend emanating from CwCc plants. ns indicates that there was no significant preference (binomial test, two-sided, P=0.42). n is the total number of wasps tested. Numbers in the bar segements represent the number of wasps showing the indicated behaviour.

3.2 Response of *D. semiclausum* in a choice situation between mechanically damaged *Bt*×LIS (BwLc transgenic line) and mechanically damaged WS×Col (CwCc control line)

When plants were mechanically damaged by making 3 holes no significant preference was found for either the *Bt*- and LIS-transgenic plants (BwLc) or the control plants (CwCc) in the Y-tube olfactometer bioassay. When mechanically damaged by punching 6 holes *D. semiclausum* females clearly preferred the control plants over the transgenic plants (binomial test, two-sided; P<0.006). The wasps did not display a significant preference when BwLc transgenic plants and control plants, both damaged by making 9 holes per plant, were compared.

The linalool synthase is expressed under the control of a constitutive promoter and thus linalool emission rates are expected to be independent of the degree of wounding. Comparing the data for the 3 holes and 6 holes damage levels, it is expected that larger amounts of wound-induced volatiles are mixed with linalool in the 6 holes-treatment. This would mean that when this blend with linalool was emitted at this elevated level, a repellent effect to *D. semiclausum* occurred. However, for the 9 holes damage level, even more wound-induced

volatiles are expected to be emitted but no significant preference was observed (Fig. 4). Response of *D. semiclausum* in a choice situation between LIS transgenic plants damaged by *Plutella xylostella* larvae versus undamaged LIS transgenic plants.

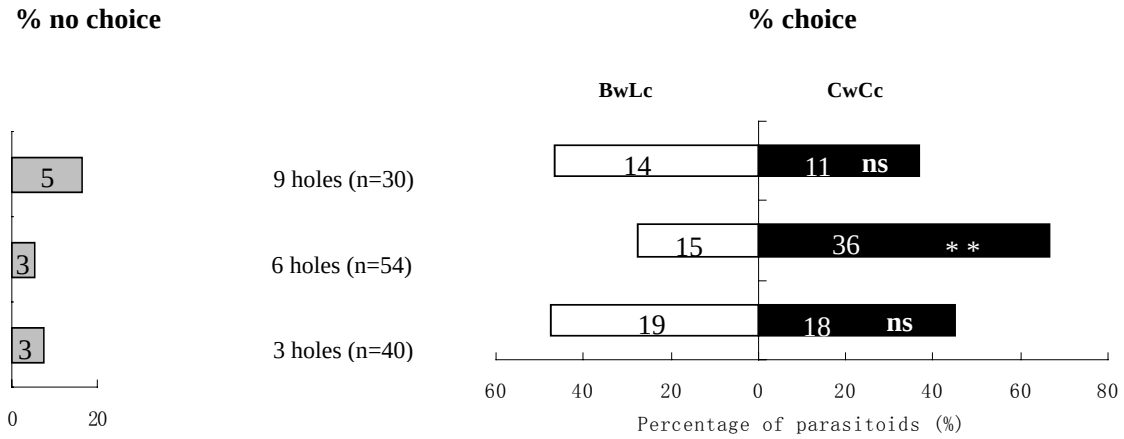

Figure 4. Response of *D. semiclausum* to mechanically damaged LIS transgenic and mechanically damaged non-transgenic *Arabidopsis* in a Y-tube olfactometer. Naive *D. semiclausum* females were given a choice between transgenic linalool emitting plants and control plants not producing linalool. The left panel of the figure shows the percentage of wasps that did not make a choice for one of the two odour sources within 5 min in area 1 or within 10 min in area 2 (Fig. 1). The white bar (left hand side) represents the percentage of wasps choosing for the volatile blend emitted by BwLc plants. The black bar (right hand side) represents the percentage of wasps choosing for the volatile blend emanating from CwCc plants. ns indicates that there was no significant preference. Asterisks indicate a significant preference ** P<0.006 (binomial test, two-sided). n is the total number of wasps tested. Numbers in the bar segements represent the number of wasps showing the indicated behaviour.

A significant preference was found for the odour emitted by both the BwLc- and the LcCw-plants damaged by *Plutella* larvae compared with undamaged plants of the respective genotypes (binomial test, two-sided; P<0.001). We therefore conclude that constitutive linalool emission does not interfere with odour-guided host location by *D. semiclausum* (Fig. 5).

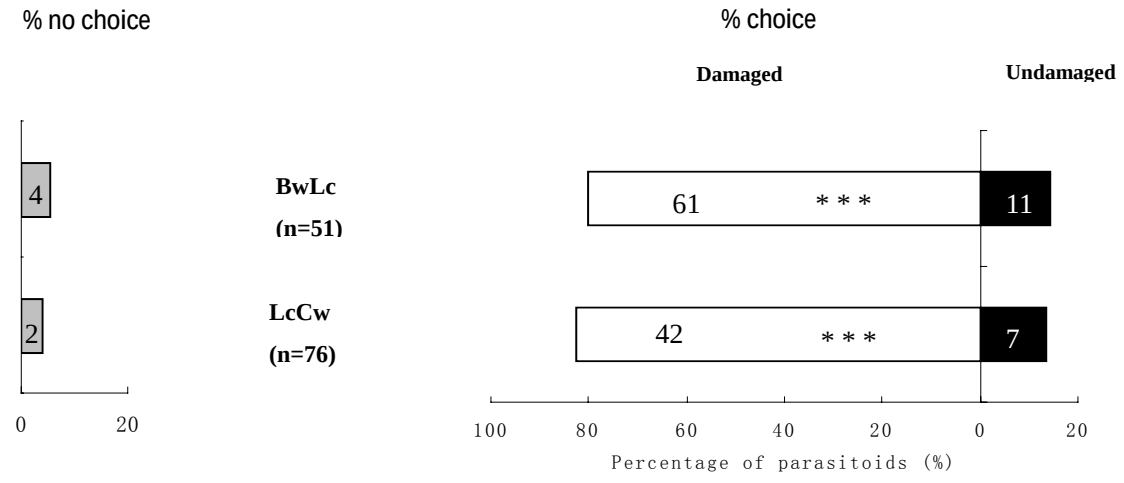

Figure 5. Response of *D. semiclausum* to LIS-transgenic *Arabidopsis* plants damaged by *P. xylostella* larvae and undamaged LIS-plants in a Y-tube olfactometer. Naive *D. semiclausum* females were given a choice between LIS-plants damaged by *Plutella* larval feeding and non-damaged LIS-plants, both in BwLc and LcCw line. The left panel of the figure shows the percentage of wasps that did not make a choice for one of the two odour sources within 5 min in area 1 or within 10 min in area 2 (Fig. 1). The white bar (left hand side) represents the percentage of wasps choosing for the volatile blend emitted by LIS-plants damaged by *P. xylostella*. The black bar (right band side) represents the percentage of wasps choosing for the odour from undamaged LIS-plants. Asterisks indicate a significant preference ***P<0.001 (binomial test, two-sided). n is the total number of wasps tested. Numbers in the bar segements represent the number of wasps showing the indicated behaviour.

3.4 Response of *Diadegma semiclausum* in a choice situation between LIS transgenic plants damaged by *Plutella xylostella* larvae and non-transgenic larval-damaged plants

A significant preference was observed for control plants (CwCc) damaged by *P. xylostella* larvae over LIS transgenic plants (LcCw) damaged by the same number of larvae (binomial test, two-sided; P<0.005). Thus, the larval-damaged linalool-emitting plants are less attractive to naive *D. semiclausum* compared with larval-damaged control plants, which indicates that there is a repellent effect of the linalool emission (Fig. 6).

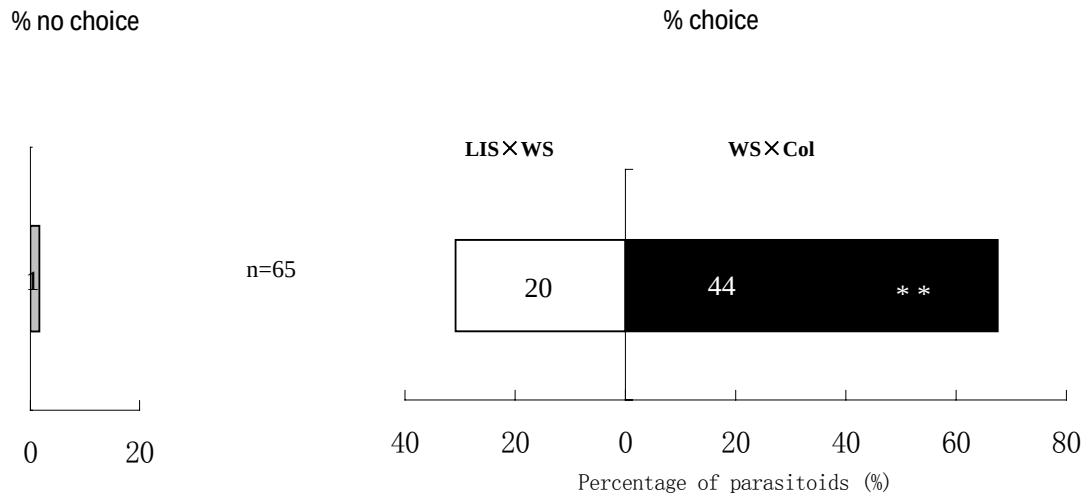

Figure 6. Response of *D. semiclausum* to *P. xylostella* larval damaged LIS transgenic *Arabidopsis* and same larval damaged non-transgenic plants in a Y-tube olfactometer. Naïve *D. semiclausum* females were given a choice between *Plutella* larval damaged LIS plants and larval damaged control plants. The left panel of the figure shows the percentage of wasps that did not make a choice for one of the two odour sources within 5 min in area 1 or within 10 min in area 2 (Fig. 1). The white bar (left hand side) represents the percentage of wasps choosing for the volatile blend emitted by LcCw plants infeasted by the larvae of *P. xylostella*. The black bar (right hand side) represents the percentage of wasps choosing for the volatile blend emanating from CwCc plants infeasted by the conspecies larvae. Asterisks indicate a significant preference ** P<0.005 (binomial test, two-sided). n is the total number of wasps tested. Numbers in the bar segements represent the number of wasps showing the indicated behaviour.

