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Review article

Effects of transgene products on honey bees (*Apis mellifera*) and bumblebees (*Bombus* sp.)

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Abstract – As more transgenic crop plants become commercialised, there is an increasing need for information on their impacts on honey bees and bumblebees. Direct effects on bees may arise upon ingestion of proteins encoded by transgenes, if they are expressed in pollen, nectar or resin. Indirect effects may occur if plant transformation inadvertently changes flower phenotype. This review summarises current findings on effects of purified transgene product ingestion on adult bee gut physiology, food consumption, olfactory learning behaviour and longevity. Bt, protease inhibitor, chitinase, glucanase and biotin-binding protein genes are discussed. Results from tests conducted in the laboratory with individual adult bees and with colonies in the field are presented. Observations of bee foraging on transgenic plants kept under containment are also summarised. Results so far suggest that transgenic plant impacts on pollinators will depend on a case-by-case analysis of the gene concerned and its expression in the parts of the plant ingested by bees.

***Apis mellifera* / *Bombus terrestris* / transgenic plants / *Bacillus thuringiensis* / protease inhibitor**

1. INTRODUCTION

In 1998 there were nearly 70 million acres of transgenic commercial crops growing worldwide (excluding China) (James, 1998). Most were grown in the United States, but there were also sizeable acreages in Argentina and Canada, and smaller but still significant areas planted in Australia, Mexico, Spain, France and South Africa (James, 1998). Soybeans, corn, cotton, oilseed rape and potato, carrying herbicide-tolerance or pest-resistance transgenes, accounted for the vast majority of these crops (James, 1998). The list of transgenic crop plants ready to be commercialised is

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longer and includes a wider range of traits. For example, in the United States, tomatoes with delayed ripening characteristics, virus-resistant squash and papaya, and engineered *Bacillus thuringiensis* strains have been approved for commercialisation (Anon, 1997). Other transgenic plants being field-tested include field crops (e.g. tobacco, alfalfa and rice), fruits (e.g. apple, kiwifruit, cranberry, grape, melon, plum, raspberry and strawberry), vegetables (e.g. broccoli, carrot, pea, eggplant and cucumber) and flowers (e.g. gladiolus, petunia and chrysanthemum) (Anon, 1997).

Many of these crops require bees for pollination. For some, crop yield is directly related to bee pollination success (e.g. apples, kiwifruit, plums, strawberries and tomatoes). In others, seed production is wholly or partially dependent on visits from bees (e.g. oilseed rape, broccoli and carrots). Additionally, there are many plants that, strictly speaking, do not require bee pollination, but are important food sources for bees (e.g. cotton) and others that are visited by bees if there is no better forage available (e.g. corn and potato) (Louveaux et al., 1980; Crane and Walker, 1984). Consequently there is a need for information about the impacts of transgenic plants on bees as pollinators and as honey producers.

Transgenic plants may have direct or indirect effects on bees. Direct effects may be defined as those that arise when a bee ingests the protein that a transgene encodes. Indirect effects may arise if the process of introducing the transgene into the plant results in inadvertent changes to plant phenotype affecting its attractiveness or nutritive value to bees.