3.5 Response of *Cotesia glomerata* in a choice situation between LIS plants and non-transgenic plants, both damaged by *Pieris brassicae* larvae

Adult female *Cotesia glomerata* parasitoids were marginally more attracted to LIS transgenic plants damaged by *Pieris brassicae* larvae than to *P. brassicae*-infested control plants in a greenhouse flight bioassay (binomial test, two-sided; P=0.07; Fig. 7).

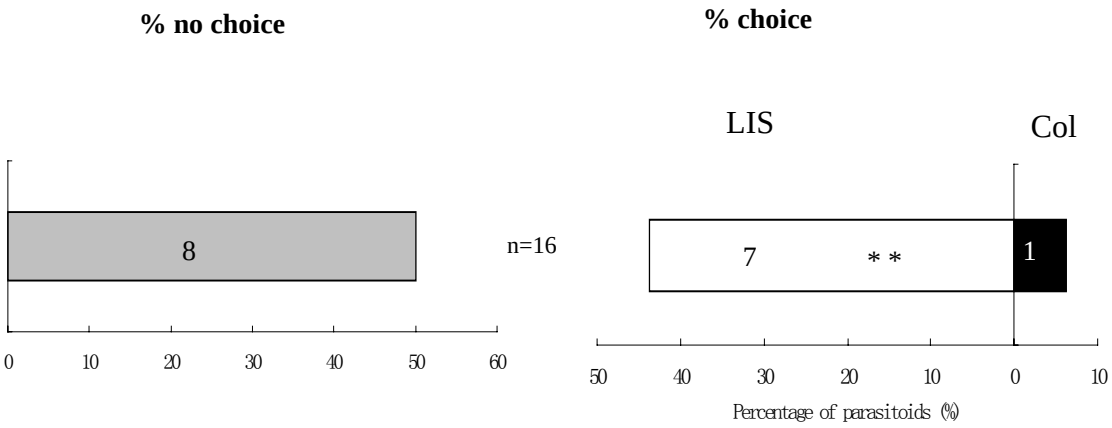

Figure 7. Response of *C. glomerata* in a two choice flight chamber experiment in a greenhouse. Naïve *C. glomerata* females were given a choice between hemizygote LIS transgenic plants and control plants (Col-0) both damaged by two *P. brassicae* larvae. The left panel of the figure shows the percentage of wasps that did not make a choice for one of the two odour sources within 6 min after release. The white left hand side bar represents the choice to *P. brassicae* larval damaged control plants and the black right hand side bar represents to same larval damaged LIS plants in the right panel of the figure.

3.6 Actual parasitisation by *D. semiclausum* on *P. xylostella* larvae

When *D. semiclausum* parasitoids were offered plants with feeding *P. xylostella* larvae, there was no significant difference in the average percentage of parasitization on the LIS transgenic line (LcCw) and the non-transgenic line (CwCc) (Mann-Whitney U-test with correction for continuity; U=125 > (critical value of U (16:16) = 75)). On both plant lines almost 50% of the *P. xylostella* larvae yielded *Diadegma* cocoons (Table 1). Overall survival rates of the 10 *Plutella* larvae inoculated on the plants at the start of the experiment were 47.5 % and 46.9 % respectively.

Table 1. Results of parasitization success of *Diadegma semiclausum* in *Plutella xylostella* larvae on hemizygous LIS transgenic plants and control plants

Plant line	N*	Nr of *Diadegma* cocoons	Nr of *Plutella* pupae	% average parasitisation	SE	P-value
LcCw	16	39	37	51.3	7.72	0.926
CwCc	16	35	40	46.6	6.42	

***** N= the number of replicate plants. The percentage of parasitization was calculated per plant and the average percentage calculated over the 16 plants is presented in the table.

4. Discussion

An increasing number of studies focused on the attraction of carnivorous arthropods by plant volatiles (e.g. Dicke et al., 1990a; 1990b; Takabayashi et al., 1994; 1995; Baldwin & Preston, 1999; Kessler & Baldwin, 2001; de Boer & Dicke, 2004; Wei & Kang, 2006). Linalool was proven to attract predators (Takabayashi et al., 1994; Turlings et al., 1995; Takabayashi & Dicke, 1996; Baldwin & Preston, 1999; Paré & Tumlinson, 1999; Dicke & van Loon, 2000) and parasitoids (Takabayashi et al., 1995; Turlings et al., 1995; Takabayashi & Dicke, 1996; De Moraes et al., 1998; Baldwin & Preston, 1999; Paré & Tumlinson, 1999; Kahl et al., 2000; Colazza et al., 2004; Yan & Wang, 2006). Other studies showed that linalool is repellent to sucking herbivores (Koschier et al., 2002; Jongsma, 2004) and chewing herbivores (De Moraes et al., 2001; van Tol et al., 2007). Herbivore-induced volatiles manipulated through genetic engineering were shown to be attractive to the natural enemies of herbivores, e.g. the innate attraction of the predatory mite *Phytoseiulus persimilis* to the sesquiterpene alcohol (3S)-(*E*)-nerolidol and the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene produced by transgenic *Arabidopsis* overexpressing a strawberry nerolidol synthase (Kappers et al., 2005) and the attraction after associative learning by the parasitic wasp *Cotesia marginiventris* to a mixture of herbivore-induced sesquiterpenes including (*E*)-β-farnesene and (*E*)-α-bergamotene with transgenic *Arabidopsis* overexpressing a maize terpene synthase TPS10 (Schnee et al., 2006).

In the present study, linalool, which was emitted in large amounts by LIS transgenic *Arabidopsis* plants (Chapter 4), makes the plants less attractive to the parasitoid *D. semiclausum* when the plants were infested with *P. xylostella* in comparison to larval infested non-transgenic control plants. The plants used in the experiments here have been checked for linalool emission through SPME-GC-MS. When plants were undamaged, *D. semiclausum* did not show a preference for either LIS transgenic plants or non-transgenic control plants. So in the latter choice situation linalool, which is constitutively emitted, did not affect *D. semiclausum* behaviour. When the plants were damaged by the herbivore *P. xylostella* (the specific host of *D. semiclausum*), LIS plants were less attractive to *D. semiclausum* compared with *P. xylostella*-infested control plants. Also mechanically damaged LIS plants were less attractive than mechanically-damaged control plants, although this was only recorded for one level of mechanical damage. However, *P. xylostella* larval-damaged LIS transgenic plants were more attractive to *D. semiclausum* compared with undamaged LIS transgenic plants. It seems that feeding damage, especially from the host herbivore *P. xylostella* plays a key role in the parasitoid´s olfactory preferences. The study by Bukovinszky et al. (2005) showed that *D. semiclausum* discriminates between *P. xylostella*-infested and uninfested cabbage plants based on subtle differences in the composition of odor blends from the plants.

In the parasitization experiments with *D. semiclausum* and *P. xylostella* the parasitization rate on LIS transgenic and non-transgenic control plants was not significantly different. This means that LIS-transgenic plants do not affect the oviposition behaviour of adult *D. semiclausum* females on the plants. The attraction of *D. semiclausum* to LIS plants infested by *P. xylostella* is meaningful for pest control on *Bt*-and-LIS dual transgenic plants. The target pest, *P. xylostella,* can be suppressed by three approaches: 1) toxicity to larvae of *P. xylostella* (effect of *Bt*) (Chapter 4); 2) repellence to female moths of *P. xylostella* (effect of LIS) (Chapter

4); and 3) attraction of *D. semiclausum*, the parasitoid of *P. xylostella* (effect of LIS) (this chapter). When the wasp *Cotesia plutellae*, a solitary endoparasitoid of *P. xylostella,* attacked *Bt*-resistant *P. xylostella* larvae on transgenic plants the wasp behaviour was not influenced by the *Bt* toxin, nor was the survival of her offspring that developed in the *P. xylostella* larvae (Schuler et al., 1999). Carnivorous arthropods may be indirectly exposed to the *Bt* toxin by feeding on herbivorous insects that have ingested the toxins from the plant. However, in target insect herbivores, the toxins are usually bound to receptors in the midgut epithelium, after which they most likely lose their toxicity. So, the parasitoids may not come into contact with *Bt* toxins via target herbivores (Groot & Dicke, 2002). The continued ability of parasitoids to locate and parasitize *Bt*-resistant target hosts on transgenic crops might even help to constrain the spread of genes for *Bt* resistance in *P. xylostella*.

Cotesia glomerata attacks *P. brassicae* and the related species *Pieris rapae*. The behavioural ecology and physiology of *C. glomerata* in host-parasitoid interactions has been thoroughly characterized (e.g. Geervliet et al., 1996; Tagawa, 2000; van Driesche et al., 2003). This species has also received attention as a parasitoid able to cue in on herbivore-induced plant volatiles (Mattiacci et al., 1994; 2001). Cabbage leaves treated with *P. brassicae* regurgitant and its mimic, almond beta-glucosidase, are highly attractive to the parasitic wasp *C. glomerata* (Mattiacci et al., 1995). LIS-transgenic *Arabidopsis* plants were marginally more attractive to *C. glomerata* when damaged by *P. brassicae* compared with *P. brassicae*-infested control plants. A potential attractant effect of linalool, the product of the LIS transgene, to *C. glomerata*, may be beneficial for the dual transgenic plants that harbour the *Bt* and LIS transgenes by suppressing the pest with *Bt* toxin and attracting the natural enemy of pest with volatile linalool emission.

In conclusion, we have shown that insertion of the LIS gene into *Arabidopsis* results in different effects on attraction of different parasitoids that parasitize herbivores on brassicaceous crops. The rate of parasitism of *P. xylostella* larvae by *D. semiclausum* was not affected by the presence of the linalool emission caused by the LIS transgene.

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Chapter 6

General Discussion

Yang, L.M

Plant defence against insect pests plays an important role in agricultural systems. Direct defence, having direct effects on the performance of herbivores has been exploited in conventional breeding for host plant resistance. In recent years plant breeding for insect resistance has been further enhanced by the adoption of genetically engineered plants with genes conferring direct resistance (Fernandez-Cornejo & McBride, 2000; Groot & Dicke, 2002; Babu et al., 2003; Rice, 2004; Wu & Guo, 2005; Ferry et al., 2006). Among these especially *Bacillus thuringiensis (Bt)* transgenes have been commercialized and *Bt* crops have been planted on a large-scale by now, reaching a world total of *ca.* 30 million hectares in 2007 (Zhao et al., 2005; Ferry et al., 2006; Christou et al., 2006; James, 2007). Indirect defence, promoting the effectiveness of natural enemies of the herbivore *e.g.* by the production of volatiles in response to herbivory that can attract predators or parasitoids, has been studied widely (Dicke, 1999; Kessler & Baldwin, 2001; Schnee et al., 2002; Dicke et al., 2003; Degenhardt et al., 2003; Schnee et al., 2006; D'Alessandro & Turlings, 2006; van Poecke, 2007). Indirect defence is represented in pest management by biological control. So far, plant breeders have not included indirect defence in breeding practices. However, there may be promising transgenic approaches to do so (Kappers et al., 2005, Schnee et al., 2006). The incorporation of transgenic crops in integrated approaches to pest management may be used to ensure their long-term sustainability and maximize their environmental and human health benefits. It is important to understand the impact of incorporating a single transgene and even more so of combining two transgenes on pest insects and their natural enemies in the context of integrated pest management. This thesis focused on the possibility of integrating host plant resistance with biological control in transgenic plants.

1. Plant resistance to insects

Plant resistance to insects refers to the ability of plants to withstand or tolerate the infestation by herbivores, where under similar growing conditions the resistant plant has a higher yield compared with the susceptible plant. Plant genetic engineering allows to insert foreign genes into a plant and have them functionally expressed. Two main approaches have been applied to develop insect-resistant transgenic crops. One is relying on the adoption of non-plant genes such as the bacterial *Bt* genes encoding for δ-endotoxins and the other focusses on plant-derived genes, such as those encoding enzyme inhibitors or lectins. To date, *Bt* transgenic crops are the only commercialized insect-resistant transgenic crops and they are planted worldwide. However, as the cultivation area of transgenic crops increases, the potential risk of insect resistance increases too. In fact, just recently the first evidence for insect resistance to a *Bt*-crop in an insect herbivore has been recorded under field conditions (Tabashnik et al. 2008).

1.1 Transgenic *Bt* plants

The first examples of genetically engineered insect resistance into tobacco and tomato plants with the expression of modified *Bt* toxin genes were successfully performed in the second half of the 1980s (Barton et al., 1987; Fischhoff et al., 1987; Vaeck et al., 1987). Since then, *Bt* genes have been transferred to a number of other crop species, including cotton, maize, rice, and potato, with target pests in the Lepidoptera and Coleoptera (e.g. Huang et al., 2002; Naimov et al., 2003).

Transgenic potato expressing SN19, a hybrid *Bt* gene of the two *Bt* genes *cry1Ba* and *cry1Ia*, was shown to be resistant to Colorado potato beetle larvae and adults, potato tuber moth larvae, and European corn borer larvae (Naimov et al., 2003). Transgenic *Arabidopsis* expressing the *Bt* gene pTC12, which is a modified SN19 with a number of single nucleotide changes in fragments encoding domain III of the gene, were demonstrated to be highly toxic to the diamondback moth (DBM), *Plutella xylostella,* in subsequent plant generations (Chaidamsari, 2005; Chapter 4). Other *Bt cry* genes have also been shown to confer resistance to *P. xylostella*, e.g. *cry1Ac, cry1Ab, cry1C* (Cao et al., 2002; 2005; 2006; 2008).

Since the first *Bt* transgenic crop was commercialized in 1996 the planting area increased rapidly worldwide, by 10-15% per year. Preventing the evolution of resistance by insects is important for the sustainable use of *Bt* crops. A number of studies focussed on insect resistance management among which the gene-pyramiding and high dose-refuge strategies have been recommended most frequently (Roush, 1998; Bates et al., 2005; Zhao et al., 2005; Ferry et al., 2006). However, both strategies have their limitations. The results of greenhouse experiments indicated that simultaneous use of one-gene and pyramided two-gene plants will select for DBM resistance to pyramided *Bt* plants more rapidly than the use of two single-gene plants (Zhao et al., 2005). A series of factors may impact the efficiency of the high-dose refuge strategy, for example the appropriate size, placement and management of refuges (Bates et al., 2005). The refuge strategy is sufficient for delaying the evolution of insect resistance in most cases but one recent study showed that in some field populations of *Helicoverpa zea* the resistance alleles had increased substantially. The possible reasons for this were estimated to be that the inheritance of resistance in *H. zea* is dominant and that the size of the refuge was not large enough for the situation of a dominant resistance allele (Tabashnik et al., 2008).

For the purpose of integrating direct and indirect plant defence, I combined a linalool synthase gene and a *Bt* gene in a single *Arabidopsis* plant and investigated the effect of the dual transgene on both larvae and adults of *P. xylostella.* It was shown that *Bt* and LIS dual transgenic plants negatively impact the larvae of *P. xylostella* by the toxin and repel the adults by the linalool emission (Chapter 4). Combining the repellent odour with *Bt* toxin in a transgenic plant may increase the probability that potentially resistant insects from *Bt* plants will mate with susceptible insects on non-transgenic plants in refuges. This may reduce the selection pressure on *P. xylostella* for developing resistance to the *Bt* toxin.

1.2 Transgenic protease-inhibitor-expressing plants

A number of studies support the general hypothesis that transgenic plants which overexpress proteinase inhibitors (PIs), including serine PIs, have the potential to protect plants against herbivorous insects by interfering with protein digestion, causing delayed growth, increased mortality, and reduced fecundity (Broadway, 1994; De Leo et al., 1998; De Leo et al.,

2001; De Leo et al., 2002; Zavala et al., 2004; Jongsma, 2004; Ferry et al., 2005; Ren & Lu, 2006). However, insects can adapt to plant protease inhibitors in different ways (Jongsma et al., 1995; Jongsma & Bolter, 1997; Giri et al., 1998; Volpicella et al., 2001; Moon et al., 2004; Bayés et al., 2005). Although mustard trypsin inhibitor-2 (MTI2), a serine protease inhibitor, was reported to have a deleterious effect on *P. xylostella* (De Leo et al., 2001; Ferry et al., 2005), I found that MTI2 in transgenic *Arabidopsis* was not active against *P. xylostella* (Chapter 2). This was verified for both a European and a Chinese population of *P. xylostella.* Biochemical investigations demonstrated that trypsins in *P. xylostella*'s gut are sensitive to MTI2 both *in vitro* and *in vivo* (80% inhibition), but that the gut proteases can rapidly degrade MTI2 as well. As a result MTI2 is ineffective. This process was specific to this inhibitor of the host plant of DBM as it did not happen to a PI from soybean, i.e. the soybean Kunitz trypsin inhibitor (SKTI). Jongsma & Bolter (1997) pointed out that to understand the effects of protease inhibitors in insects it is crucial to calculate dissociation constants for inhibitors and to convert the *in vitro* assay results back to the *in vivo* situation. It is demonstrated that the enzyme activity which would be completely inhibited *in vivo* could be largely active under dilute *in vitro* assay conditions (Chapter 2). A number of early studies base their conclusions, however, purely on the *in vitro* observations without calculating and mimicking the actual *in vivo* situation leading to potentially false conclusions about the consequences for the insects (e.g. Giri et al., 1998; Girard et al., 1998; Lu et al., 2006). My study shows that MTI2 is not an effective PI against the diamondback moth due to rapid inactivation of the inhibitor. As MTI2 originates from mustard plants that belong to the Brassicaceae family, this is likely to reflect an adaptation of DBM to one of its host plants.

2. Plant indirect defence against herbivorous insects

Plants can defend themselves indirectly by promoting the activities of natural enemies of herbivores through different traits, e.g. plant volatiles that help carnivores to find their herbivorous prey. The natural enemies include predators and parasitoids of the herbivores. However, herbivores may use the induced plant volatiles to their own benefit, e.g. to avoid plants expressing indirect defence (De Moraes et al., 2001).

2.1 Volatiles act as infochemicals in plant defence

Plant volatiles mediate the communication between plants and the insects in their surroundings and can strongly influence the preference and performance of insects. Several studies showed that plant volatiles are repellent to herbivores, either by deterring oviposition of the adult females or by repellence of the herbivorous stages (Harrewijn et al., 1995; Birkett et al., 2000; De Moraes et al., 2001; D' Alessandro & Turlings, 2006).