Direct effects on bees may arise if transgene products (proteins) occur in the pollen, nectar or resin of a transgenic plant. These effects will depend on the nature of the transgene product and on the amount of it consumed by the bee. Of the plant products that bees collect, pollen represents the most likely vehicle for a transgene product. Pollen is a plant tissue composed of 8 to 40% protein (Herbert, 1992), whereas nectar and resin are plant secretions without significant protein content (Baker and Baker, 1977; Schmidt and Buchmann, 1992). There are surprisingly few published measurements of transgene expression levels in the pollen or nectar of transgenic plants and none for the resins, gums or exudates that bees collect for propolis manufacture. Transgenic corn containing a Bt gene controlled by a pollen-specific promoter was found to have pollen containing 260–418 ng of Bt toxin per mg of total soluble protein (Kozeil et al., 1993). However, transgenic corn plants containing the same Bt gene on a different promoter (cauliflower mosaic virus or CaMV 35S) did not produce measurable quantities of the toxin in pollen (Kozeil et al., 1993). Transgenic Bt-cotton plants (commercial cultivar, Bollgard™, with *cry*1Ac gene driven by CaMV 35S promoter) had 0.6 µg of Bt toxin in their pollen (per g fresh weight), whereas the petals of the same plants contained 3.4 µg of toxin per g (Greenplate, 1997). Indirect evidence of Bt gene expression in pollen has also been provided by a report of insecticidal activity of pollen from Bt-transgenic N4640 maize (Losey et al., 1999). Transgenic oilseed rape plants containing a gene encoding the proteinase inhibitor, oryzacystatin I (OC-I), under the control of the CaMV 35S promoter had measurable quantities of this transgene product in their leaves (0.2–0.4% of total soluble protein), but not in their pollen (Bonadé Bottino et al., 1998). This finding was confirmed by Jouanin et al. (1998) and these authors also noted that Bowman-Birk soybean trypsin inhibitor (BBI) could not be detected in the nectar or pollen of transgenic oilseed rape plants with measurable expression levels in leaves (gene also on CaMV 35S promoter). Clearly, more information is required before any generalisations can be made about transgene expression levels in the parts of plants ingested by bees. Without this information we are somewhat limited in the conclusions we can draw

from experiments to test the impacts of transgene products and transgenic plants on bees.

Data on pollen grain dispersal is available from various studies designed to assess gene flow from transgenic plants (e.g. Lavigne et al., 1998; Foueillassar, unpublished data). From this one may deduce the potential risks to non-target insects of exposure to proteins which could be expressed in transgenic pollen. Unfortunately, current predictive mathematical models of pollen dispersal take into account pollen carried only by wind and not by insects. More information is needed on insect-mediated pollen movement in order to make reasonable predictions about the levels of transgenic pollen to which bees may be exposed in the field.

Indirect effects of transgenic plants on bees may occur when genetic modification results in an unexpected change in the plant's phenotype. Insertional mutagenesis is one such change. In this case, the random positioning of the transgene in the plant's genome interferes with a gene or suite of genes needed for a "normal" phenotype. For example, an insertional mutagenesis event that resulted in plants without flowers would have a definite negative impact on bees. Less obvious changes, such as alterations in nectar quality or volume would be harder, but not impossible, to detect. Effects due to insertional mutagenesis will vary among different lines of plants derived from separate transformation events and can easily be eliminated by line selection. Pleiotropic effects represent a second type of inadvertent phenotypic change. In this case, it is not the position of the transgene, but its product, which interferes unexpectedly with a biochemical pathway in the plant to create a phenotypic change. Such changes would occur in all lines of the transgenic plant and could not be remedied by line selection.

Test methodologies are currently being developed to assess the impacts of transgenic plants on bees. Purified transgene products can be used in conventional tests for oral and contact toxicity, similar to those developed for chemical pesticides. These proteins can also be used in behavioural assays with individual bees and can be fed to colonies of bees to assess colony-level and sub-lethal effects. (See Tab. I for a summary of such experiments.) However, it is important when designing these tests to take into account the biological activity of the transgene product in question and the dosage levels likely to be encountered by bees foraging on transgenic plants expressing the gene. The mechanisms of action of transgene products, such as Bt toxins, chitinases or protease inhibitors, vary considerably and also differ from those of conventional insecticides, which tend to fall into several well-defined categories such as nervous system inhibitors or insect growth regulators. As mentioned previously, a lack of information on transgene expression levels in pollen (or nectar or resin) limits our ability to design meaningful toxicity tests. Because of this, the dosage levels used in tests so far are estimates of the range of concentrations of a transgene product that a bee might be expected to encounter when foraging on a transgenic crop. Sometimes very high doses are included to simulate a "worst-case scenario" for the bee. These tests can provide useful information on bee impacts, in advance of the actual production of transgenic plants for testing, thus saving time and effort. Ultimately however, tests with whole transgenic plants must be conducted to confirm in vitro test results, to learn of any pleiotropic effects and to check for line effects such as those caused by insertional mutagenesis.

2. RECENT RESEARCH RESULTS

2.1. *Bt* genes

Bt genes are isolated from *Bacillus thuringiensis*, a soil-dwelling bacterium which produces a range of insect-specific toxic proteins. Different strains of *B. thuringiensis*

Table I. Summary of tests with bees and purified protein transgene products.

Protein tested	Type of experiment, situation, stages tested	Doses tested	Reference
Bt toxins			
Cry1Ac	Toxicity, laboratory, larvae and adults	20 µg/ml	Sims, 1995; Anon, 2000
Cry1Ab	Toxicity, laboratory, larvae	20 µg/ml	Anon, 2000
Cry9C	Toxicity, laboratory, larvae	20 µg/ml	Anon, 2000
Cry3A	Toxicity, laboratory, larvae	Not given	Anon, 2000
Cry3B	Toxicity and growth effects, field colony, larvae and pupae	0.066 or 0.332%	Arpaia, 1996
Cry1Ba	Toxicity and food consumption, laboratory, adults	10, 2.5, 0.25 mg/g	Malone et al., 1999
Cry1Ba	Toxicity and flight activity, field colony, adults	625 µg/g	Malone et al., 2001
Serine protease inhibitors			
Bowman-Birk soybean trypsin inhibitor (BBI)	Short-term toxicity, laboratory, adults	1, 0.1, 0.01, 0.001 mg/g	Belzunces et al., 1994
BBI	Short- and long-term toxicity, laboratory, adults	11 µg per bee (short-term); 26 µg/ml (long-term)	Girard et al., 1998
BBI	Long-term toxicity, laboratory, adults	1, 0.1, 0.01 mg/ml	Sandoz, 1996; Pham-Delègue et al., 2000
BBI	Conditioned proboscis extension assay, laboratory, adults	1, 5, 10 µg/ml (reward); 1, 0.1, 0.026, 0.01 mg/ml (dose)	Girard et al., 1998; Jouanin et al., 1998; Pham-Delègue et al., 2000
Aprotinin	Long-term toxicity, laboratory, adults	10, 5, 1, 0.1, 0.01 mg/ml	Malone et al., 1995; Burgess et al., 1996
Aprotinin	Toxicity, laboratory, bumblebee adults	10, 5, 1, 0.1, 0.01 mg/g	Malone et al., 2000
Kunitz soybean trypsin inhibitor (SBTI)	Long-term toxicity, laboratory, adults	10, 5, 1, 0.1, 0.01 mg/ml	Malone et al., 1995; Burgess et al., 1996
SBTI	Long-term toxicity, laboratory, adults	1, 0.1, 0.01 mg/ml	Sandoz, 1996; Pham-Delègue et al., 2000
SBTI	Toxicity and food consumption, laboratory, adults	10, 5, 0.5 mg/g	Malone et al., 1999
SBTI	Toxicity, laboratory, bumblebee adults	10, 5, 1, 0.1, 0.01 mg/g	Malone et al., 2000
SBTI	Toxicity and flight activity, field colony, adults	2.5 mg/g	Malone et al., in press
SBTI	Conditioned proboscis extension assay, laboratory, adults	1, 0.1, 0.01 mg/ml (dose)	Jouanin et al., 1998; Pham-Delègue et al., 2000

Table I. Continued.