Volatiles produced by plants after herbivory are reliable cues for carnivores to locate the herbivores. Many carnivores can discriminate between plants infested with different herbivore species. The dominant volatiles emitted by plants are fatty acid-derivatives, terpenoids, and phenolics (e.g. Dicke et al. 1990; van Poecke et al., 2001). Volatile emission in crops can possibly be manipulated as a practical strategy to attract natural enemies of herbivores and thus to control pests in an environmentally safe way. Terpenoids represent the largest and most diverse chemical group of plant volatiles (Gershenzon & Dudareva, 2007). The majority

of terpenoids released by induction of herbivore feeding are monoterpenes and sesquiterpenes and many of them are emitted specifically in response to herbivore feeding, as often the same emission can not be found in response to mechanical damage. The emission of terpenoids may occur from various parts of the plant and is normally systemic and prolonged. The cues that attract carnivorous arthropods are often complex mixtures of herbivore-induced plant volatiles, making it difficult to demonstrate the role of specific compounds. Engineering terpene emission is highly helpful to study the effect of individual terpenoid volatiles on the behaviour of insects (Aharoni et al., 2003; Kappers et al., 2005).

2.2 Transgenic plants emitting terpenoids

Terpenoids play an important role in various aspects of plant-insect interactions. Therefore, increasing attention can be noted for terpenoid metabolic engineering to enhance direct or indirect plant defence (Aharoni et al., 2003; 2005; Kappers et al., 2005; Schnee et al., 2006; Beale et al. 2006). Up to now, only constitutive or tissue-specific promoters have been used in these researches. There have been no reports on genetic engineering of monoterpene synthases with wound-inducible promoters. Inducible promoters can regulate gene expression depending on chemical elicitation, mechanical wounding, herbivore damage, or pathogen invasion. The use of inducible promoters will be more representative of the actual response of plants to insect damage. My results present the first example to couple a wound-inducible promoter to a terpenoid synthase. As a result methyl-jasmonate inducible linalool expression was achieved in *Arabidopsis,* but remarkably, the gene failed to be expressed in response to induction by DBM herbivory (Chapter 3). One possible reason for this could be that *P. xylostella* is capable of suppressing (part of) the jasmonate-inducible pathways in Brassicaceous plants and in this way, blocks the plant's indirect defence. However, *P. xylostella* herbivory on *Brassica* species is well-known to result in the induction of indirect plant defence (Bukovinszky et al. 2005). Alternatively, *P. xylostella*-induced indirect defence in *Arabidopsis* is not (exclusively) dependent on jasmonic acid induction. It was shown for other herbivores that their effects on global gene transcription in *Arabidopsis* were not identical to the response to jasmonic acid treatment (De Vos et al. 2005). In *Arabidopsis* plants with constitutive terpenoid emission a retardation of plant growth was observed (Aharoni et al., 2003; Kappers et al., 2005; chapter 4) but this was not observed in my plants in which the expression of the linalool synthase was under the control of the wound-inducible promoter. So the use of an inducible promoter can in principle overcome the negative effects of constitutive promoters on plant phenotype. My results show that inducible linalool synthase gene expression could be successfully developed through a transgenic approach. A next step in this research will be to develop herbivore-inducible gene expression, e.g. inducible by herbivory by *P. xylostella.*

2.3 Integration of host plant resistance and volatile emission in transgenic plants

Various *Bt* genes have been utilized in a number of agricultural crops at a large scale for pest management since the first transgenic plants were constructed in the 1980s. Several plants with terpenoid-synthase transgenes have been demonstrated to have impacts on the behaviour of both herbivores and carnivores (Aharoni et al., 2003; Jongsma, 2004; Kappers et al., 2005; Schnee et al., 2006). Currently, combining two *Bt* genes in a single crop is common

practice and also combining a *Bt* gene with a protease inhibitor gene has been conducted (Halpin, 2005; Zhao et al., 2005; Cao et al., 2008). A number of studies have been concerned with the impacts of *Bt* plants on natural enemies of pests (Schuler et al., 1999; Romeis et al., 2006). However, so far commercialization of transgenic crops has been restricted to employing direct insect resistance genes only. In the natural situation, plants can balance the deployment of direct and indirect defences. For example, when attacked by specialist tobacco hornworm larvae which are adapted to nicotine, wild tobacco plants reduce their induced nicotine production and release volatile terpenoids to attract enemies of the herbivore (Kahl et al., 2000).

For the purpose of integration of direct and indirect plant defence in a single plant genotype, in this study I combined in transgenic *Arabidopsis* for the first time two genes which are representative for those two strategies, the *Bt* SN19 and the linalool synthase FaNES1 (LIS) genes. The combination of the LIS gene encoding a biosynthetic enzyme leading to linalool emission with the *Bt* gene in *Arabidopsis* has been helpful for investigating the effects of the dual transgene on both herbivores and their natural enemies. The most important conclusions in this respect will be discussed in the next paragraphs.

3. The impact of integrated transgenic plants on herbivores and carnivores

3.1 The impact of integrated transgenes on a specialist herbivore (*P. xylostella*)

The dual transgene combination of *Bt* SN19 and linalool synthase resulted in a toxic effect on larvae of *P. xylostella* and a repellent effect on the oviposition by adult moths. Both the homozygous and the hemizygous *Bt* transgenic plants were toxic to the larvae, while the LIS transgene did not affect the larval survival of *P. xylostella.* This confirms that the toxic effect of the dual transgenic plant is due to the *Bt* gene alone. The repellent effect to *P. xylostella* moths of hybrid transgenic plants with *Bt* and LIS genes or LIS genes alone suggests that the repellence is a function of the LIS gene rather than *Bt* gene (Chapter 4). It is clear that the dual transgenes in this case result in dual effects which enhance each other: fewer herbivore offspring with an increased mortality rate as a result of the *Bt* toxin.

It has been reported that the DBM is attracted to plant volatiles induced by herbivores and shows increased oviposition on such plants (Reddy & Guerrero, 2000; Shiojiri et al., 2002). In other studies it was observed that leaf extracts from the syringa tree (*Melia azedarach* L.) have a repellent effect on oviposition behaviour of DBM under greenhouse and field conditions (Charleston et al., 2005; Charleston et al., 2006). In the oviposition experiment I conducted, DBM showed an avoidance of linalool emitting plants in favour of *Bt* transgenic and non-transgenic control plants. However, in an agricultural setting an alternative oviposition site needs to be available for a repellence strategy to be effective. When combining a linalool synthase transgene with a *Bt* transgenic crop this will confer DBM repellence to the crop, and therefore a refuge is necessary where DBM can oviposit to reduce selection pressure that might lead to insensitivity to the repellent. The target pest, *P. xylostella,* can be suppressed by three approaches: 1) toxicity to larvae of *P. xylostella* (effect of *Bt*) (Chapter 4); 2) repellence of female *P. xylostella* moths (effect of LIS) (Chapter 4); and 3) attraction of *D. semiclausum*, the parasitoid of *P. xylostella* (effect of LIS) (Chapter 5). Thus, the two transgenes have a set of effects that collectively help to combat this important pest.

3.2 The impact of the two transgenes on parasitoids

The results presented in this thesis show that LIS-transgenic *Arabidopsis* plants with constitutively high emission of linalool affected the behaviour of two parasitoids. When plants were undamaged, *D. semiclausum* did not show a preference for either LIS transgenic plants or non-transgenic control plants; when plants were damaged by the herbivore *P. xylostella* or mechanically damaged, LIS plants were less attractive to *D. semiclausum* compared with non-transgenic control plants. However, when *P. xylostella*-infested LIS and LIS-with-*Bt* double transgenic plants were compared with undamaged plants of the same genotype, *D. semiclausum* was attracted to the former odour source. Moreover, *Cotesia glomerata* is more attracted to *Pieris brassicae*-infested LIS transgenic plants when compared with infested non-transgenic control plants (Chapter 5). Thus the two parasitoid species studied respond differently to the increased emission of linalool. However, in both cases the parasitoids are able to locate their hosts feeding on the transgenic LIS plants.

Some previous studies investigated the effect of additional volatiles on the attraction of predators and parasitoids to host-induced or prey-induced plant volatiles (Dicke et al., 2003; Perfecto and Vet, 2003; De Boer et al., 2004; Moayeri et al., 2007). The “additional volatiles” could come from the constitutive emission resulting from the insertion of a foreign gene or from neighbouring plants. The results of Perfecto and Vet (2003) demonstrated that additional volatiles from neighbouring non-host plants could change the behaviour of the two parasitoids, *Cotesia glomerata* and *Cotesia rubecula*. *Cotesia glomerata*, a parasitoid accepting a wider range of hosts, had a reduced foraging efficiency by the presence of non-host plants, whereas foraging efficiency of *C. rubecula*, with a narrower host range, was enhanced. The wasps may be attracted to general green leaf volatiles in the damaged non-host plants that are similar to those also released from host plants.

Several studies on transgenic plants producing novel terpenoids involved in tritrophic interactions have been reported and the role of terpenoids in the attraction of carnivores was demonstrated (Kappers et al., 2005; Schnee et al., 2006; Beale et al., 2006). However, no examples of combining a terpene synthese gene with a *Bt* transgene and subsequently investigating the effects on herbivores and their enemies were reported. *Diadegma semiclausum* was attracted to *P. xylostella*-infested LIS and *Bt*×LIS (BwLc) transgenic plants compared with non-infested transgenic plants of the same transgenic line in my study. This indicates that this new approach for enhancing pest control in *Bt* transgenic crops, by inserting not only toxicity to herbivores but also attraction of the natural enemies of herbivores, can provide a new approach. The natural enemies may be especially valuable for controlling pests which are tolerant to the *Bt* toxin. The study of Schuler et al. (1999) showed that the parasitoid *Cotesia plutellae* could develop normally in *Bt*-resistant *P. xylostella* larvae feeding on *Bt* transgenic plants (Schuler et al., 1999).