Protein tested	Type of experiment, situation, stages tested	Doses tested	Reference
Potato proteinase inhibitor I (POT-1)	Long-term toxicity, laboratory, adults	2, 0.1 mg/ml, 10, 2 mg/g	Malone et al., 1998
Potato proteinase inhibitor II (POT-2)	Long-term toxicity, laboratory, adults	2, 0.1 mg/ml, 10, 2 mg/g	Malone et al., 1998
POT-1	Toxicity, laboratory, bumblebee adults	10, 5, 1, 0.1, 0.01 mg/g	Malone et al., 2000
POT-2	Toxicity, laboratory, bumblebee adults	10, 5, 1, 0.1, 0.01 mg/g	Malone et al., 2000
Cowpea trypsin inhibitor (CpTI)	Acute toxicity laboratory, adults	11 µg per bee (oral); 0.5 µg per bee (injected)	Picard-Nizou et al., 1997
CpTI	Conditioned proboscis extension assay, laboratory, adults	1, 5, 10 µg/ml (reward); 0.026 mg/ml (dose)	Picard-Nizou et al., 1997
Cysteine protease inhibitors			
Oryzacystatin I (OC-I)	Short- and long-term toxicity, laboratory, adults	11 µg per bee (short-term); 26 µg/ml (long-term)	Girard et al., 1998
OC-I	Long-term toxicity, laboratory, adults	1, 0.1, 0.01 mg/ml	Sandoz, 1996
OC-I	Conditioned proboscis extension assay, laboratory, adults	1, 5, 10 µg/ml (reward); 1, 0.1, 0.01, 0.026 mg/ml (dose)	Girard et al., 1998; Jouanin et al., 1998; Pham-Delègue et al., 2000
Chicken egg white cystatin	Short- and long-term toxicity, laboratory, adults	11 µg per bee (short-term); 26 µg/ml (long-term)	Girard et al., 1998
Other proteins			
Chitinase	Acute toxicity laboratory, adults	11 µg per bee (oral); 1.69 µg per bee (injected)	Picard-Nizou et al., 1997
Chitinase	Conditioned proboscis extension assay, laboratory, adults	1, 5, 10 µg/ml (reward)	Picard-Nizou et al., 1997
Chitinase	Foraging activity on artificial flower, flight room, adults	1.3 mg/ml, 13, 1.3 µg/ml (reward)	Picard et al., 1991
β-1,3 glucanase	Short-term toxicity, laboratory, adults	11 µg per bee (oral); 0.3 µg per bee (injected)	Picard-Nizou et al., 1997
β-1,3 glucanase	Conditioned proboscis extension assay, laboratory, adults	1, 5, 10 µg/ml (reward)	Picard-Nizou et al., 1997
β-1,3 glucanase	Foraging activity on artificial flower, flight room, adults	1.3 mg/ml, 13, 1.3 µg/ml (reward)	Picard et al., 1991
Avidin	Long-term toxicity and food consumption, laboratory, adults	6.7, 20 µM	Christeller et al., 1999

produce different suites of toxins. Usually each toxin is specific to a particular order of insects and Bt genes encoding toxins with lepidopteran, dipteran or coleopteran activity have been isolated. Cultured *B. thuringiensis* spores and vegetative stages have been used for many years in biopesticide preparations where their lack of hymenopteran activity has ensured a good safety record with bees. Transgenic cotton and corn plants containing lepidopteran-active Bt genes are commercially available, as are coleopteran-active Bt-transgenic potatoes (Anon, 1997, 2000). These plants present single toxins to the insect in a pure and "activated" form, whereas the biopesticide preparations, containing whole bacteria and spores, usually present the insects with mixtures of toxins that need to be activated by conditions in the insect's gut. Because of this, additional testing needs to be undertaken to ensure the safety of transgenic Bt-plants to beneficial insects such as bees. Fortunately, Bt toxins can be purified and activated to resemble the state in which they are expressed in transgenic plants (e.g. Simpson et al., 1997) and these can be used in trials with bees.