4. Future perspectives

This thesis represents the first study on integrating direct and indirect defences in a transgenic plant. The *Bt* toxin gene and the linalool synthase gene were successfully combined in a transgenic *Arabidopsis* genotype and several effects of the dual transgene on

both herbivore and parasitoids were revealed. The results presented in this thesis indicate that the double transgenic plant can impact the herbivore through toxicity and repellence, and can impact the behaviour of parasitoids in a positive way. Both parasitoids were able to locate their hosts in the herbivore-infested transgenic crop. It should be noted that the parasitoids used in this thesis were naïve, i.e. they had no oviposition experience. It is well-known that many parasitoids can learn to respond to novel odours (Vet & Dicke 1992). Thus, even when the transgenic plants are less attractive to parasitoids than wildtype plants, after finding hosts in the transgenic crop the parasitoids may learn to respond to the novel odour blend. This deserves investigation in the future. Thus, genes encoding *Bt* toxin and linalool synthase integrated into *Arabidopsis* may act as a model to investigate new methods to improve the control of the diamondback moth. Further research on induced linalool emission in response to insect attack will be relevant for the engineering of indirect plant defence. Compared with constitutive production, the use of herbivore-inducible terpenoid expression will result in a more reliable cue to affect the behaviour of the natural enemies of herbivores, will save metabolic resources for plant development and reduce any negative phenotypes associated with the constitutive expression of the gene. On the other hand we have shown that constitutive expression of the linalool gene has the additional benefit of repelling moths from their host plants while it does not interfere with or even enhances, in the case of *C. glomerata*, host-finding by the parasitic wasps.

This study is an initial step towards combining direct and indirect defences in a transgenic plant and this line of research is worth to be continued. The most important aspects to be included in future studies are 1) what are the effects of the *Bt*-and-LIS dual transgenic plant on other combinations of herbivores and parasitoids? 2) what are the effects on pests that cannot be controlled with *Bt* toxins, such as aphids and thrips? It is interesting to note that aphids are repelled by *Arabidopsis* plants expressing the LIS transgene (Aharoni et al. 2003). 3) What are the effects of linalool emission in terms of herbivore repellence and parasitoid attraction when the LIS gene is under the control of an inducible promoter? 4) How does learning by parasitoids affect the behavioural responses of parasitoids to LIS-transgenic plants? Does this result in (further) enhanced attraction of the parastioids? 5) A next step will have to be to take this approach to a crop species. Subsequently, studies in the field will need to be done to investigate the effects of inserting genes mediating direct and indirect defences in commercial crops. This will indicate whether this approach is promising and compatible with horticultural practice.

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Summary

Host plant resistance and biological control are two important components of integrated pest management. They are usually developed in isolation. However, host plant characteristics can decisively affect the effectiveness of biological control agents, and therefore when altering plant characteristics in a breeding programme, the implications for biological control should be studied as well.

Plant genetic engineering makes it possible to transfer a foreign gene to a host plant to introduce resistance against insects or to produce a volatile which can attract the natural enemy of the pest after the host plant is wounded by insect herbivores. In this thesis I have modified direct and indirect defence to herbivores in *Arabidopsis* plants through a transgenic approach. In order to evaluate transgenic *Arabidopsis* with different genes that influence direct and indirect defence to the herbivore *Plutella xylostella (*diamondback moth, DBM), I studied the following four aspects:

(1) Effect of overexpression of Mustard Trypsin Inhibitor 2 (MTI2) in transgenic *Arabidopsis* on larvae of the diamondback moth, *Plutella xylostella*

Direct plant defence includes the production of anti-digestive compounds such as protease inhibitors (PI). Mustard, a brassicaceous plant, produces Mustard Trypsin Inhibitor 2 and transgenic *Arabidopsis* plants expressing the gene encoding for this PI were used in this study. I investigated the effect of MTI2 transgenic *Arabidopsis* on larvae of DBM. The performance of a Dutch and a Chinese strain of DBM was assessed. In contrast to earlier reports, we did not find significant differences in larval mortality or development rate relative to the untransformed control, whereas PI-expression levels were similar or higher. This indicated that these strains of *P. xylostella* are resistant to the protease inhibitor MTI2. Biochemical experiments for understanding the mechanism of adaptation in DBM were conducted. Trypsin activity in gut extracts from larvae feeding on either control or transgenic plants were titrated with MTI2 and the protease inhibitor Soybean Kunitz Trypsin Inhibitor (SKTI) to assess the basis of the insensitivity to MTI2. The specific trypsin activity per larval gut for larvae reared on MTI2 plants was not significantly higher compared to the control, and ca. 80% of trypsin activity could be inhibited by both inhibitors in both treatments, suggesting no specific induction of PI-insensitive activity in response to MTI2 in the diet. I found that a pre-incubation of MTI2 and SKTI with gut proteases for 3 hours resulted in complete loss of inhibitory activity of MTI2, but not of SKTI, at the concentration ratios found *in planta*. Gut extracts of larvae reared on control or MTI2 leaves were equally well capable of this degradation indicating that the inactivating enzymes are constitutively expressed. In conclusion, it appears that the insensitivity of the gut enzymes of DBM to MTI2 can be sufficiently explained by the specific inactivation of MTI2. DBM has apparently developed MTI2 inactivation as a way to protect itself against this protease inhibitor. This makes ecological sense as MTI2 is part of the defense of its brassicaceous host plants.

(2) Expression of a linalool synthase gene controlled by the wound-inducible potato PI2 promoter in *Arabidopsis*

Terpenoids are abundantly present among plant volatiles and are involved in indirect defence in many plant species. I exploited the availability of a linalool synthase gene to

develop transgenic herbivore-inducible linalool-producing *Arabidopsis* plants. The FaNES1 linalool synthase (LIS) cDNA from strawberry, with plastid targeting and a synthetic intron (LIS') was placed under the control of the wound-inducible Proteinase Inhibitor 2 (PI2) promoter from potato. The construct pBin-P_{PI2}-LIS' was transformed to *Arabidopsis thaliana* ecotype Columbia 0. The expression of active linalool synthase by the P_{PI2}-LIS' gene construct in the transgenic lines was confirmed by measuring linalool emission using solid phase micro-extraction (SPME) and GC-MS measurements after induction with methyl jasmonate. The results show that the use of the PI2 promoter from potato resulted in methyl-jasmonate inducible linalool expression in *Arabidopsis,* although it failed to yield a response to herbivory by *Plutella xylostella* larvae.

(3) Effects of transgenic *Arabidopsis* expressing a combination of *Bacillus thuringiensis* (*Bt*) endotoxin and linalool on larvae and adults of *P. xylostella*

A gene encoding a *Bt* toxin and a gene encoding linalool synthase were integrated into *Arabidopsis* as a model to investigate new methods to improve the control of DBM. Homozygous transgenic *Arabidopsis* expressing the *Bt* toxin gene SN19 and the strawberry linalool synthase gene FaNES1 (LIS) were crossed to wildtypes and each other to obtain hybrid lines for these traits in different combinations. These hybrid *Arabidopsis* plants were used for larval feeding and oviposition preference bioassays. The results show that the expression of the *Bt* gene SN19 resulted in toxicity to the larvae of DBM in both homozygous and hemizygous dose whereas linalool emitting LIS transgenic *Arabidopsis* did not impact the development of DBM larvae. However, oviposition choice experiments showed for the first time that linalool emission made the plant repellent to DBM females. The moths laid fewer eggs on LIS-transgenic plants compared to non-transgenic control plants. Oviposition behaviour was not affected by the presence of the *Bt* toxin, so that *Bt* plants were not deterrent relative to control hybrid plants. In conclusion, the experiments demonstrate that hybrid lines carrying *Bt* and LIS genes in *Arabidopsis* combine toxicity to the larvae and repellence to the adults of DBM. This repellence (i) may reduce the incidence of resistant insects, when avoiding linalool expressing plants which co-express Bt toxins is also observed in the field; (ii) may increase the probability that potentially resistant insects from *Bt* plants will mate with susceptible insects colonising non-transgenic plants that constitute refuges; and (iii) may favor susceptible insects, when avoiding linalool expressing plants provides a selective advantage, as it will when these plants express Bt toxins. Thus, the co-expression of linalool provides a novel option to reduce selection of *Bt*-resistant larvae.

(4) Effect of the combined constitutive emission of linalool and expression of Bt toxin in transgenic *Arabidopsis* on parasitoid attraction and survival

Transgenic *Arabidopsis* plants with a high emission rate of linalool and the expression of a *Bt* gene were used in a Y-tube olfactometer and greenhouse flight-bioassay to study their effect on the behaviour of the parasitoids *Diadegma semiclausum* and *Cotesia glomerata*. Undamaged *Bt* x linalool synthase (LIS) transgenic plants were neither attractive nor repellent to the female wasps of *D. semiclausum* compared with undamaged non-transgenic control plants. When LIS plants were damaged by the larvae of DBM or mechanically they showed a repellent effect on *D. semiclausum* compared with similarly treated non-transgenic control

plants. Yet, when LIS plants were damaged by DBM larvae, the plants were more attractive to the parasitoids than undamaged LIS plants. LIS plants that were damaged by *Pieris brassicae* lavae were more attractive to adult female *Cotesia glomerata* wasps when compared with *P. brassicae*-damaged non-transgenic control plants.

Experiments addressing the actual parasitization rate of *P. xylostella* by *D. semiclausum* showed that there is no significant difference between the parasitization rates on the hemizygous LIS-transgenic plants and the non-transgenic control plants. In conclusion, the data show that insertion of the LIS gene into *Arabidopsis* results in different effects on attraction of different parasitoids that parasitize herbivores on brassicaceous crops. The rate of parasitism of *P. xylostella* larvae by *D. semiclausum* was not affected by the presence of the linalool emission caused by the LIS transgene.