Purified Cry 1Ac (= CryIA(c), lepidopteran-active) toxin fed at a concentration of 20 µg/ml to 1–3 day-old larvae and adults of *Apis mellifera* had no significant effect on the survival of these insects (Sims, 1995). This toxin concentration was more than "100 times the concentration of CryIA(c) protein found in the field as present in pollen and nectar of transgenic cotton" (Sims, 1995), but the author did not give details of these gene expression measurements. Similar toxicity test results were submitted to the United States' Environmental Protection Agency (EPA) for registration of Bt-cotton. No toxicity was noted in honey bee larvae or adults fed purified Cry1Ac at levels "1 700 or 10 000 times the levels found in pollen and nectar, respectively, of transgenic insect resistant cotton plants" (Anon, 2000). Honey bee larval tests for the EPA have also revealed no bee toxicity for Cry1Ab and Cry9C (both lepidopteran-active toxins for expression in corn) or for Cry3A (coleopteran-active toxin for potatoes) (Anon, 2000). Similarly, purified Cry3B toxin (= CryIIIB, coleopteran-active) fed in sugar syrup at concentrations of 0.066% or 0.332% to colonies of honey bees over a two-month period had no effect on larval survival or pupal dry weight (Arpaia, 1996). Purified Cry1Ba toxin (lepidopteran-active), mixed into a pollen-based food at 10, 2.5 or 0.25 mg/g and fed to adult honey bees for seven days post-emergence, had no significant effect on the rate at which each food was consumed or on the longevity of the bees (Malone et al., 1999). A similar lack of effect was noted with two Bt biopesticide preparations (Foray 48B and Dipel 2X) fed to bees in the same experiment at 2.5 mg/g. However, an extremely high concentration of Dipel (10 mg/g) resulted in significantly reduced food consumption and survival, although whether this was due to the Bt toxin or some of the "inert" ingredients in the preparation was not ascertained (Malone et al., 1999). Worker honey bees fed for seven days post-emergence with 625 µg/g purified Cry1Ba toxin mixed into pollen-based food and then returned to their hives had similar longevity and flight activity to control bees (Malone et al., 2001).

A semi-field study in Germany (Schur et al., 2000, reported in Anon, 2000), using field-grown Cry1Ab Bt-corn plants and honey bee colonies placed inside gauze tents covering parts of the cornfields, showed no adverse effects of Bt-corn pollen containing high levels of Cry1Ab protein on bee survival, foraging frequency, behaviour or brood development during the seven-day period of pollen shed. Subsequent observation for a further 30 days revealed no effects on brood development.

In response to public concerns raised by the monarch butterfly/Bt-corn pollen study (Losey et al., 1999), tests are also under way to compare the respective effects of purified lepidopteran-active Bt toxins, of a

Bt biopesticide preparation, and of a conventional chemical pesticide commonly used on corn and potentially present when pollen is produced (MHP-D, unpublished data). Such work is expected to assess the biosafety correlates of the different methods used for crop protection, rather than simply comparing the effects of transgenic plants versus control plants, which is not agronomically realistic.

There are no published reports of effects of purified Bt toxins on bumblebees and no published studies specifically describing the effects of transgenic Bt plants on bumblebees.

Results so far suggest that the specificities of different Bt toxins are retained in their activated form and, with the possible exception of those derived from hymenopteran-active Bt strains (e.g. Benz and Joeressen, 1994), Bt transgene products are very likely to be safe for honey bees and bumblebees. Further studies with whole transgenic Bt-plants will still be required to check for (and eliminate) any inadvertent alterations to plant phenotype that might affect bees.

2.2. Protease inhibitor genes

2.2.1. Tests with purified protease inhibitors

Protease inhibitors (PIs) can be isolated from a great number of natural sources, representing plants, animals and microbes. As their name suggests, they are proteins which inhibit protease activity. When ingested, some PIs can inhibit insect digestive proteolytic enzymes and cause starvation and death of the insect (e.g. Steffens et al., 1978); Gatehouse et al., 1979; Burgess et al., 1991, 1994; Johnston et al., 1991, 1993, 1995). Transgenic plants expressing PIs have been shown to be protected from pest insect attack (Boulter et al., 1990; Hilder et al., 1987; Johnson et al., 1989; McManus and Burgess, 1999). PIs vary in their ability to inhibit specific proteases. For example, cysteine proteases respond to one set of PIs and serine proteases to another. Some PIs bind strongly to only one type of protease; others have dual specificity. The impact of a PI on a particular insect will depend on the insect's gut protease profile and the specific activity (or activities) of the PI in question. Because their mechanism of action involves molecule-to-molecule binding, the impacts of PIs on insects are often dose-dependent.