Taken together my data show that a gene that mediates insect resistance and a gene that mediates volatile production can be simultaneously expressed in a transgenic plant genotype. The effects of the dual transgene to the DBM and two parasitoid species show that the transgenic *Arabidopsis* plants developed in this project are resistant to the herbivore as a result of the Bt-toxin gene and at the same time repel the herbivore as a result of expression of the linalool synthase gene. The effects of transgenic linalool expression on parasitoids depend on the species studied, but appear to be either neutral or beneficial for attraction and neutral with regard to parasitization. Thus, genes encoding for *Bt* toxin and linalool synthase integrated into *Arabidopsis* may act as a good model to investigate new methods to improve the control of the diamondback moth, *P. xylostella*. Furthermore, these results provide interesting options for the development of transgenic crops that interfere with the biology of pests and enhance the effectiveness of the pest's natural enemies. In this way transgenic crops that integrate host plant resistance with biological control may be developed.

Samenvatting

Waardplantresistentie en biologische bestrijding zijn twee belangrijke bestanddelen van geïntegreerde plaagbestrijding. Ze worden veelal afzonderlijk van elkaar ontwikkeld. Echter, waardplantkenmerken kunnen een beslissende invloed uitoefenen op de effectiviteit van organismen die voor biologische bestrijding worden ingezet. Daarom moeten ook de gevolgen bestudeerd worden die het veranderen van plantkenmerken in een veredelingsprogramma kunnen hebben voor biologische bestrijding.

Genetische modificatie van planten maakt het mogelijk om een soort-vreemd gen over te brengen naar een waardplant om resistentie tegen insecten te introduceren of om een vluchtige stof te produceren welke de natuurlijke vijand van de plaag kan aantrekken wanneer de waardplant door plantenetende insecten wordt aangetast. In dit proefschrift heb ik directe en indirecte verdediging van Arabidopsis planten tegen plantenetende insecten met behulp van een transgene benadering gemodificeerd. Teneinde de verdediging van transgene Arabidopsis, uitgerust met verschillende genen welke directe en indirecte verdediging beïnvloeden, tegen het plantenetend insect Plutella xylostella (het koolmotje, in het Engels 'diamondback moth', hierna afgekort als DBM) te evalueren, heb ik de volgende vier aspecten bestudeerd:

(1) Effect van over-expressie van Mustard Trypsin Inhibitor 2 (MTI2) in transgene Arabidopsis op de larven van de Diamondback moth, Plutella xylostella.

Directe verdediging van planten omvat onder meer de productie van verteringsremmende stoffen zoals protease-remmers. Mosterd, een plant die tot de Brassicaceae behoort, produceert MTI2 en transgene Arabidopsis planten, die het gen dat codeert voor deze protease-remmer tot expressie brachten, zijn in deze studie gebruikt. Ik heb onderzocht welk effect MTI2-transgene Arabidopsis-planten uitoefenden op DBM-rupsen. Sterfte en groei werden bepaald van een Nederlandse en een Chinese DBM-stam. In tegensteling tot eerdere publicaties werden geen significante verschillen in sterfte of ontwikkeling gevonden vergeleken met de niet-getransformeerde controle-planten, terwijl daarentegen de expressie van de protease-remmer gelijk of hoger was. Dit liet zien dat deze stammen resistent zijn tegen de protease-remmer MTI2. Biochemische experimenten werden uitgevoerd om het aanpassingsmechanisme van DBM te ontrafelen. Trypsine-activiteit in darm-extracten afkomstig van rupsen die hadden gevreten van controle - of transgene planten werden getitreerd met MTI2 en de protease-remmer Soybean Kunitz Trypsin Inhibitor (SKTI) om de biochemische basis van de ongevoeligheid voor MTI2 te achterhalen. De specifieke trypsine-activiteit per rupsendarm voor rupsen die waren opgekweekt op MTI2-planten was niet significant hoger vergeleken met de controle en ca. 80% van de trypsine-activiteit kon geremd worden door beide remmers in beide behandelingen, hetgeen suggereert dat er geen specifieke inductie van proteaseremmer-ongevoelige activiteit optrad als reactie op MTI2 in het voedsel. Ik vond dat een pre-incubatie van MTI2 en SKTI met darm-proteasen gedurende 3 uur een volledig verlies van remmende activiteit van MTI2, maar niet van SKTI, tot gevolg had bij de concentratie-verhoudingen welke gevonden werden in planta. Darmextracten van rupsen opgekweekt op bladeren van controle – of MTI2-planten waren in gelijke mate in

staat om deze afbraak tot stand te brengen, hetgeen aangaf dat de inactiverende enzymen constitutief tot expressie komen. Concluderende lijkt het erop dat de ongevoeligheid voor MTI2 van de darm-enzymen van DBM afdoende verklaard kan worden door de specifieke inactivatie van MTI2. DBM heeft klaarblijkelijk inactivatie van MTI2 ontwikkeld als een manier om zich te beschermen tegen deze protease-remmer. Vanuit ecologisch opzicht is dit functioneel aangezien MTI2 een onderdeel is van de verdediging van de waardplanten van DBM, de Brassicaceae.

(2) Expressie in Arabidopsis van een linalool-synthase gen dat onder controle staat van een wond-geïnduceerde PI2-promotor uit aardappel

Terpenoïden zijn een belangrijke groep binnen de vluchtige plantenstoffen en spelen een rol in de indirecte verdediging van veel plantensoorten. Ik maakte gebruik van de beschikbaarheid van een linalool synthase-gen om transgene Arabidopsis-planten te ontwikkelen die linalool produceerden na inductie door vraat. Het FaNES1 linalool synthase (LIS) cDNA van aardbei, tot expressie gebracht in de chloroplasten en voorzien van een synthetisch intron (LIS'), werd onder controle geplaatst van een door vraat geïnduceerde Protease-remmer 2 (PI2) promotor afkomstig uit aardappel. Het construct pBin-PPI2-LIS' wered getransformeerd naar Arabidopsis thaliana ecotype Columbia 0. De expressie van actief linalool synthase door het PPI2-LIS' genconstruct in de transgene lijnen werd bevestigd door de linaloolemissie te meten met behulp van 'solid phase micro-extraction' (SPME) en GC-MS metingen na inductie met methyljasmonaat. De resultaten laten zien dat de toepassing in Arabidopsis van de PI2-promotor uit aardappel, expressie van linalool tot gevolg had, welke induceerbaar was door methyljasmonaat, hoewel het niet lukte om een reactie te induceren met vraat door DBM-rupsen.

(3) Effecten van transgene Arabidopsis welke een combinatie van Bacillus thuringiensis (Bt) endotoxine en linalool tot expressie brengen, op rupsen en motten van Plutella xylostella

Een gen dat codeert voor een Bt toxine en een gen coderend voor linalool synthase werden geïntegreerd in Arabidopsis als een model om nieuwe methoden te onderzoeken om de beheersing van DBM te verbeteren. Homozygote transgene Arabidopsis welke het Bt toxine-gen SN19 en het linalool synthase-gen FaNES1 (LIS), afkomstig uit aardbei, tot expressie brachten, werden gekruist met wildtypen en met elkaar om hybride lijnen voor deze eigenschappen te verkrijgen in verschillende combinaties. Deze hybride Arabidopsis planten werden gebruikt voor biotoetsen waarin de effecten op rupsenvraat en eilegvoorkeur werden bestudeerd. De resultaten laten zien dat de expressie van het Bt-gen SN19 toxiciteit voor rupsen van DBM tot gevolg had in zowel homozygote als hemizygote dosis, terwijl daarentegen de emissie van linalool door LIS-transgene Arabidopsis de ontwikkeling van DBM rupsen niet beïnvloedde. Eilegvoorkeur-experimenten lieten echter voor het eerst zien dat de emissie van linalool de plant afstotend maakte voor DBM-vrouwtjes. De motten legden minder eieren op LIS-transgene planten vergeleken met niet-transgene controleplanten. Eileggedrag werd niet beïnvloed door de aanwezigheid van het Bt toxine, zodat Bt-planten niet afstotend waren vergeleken met hybride controleplanten. Concluderend tonen de experimenten aan

dat hybride Arabidopsis-lijnen uitgerust met Bt- en LIS-genen toxiciteit voor DBM-rupsen combineren met een afstotende werking op DBM-motten. De afstotende werking (1) kan het vóórkomen van resistente insecten terugbrengen, indien de vermijding van linalool-producerende planten welke ook Bt toxinen tot expressie brengen, ook onder veldomstandigheden wordt waargenomen; (2) kan de waarschijnlijkheid verhogen dat mogelijk resistente insecten afkomstig van Bt-planten zullen paren met vatbare insecten die niet-transgene planten koloniseren welke als refugia dienst doen; en (3) kan vatbare insecten bevoordelen, als de vermijding van linalool-producerende planten een selectief voordeel biedt, hetgeen het geval is als deze planten Bt toxinen tot expressie brengen. Dientengevolge biedt de co-expressie van linalool een geheel nieuwe optie om de selectie van Bt-resistente rupsen te verminderen.

(4) Effect van de gecombineerde constitutieve expressie van linalool en Bt toxine in transgene Arabidopsis op de aantrekking en overleving van parasitoïden

Transgene Arabidopsis planten die een hoge emissie van linalool vertoonden en een Bt-gen tot expressie brachten, werden gebruikt in een Y-buis olfactometer en een vlieggedrag-biotoets in een kas om hun effect op het gedrag van de parasitoïden Diadegma semiclausum en Cotesia glomerata te bestuderen. Onbeschadigde transgene Bt x linalool synthase (LIS) planten waren niet aantrekkelijk noch afstotend voor vrouwelijke D. semiclausum wespen vergeleken met onbeschadigde controleplanten. Wanneer LIS planten vraatschade ondergingen door DBM-rupsen of mechanisch beschadigd werden, lieten ze een afstotend effect zien op D. semiclausum vergeleken met niet-transgene controleplanten die op dezelfde manier waren behandeld. Echter, wanneer LIS planten vraatschade ondergingen door DBM-rupsen, waren de planten aantrekkelijker voor de parasitoïden dan onbeschadigde LIS-planten. LIS-planten die door Pieris brassicae rupsen beschadigd werden waren aantrekkelijker voor adulte vrouwelijke Cotesia glomerata wespen vergeleken met door P. brassicae aangevreten niet-transgene controle-planten.