Honey bees and bumblebees use proteolytic enzymes to digest dietary protein (Winston, 1987; Malone et al., 1998, 2000) and so it is not surprising that some PIs at some concentrations have been demonstrated to have effects on these insects. Serine proteases predominate in these insects and serine PIs, such as soybean trypsin inhibitor, may affect bees more than cysteine PIs, such as oryzacystatin.

In the absence of quantitative data on pollen PI expression levels, one must be cautious in drawing conclusions about the effects of transgenic plants on bees based on results of laboratory experiments with purified transgene products. However, if we assume that the bees in the experiments described below received a diet which was 25% protein, then the doses of PIs administered ranged from 0.004% to 4% of total protein received. PI-transgenic plants which are effectively protected from pest attack typically have leaf expression levels ranging from 0.05% to 2.5% of total protein. For example, rice expressing 0.5 to 2% of a potato PI was resistant to pink stem borer (Duan et al., 1996), *Spodoptera litura* were killed by feeding on leaves of tobacco expressing 0.4 to 1% soybean trypsin inhibitor (McManus and Burgess, 1999), rice expressing 0.05 to 2.5% soybean trypsin inhibitor had improved resistance to brown planthopper (Lee et al., 1999), and *Wiseana* spp. growth was reduced on white clover expressing 0.07% aprotinin (Voisey et al., 1999).

Purified Bowman-Birk soybean trypsin inhibitor (BBI) fed to foraging (older)

honey bees at dose levels of 1, 0.1, 0.01 or 0.001 mg/g of sugar syrup had no effect on bee survival over four days (Belzunces et al., 1994). However trypsin activity levels in foraging bees fed three different doses of BBI in syrup for 3.5 days were significantly different from those in control bees. The lowest BBI dose (0.001 mg/g) resulted in a slight but significant increase in trypsin activity, while the two other doses (0.1 and 1 mg/g) resulted in significant reductions in activity. In vitro tests in which enzyme extracts from control bee guts were incubated with BBI at a range of concentrations showed an 80% reduction in non-specific protease activity and a 100% reduction in trypsin activity.

Some other studies on the direct effects of PIs on bees have used newly-emerged adult bees (Malone et al., 1995, 1998; Burgess et al., 1996). It is only during these first few days of adulthood that honey bees consume and need to digest significant amounts of protein-rich pollen (Crailsheim and Stolberg, 1989), so one would expect the impacts of PIs to be greater at that time. When fed to young adult bees, four different serine endopeptidase inhibitors had dose-dependent effects on bee survival and many of the PI treatments significantly altered protease activity levels in the midguts of these bees (Malone et al., 1995, 1998; Burgess et al., 1996).

Aprotinin (also known as bovine pancreatic trypsin inhibitor or BPTI) and SBTI (= SKTI; soybean Kunitz trypsin inhibitor) both significantly reduce the survival of bees fed these PIs ad lib in sugar syrup at 10, 5 or 1 mg/ml, but not at 0.1 or 0.01 mg/ml (Malone et al., 1995; Burgess et al., 1996). In vivo activity levels of three midgut endopeptidases (trypsin, chymotrypsin and elastase) and the exopeptidase leucine aminopeptidase (LAP) were determined for these bees at two time points: the 8th day after emergence and when 75% of bees had died. LAP activity levels increased significantly in bees fed with either inhibitor at all concentrations. At day 8, bees fed BPTI at all concentrations had significantly reduced levels of trypsin, chymotrypsin and elastase. At the time of 75% mortality, bees fed BPTI at each concentration had reduced trypsin levels, but only those fed the inhibitor at the highest dose had reduced chymotrypsin or elastase activity. At both time points, only bees fed SBTI at the highest concentration had lowered trypsin, chymotrypsin and elastase activities. These results suggest that the observed reductions in bee survival at the higher PI dose levels are in fact the result of a disruption in their ability to digest protein. We may also speculate that the increased levels of LAP represent some kind of compensatory mechanism to make up for the loss of proteolytic function in the gut.