Experimenten die ten doel hadden de parasiterings-percentages van DBM door D. semiclausum te onderzoeken lieten zien dat er geen significant verschil was tussen deze percentages op de hemizygote LIS-transgene planten en de niet-transgene controle-planten. De slotsom uit de gegevens is dat insertie van het LIS-gen in Arabidopsis verschillende effecten tot gevolg heeft voor verschillende parasitoïden die plantenetende insecten parasiteren op gewassen die tot de Brassicaceae behoren. Het parasiteringspercentage van DBM-rupsen door D. semiclausum werd niet beïnvloed door de linaloolemissie veroorzaakt door het LIS-transgen.

Samengenomen laten mijn gegevens zien dat een gen dat resistentie veroorzaakt tegen insecten en een gen dat de productie van een vluchtige stof bewerkstelligt tegelijkertijd tot expressie kunnen komen in een transgeen genotype. De effecten van het tweevoudige transgeen op DBM en de twee soorten parasitoïden tonen aan dat de transgene Arabidopsis planten die ontwikkeld zijn in dit project resistent zijn tegen het plantenetend insect als gevolg van het Bt toxinegen en tegelijkertijd het plantenetend insect afstoten als gevolg van de expressie van het linalool synthase-gen. De effecten van transgene linaloolexpressie op

parasitoïden hangt af van de bestudeerde soort, maar lijken ofwel neutraal ofwel voordelig voor de aantrekking en neutraal met betrekking tot parasiteringspercentage. De conclusie is gerechtvaardigd dat genen die coderen voor Bt toxine en linaloolsynthase, geïntegreerd in Arabidopsis als een goed model kunnen functioneren om nieuwe methoden te onderzoeken ter verbetering van de beheersing van DBM, P. xylostella. Ook bieden deze resultaten interessante opties voor de ontwikkeling van transgene gewassen die interfereren met de biologie van plaaginsecten en de effectiviteit verhogen van natuurlijke vijanden van het plaaginsect. Op deze wijze kunnen transgene gewassen ontwikkeld worden welke waardplantresistentie en biologische bestrijding integreren.

中文摘要

在拟南芥－昆虫互作模式系统下综合寄主植物抗性和生物防治的研究

杨丽梅

寄主植物抗性和生物防治是害虫综合防治的两个重要方面，通常二者是独立的，但是寄主植物的性状可以影响生物防治的效果，因此在一个育种程序中当植物性状改变了，其对生物防治的潜在影响也应予以研究。

植物遗传工程使得向寄主植物导入外源基因成为可能，这包括引进抗虫基因或引进植物被植食性昆虫伤害后可以产生吸引天敌的挥发物的基因。本论文利用转基因植物改善了拟南芥对害虫的直接防卫和间接防卫，为评价含有不同基因的转基因拟南芥对害虫小菜蛾（*Plutella xylostella*）的直接防卫和间接防卫，从以下四个方面进行了研究：

1．过量表达芥菜胰蛋白酶抑制剂 2（MTI2）的转基因拟南芥对小菜蛾幼虫的影响

植物直接防卫包括产生抗消化的化合物如蛋白酶抑制剂（PI）。芥菜为芸薹属植物，可产生芥菜胰蛋白酶抑制剂 MTI2，将能编码产生这种蛋白酶抑制剂的转基因拟南芥用于本研究。首先分别利用荷兰和中国的小菜蛾种群研究 MTI2 转基因拟南芥对小菜蛾幼虫的影响，得到了与前人报道相悖的结果：尽管所用转基因植物表现出与之相似甚至更高的基因表达量，转基因植物与对照植物对小菜蛾幼虫死亡率及发育的影响表现没有显著差异，说明这些小菜蛾种群对 MTI2 具有抗性。为搞清其适应机理继而做了一系列生化试验：对用对照和转基因植物饲喂的幼虫中肠的提取液分别用 MTI2 和大豆 Kunitz 型胰蛋白酶抑制剂(SKTI)进行滴定法测定其胰蛋白酶活性以估计 MTI2 的非灵敏性，结果显示喂食芥菜胰蛋白酶抑制剂(MTI2)植物的幼虫中肠的特异性胰蛋白酶活性不比喂食对照植物幼虫的高，在两个处理中大约 80% 的胰蛋白酶活性可以被两种抑制剂所抑制，表明对于存在于其食物中的芥菜胰蛋白酶抑制剂(MTI2)没有特异诱导出对蛋白酶抑制剂不敏感的活性；对其中肠蛋白酶分别与 MTI2 和 SKTI 预培养 3 小时，结果在与活体植物相同的条件下 MTI2 完全失去抑制活性，而 SKTI 未失活；用对照植物与 MTI2 植物喂食的幼虫中肠提取物同样可以使 MTI2 降解，表明使酶失活的表达是持续的而不是诱导的。因此可以得出结论：小菜蛾中肠酶对 MTI2 的不敏感性完全能够用 MTI2 的特定失活来解释，显然小菜蛾通过使 MTI2 发生失活作为一种抵抗这种蛋白酶抑制剂的方式，这具有生态学意义，因为 MTI2 是芸苔属寄主植物自我防卫的组成部分。

2．由受伤诱导型马铃薯 PI2 启动子控制的芳香醇合成酶基因在拟南芥中的表达

萜类化合物大量存在于植物挥发物中并参与多种植物的间接防卫，本论文开创了利用芳樟醇合酶基因获得通过害虫诱导产生芳樟醇的转基因拟南芥的可用性。FaNES1 芳樟醇合酶基因（LIS）cDNA来自草莓，其以质体为靶标并带有内含子的序列（LIS’）与受伤诱导型马铃薯蛋白酶抑制剂 2 (PI2)启动子构建为pBin-P_{PI2}-LIS’， 并将其转入生态型为Columbia 0 的拟南芥中。P_{PI2}－LIS’转基因植株的活性表达通过利用固相微萃取（SPME）气象色谱－质谱法（GC－MS）测定由茉莉酮酸甲酯诱导产生的芳樟醇挥发物来确定，结果显示应用马铃薯PI2 启动子可以使转基因拟南芥在茉莉酮酸甲酯诱导下产生芳樟醇挥发物，而在小菜蛾幼虫诱导下没有该物质产生。

3．同时表达苏云金芽胞杆菌（Bt）内毒素与芳樟醇的转基因拟南芥对小菜蛾幼虫和成虫的影响

本论文将编码产生Bt毒蛋白的Bt基因和编码产生芳樟醇的芳樟醇合酶基因结合到拟南芥植物中，并以其作模型研究改进对小菜蛾防治的新方法。利用纯合表达Bt（SN19）基因和纯合表达草莓芳樟醇合酶基因FaNES1（LIS）的转基因拟南芥分别与野生型及二者互相杂交，获得这些性状间的不同组合，并用这些植物杂交系进行幼虫饲喂及成虫产卵试验，结果显示，SN19Bt基因在纯合系和杂合系中的剂量表达都对小菜蛾幼虫具有毒性，而产生芳樟醇的LIS转基因拟南芥则不影响小菜蛾幼虫的生长发育；然而小菜蛾成虫产卵试验首次显示芳樟醇的产生使得植物排斥其雌蛾，雌蛾在LIS转基因植物上的产卵量少于在非转基因对照植物上的，其产卵行为不受Bt毒素的影响，因此含有Bt的转基因植物与对照植物相比对雌蛾没有威慑作用。结论如下，试验表明同时表达Bt基因和LIS基因的杂合转基因拟南芥兼具对小菜蛾幼虫的毒性和对其成虫的驱避性，而这种驱避性（i）当具有驱避性的LIS基因与Bt基因共同表达的转基因植物在露地条件下种植时可以减少抗性害虫对Bt植物的选择几率；（ii）能够增加产生于Bt植物上、具有潜在抗性的害虫与产生于非转基因的避难所植物上的害虫的交配概率；（iii）当植物表达Bt毒性而兼有驱避性的表达芳樟醇的植物具有选择优势时，有利于敏感害虫的繁殖。因此，Bt与芳樟醇的共表达为减小对Bt具有抗性的幼虫的选择压提供了一种新的途径。

4．综合持续表达芳樟醇与表达Bt毒性的转基因拟南芥对寄生蜂行为及生存的影响

利用大量持续产生芳樟醇和表达Bt基因的转基因拟南芥进行Y－tube嗅觉计试验，研究其对半闭弯尾姬蜂（*Diadegma semiclausum*）和菜粉蝶盘绒茧蜂(*Cotesia glomerata*)行为的影响。与非转基因对照植物相比，未受损伤的Bt与LIS的杂交转基因植物对半闭弯尾姬蜂的雌蜂表现为既不吸引也不排斥；与相同处理的非转基因对照植物相比，被小菜蛾幼虫或机械损伤的LIS转基因植物对*D. semiclausum*显示排斥作用；然而与未受损伤的LIS转基因植物相比，受小菜蛾损害的LIS转基因植物对*D. semiclausum*表现为吸引作用；与同样受损的非LIS转基因植物相比，受大菜粉蝶幼虫损害的LIS转基因植物对菜粉蝶盘绒茧蜂具有较强的吸引力。实际寄生率的试验显示非纯合LIS转基因植物与非转基因对照植物之间对半闭弯尾姬蜂对小菜蛾寄生率的影响表现为差异不显著。本章结论如下，数据显示将LIS基因导入拟南芥中可对芸薹属害虫的不同寄生蜂行为产生不同的影响；*D. semiclausum*对*P. xylostella*寄生率不因为LIS基因引起产生的芳樟醇而受到影响。

综上所述，本论文结果显示植物抗虫基因和产生挥发物的基因可以在同一植物基因型中被表达，在本研究中该双基因转化体对小菜蛾及其寄生蜂的影响结果表明，对小菜蛾的抗性表现为Bt毒蛋白基因对其幼虫的毒性同时表现为由于LIS基因表达对其成虫的驱避性。表达芳樟醇的转基因植物对寄生蜂的影响因其种类不同而不同，但对其行为的影响表现为中性或吸引，对寄生率的影响表现为中性。因此，可将Bt毒性基因和芳樟醇合酶基因同时整合到拟南芥中作为一个好的模型用于改善小菜蛾防治的新方法的研究。此外这些结果引出了令人感兴趣的通过干涉害虫生物学和增强害虫天敌效力的转基因作物发展的可能性，以这种方式可以获得综合寄主植物抗性和生物防治的转基因植物。

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Curriculum vitae

Limei Yang was born on 5 January, 1960 in Zhangzhou, Fujian Province and grew up in Beijing, China. She started her undergraduate study in Beijing Agricultural University (nowadays China Agricultural University) in 1980 and graduated in 1984 with a Bachelor degree. After that she was employed by the Institute of Vegetables & Flowers (IVF, the former Vegetable Research Institute), Chinese Academy of Agricultural Sciences (CAAS) as a Research Assistant. In 1992 she received a fellowship from the government of the Netherlands and studied as an MSc student at Wageningen University. Her main topic was plant breeding and her MSc thesis was entitled “Protoplast fusion and detection of alien chromosomes in the backcross progenies through in-situ hybridization”. The MSc degree was awarded in January 1994 and she continued her career on cole crop breeding in the IVF. Limei was employed as an associate professor in IVF in 1998. She started her PhD study in the graduate school of CAAS in 2001 and was selected as a candidate for the “Joint PhD training program between WU and CAAS” registered at WU as a sandwich PhD student under the supervision of Marcel Dicke, Maarten Jongsma, and Joop van Loon at WU and Zhiyuan Fang at CAAS. She spent the first year in Wageningen for taking courses, writing the project proposal, and carrying out initial experiments. Subsequently she worked for two years in IVF and went to Wageningen twice for the lab work in 2004 for three months and 2005 for six months respectively, supported by IAC grants. The sandwich PhD project she worked in the Laboratory of Entomology and Plant Research International of WUR ended with the completion of this thesis. She will continue her job in IVF focusing on biotechnology and breeding research on cole crops.

List of Publications

Yang, L.M., van Loon, J.J.A., Dicke, M., Fang, Z.Y., Jongsma, M.A. The diamondback moth, *Plutella xylostella,* specifically inactivates Mustard Trypsin Inhibitor 2 (MTI2) to overcome host plant defence. *Submitted*

Yang, L.M., Mercke, P., van Loon, J.J.A., Fang, Z.Y., Dicke, M., Jongsma, M.A. Expression in *Arabidopsis* of a strawberry linalool synthase gene under the control of the inducible potato PI2 promoter. Agricultural Sciences in China (in press).

Fang, Z.Y., Liu, Y.M., **Yang, L.M.**, Zhuang, M., Zhang Y.Y., Sun, P.T. (2007). Review and prospect of the breeding of new variety Zhonggan series and technology of heterosis utilization in cabbage (*Brassica oleracea* var. *capitata* L.). Scientia Agricultura Sinica 40 (suppl. 1): 320-324.

Luo, J., Chen, W., Zhuang, M., Li, Y.H., Wang, X.W., Liu, Y.M., **Yang, L.M.**, Zhang, Y.Y., Fang, Z.Y. (2007). Construction and transformation of inverted repeat of Turnip Mosaic Virus coat protein gene. China Vegetables 2007(2): 10-12.

Yan, H.L., Fang, Z.Y., Liu, Y.M., Wang, Y.J., **Yang, L.M.**, Zhuang, M., Zhang, Y.Y., Sun, P.T. (2007). Genetic effect of sterility of the dominant genic male sterile material (DGMS79－399－3) in Cabbage (*Brassica oleracea* var. *capitata* L.). Acta Horticultrae Sinica 34(1): 93-98.

Wang, X.J., **Yang, L.M.**, Fang, Z.Y., Yu, Z., Cui, L. (2006). The research and utilization of *Bt* gene in Vegetable breeding. China Vegetables 2006 (9): 35-38.

Zhang, Y.Y., Fang, Z.Y., Liu, Y.M., **Yang, L.M.**, Wang, X.W., Zhuang, M., Sun, P.T. (2005). A new cabbage F1 hybrid - ‘Zhonggan 21’. China Vegetables 2005 (10/11): 28-29.

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Yang, L.M., Fang, Z.Y., Liu, Y.M., Wang, X.W., Zhuang, M., Zhang, Y.Y., Sun, P.T. (2004). ‘Zhonggan 18’ -a new cabbage hybrid variety with the hybridization of dominant male sterile line and inbred line. Acta Horticulturae Sinica 31 (6): 837.

Fang, Z.Y., Liu, Y.M., **Yang, L.M.**, Wang, X.W., Zhuang, M., Zhang, Y.Y., Sun, P.T. (2004). Breeding and seed production technique of dominant genic male sterile (DGMS) line and cytoplasmic male sterile (CMS) line in cabbage. Scientia Agricultura Sinica 37 (5): 717-723.

Zhuang, M., Zhang, Y.Y., Fang, Z.Y., Liu, Y.M., **Yang, L.M.**, Wang, X.W., Sun, P.T. (2004). Breeding of superior cross combinations and analysis of combining ability among several inbreds of purple cabbage. China Vegetables 2004 (3): 30-31.

Xiong, X.J., **Yang, L.M.**, Liu, M.Y., Fang, Z.Y. Evolvement of transgenic study in vegetable crops. Northern Horticulture 2004(1): 4-6.

Yang, L.M., Fang, Z.Y., Liu, Y.M., Zhuang, M., Zhang, Y.Y., Sun, P.T. (2003). Breeding of cabbage inbred line with micro-spore culture. China Vegetables 2003 (6): 31-32.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to:
Date:
Group:

1) Start-up phase	date	cp
▶ **First presentation of your project**		
Integration of host plant resistance and biological control in transgenic plants	Mar 09, 2002	1.5
▶ **Writing or rewriting a project proposal**		
Integration of host plant resistance and biological control in transgenic plants	2002	6.0
▶ **Writing a review or book chapter**		
▶ **MSc courses**		
Biology of Insects	2001	6.0
▶ **Laboratory use of isotopes**		
Subtotal Start-up Phase		13.5

2) Scientific Exposure	date	cp
▶ **EPS PhD student days**		
EPS PhD Student Day, Wageningen University	Jan 24, 2002	0.3
▶ **EPS theme symposia**		
EPS Theme Symposium 2 'Interactions between Plants and Biotic Agents', Leiden University	Dec 17, 2001	0.3
▶ **Seminars (series), workshops and symposia**		
Entomology WU seminars	2001-2002	0.5
PRI seminars	2001-2002	0.6
IVF-CAAS seminars	2002-2008	1.0
▶ **Seminar plus**		
▶ **International symposia and congresses**		
15th International Plant Protection Congress, Beijing, China	May 11-16, 2004	1.8
Chinese National Congress of Horticultural Plant Biotechnology, Beijing, China	Mar 18-19, 2007	0.6
▶ **Presentations**		
Max Planck Institute for Chemical Ecology, Jena	Jun 2002	0.7
Chinese National Congress of Horticultral Plant Biotechnology, Beijing, China	Mar 19, 2007	0.7
Poster in WU-CAAS Autumn School	Nov 2003	0.7
▶ **IAB interview**		
▶ **Excursions -** EPS PhD excursion Germany	Jun 2002	2.4
Subtotal Scientific Exposure		9.6

3) In-Depth Studies	date	cp
▶ **EPS courses or other PhD courses**		
WUR-CAAS Autumn School, Beijing, China	Nov 2003	1.5
Modern Scientific & Technological Revolution and Current Society - CAAS	Sept. 2002-Jan. 2003	4.5
Progress in Agricultural Science and Technology - CAAS	Sept. 2002-Jan. 2003	3.0
Vegetable Genetics and Breeding - CAAS	Feb. 2003-Jul. 2003	4.5
The study of cabbage transformation - IVF, CAAS	Feb. 2003-Jul. 2003	3.0
▶ **Journal club**		
Member of literature discussion group (Entomology lab)	Oct 2001-Sept 2002	1.0
▶ **Individual research training**		
Subtotal In-Depth Studies		17.5

4) Personal development	date	cp
▶ **Skill training courses**		
How to write an Application Grant	Sept. 2002-Jan. 2003	1.5
English Training Course for Reading, Listening, and writing	Sept. 2002-Jan. 2003	6.0
Scientific English Reading - IVF, CAAS	Feb. 2003-Jul. 2003	1.5
▶ **Organisation of PhD students day, course or conference**		
▶ **Membership of Board, Committee or PhD council**		
Subtotal Personal Development		9.0

TOTAL NUMBER OF CREDIT POINTS*		**49.6**

** A credit represents a normative study load of 28 hours of study*

Revolution and Current Society(*)

This research has been conducted at the Graduate School Experimental Sciences (EPS) and the Graduate School of the Chinese Academy of Agricultural Sciences (CAAS).

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