Very similar results were obtained with bees fed potato proteinase inhibitor I (POT-1 or PI-I) and potato proteinase inhibitor II (POT-2 or PI-II) (Malone et al., 1998). Newly-emerged bees were fed each PI in either sugar syrup (2 or 0.1 mg/ml) administered ad lib, or in a pollen-based food (10 or 2 mg/g) which was replaced with control food after eight days. In vivo activities of trypsin, chymotrypsin, elastase and LAP were determined at day 3 and at day 8. Enzyme activities were significantly lower at day 8 than at day 3, except for elastase, which did not change. Potato PI-II significantly reduced the activity of all endopeptidases at both time points, regardless of the dose level or the medium in which the inhibitor was administered. Potato PI-I acted in a similar manner, except that 0.1 mg/ml potato PI-I in syrup had no effect on bees. There was no consistent trend in changes in LAP activity. Bees fed either inhibitor at 10 mg/g in pollen or at 2 mg/ml in syrup had significantly reduced survival, with the effect of the pollen treatment being greater than the syrup treatment. Bees fed potato PI-I or potato PI-II at 2 mg/g in pollen or 0.1 mg/ml in syrup had survival similar to that of control bees.

Acute toxicity tests similar to those used to test chemical pesticides, in which 10-day-old adult honey bees were either fed or injected with cowpea trypsin inhibitor (CpTI), showed that an oral dose of 11 μg per bee and an injected dose of 0.5 μg per bee had no effect on bee survival after 24 or 48 hours (Picard-Nizou et al., 1997).

Tests of short- and long-term toxicity of BBI, oryzacystatin I (OC-I) and chicken egg white cystatin to honey bees have also been carried out (Girard et al., 1998). In the short-term test, 15-day-old worker bees were supplied with 11 μg of PI each over a period of 24 hours, and then given control syrup. None of the treatments resulted in significant bee mortality at 24, 48 or 96 hours. In the long-term test, 2-day-old bees were given a continuous supply of syrup with 26 μg/ml PI added and their longevity recorded. There was considerable variability in bee longevity in this test, but no significant effects could be attributed to the ingestion of these PIs at this low concentration and bees taken from the long-term test at 15–16 days had levels of midgut proteolytic activity that did not differ from the controls.

Sandoz (1996) conducted further long-term tests with SBTI, OC-I, BBI and a mixture of OC-I and BBI fed continuously to 2-day-old bees at concentrations of 1, 0.1 or 0.01 mg/ml. Significant mortality occurred only for bees fed SBTI, BBI or the OC-I/BBI mixture at the highest dose level. These findings were confirmed by Jouanin et al. (1998) who reported that OC-I (1, 0.1 or 0.01 mg/ml) had no effect on short- or long-term honey bee mortality. BBI at 1 mg/ml, however, reduced bee survival, altered olfactory learning performance and resulted in overproduction of the gut proteases, trypsin and chymotrypsin.

Additional work has been conducted on the effects of the two serine proteinase inhibitors, BBI and SBTI (Pham-Delègue et al., 2000). These experiments have shown that, compared to a control diet containing a neutral protein (bovine serum albumin), diets containing these PIs at 1 mg/ml, and at 0.1 mg/ml to a lesser extent, significantly increased the probability of bee death at a given time. Bee gut proteolytic activities were increased when BBI and SBTI were ingested at 1 mg/ml or 0.1 mg/ml, with trypsin activity being increased at both concentrations, and other activities only at the higher one. Interestingly, new forms of proteinases that were still sensitive to BBI and SBTI were produced. This suggests that bees ingesting high doses of BBI or SBTI will overproduce proteinases and thus will require large quantities of amino acids derived from body proteins. Such mobilisation of body proteins might explain the reduced longevity and lower behavioural responses of bees fed high doses of BBI or SBTI.

In a field experiment with a purified PI, worker honey bees fed for seven days post-emergence with 2.5 mg/g aprotinin mixed into pollen-based food and then returned to their hives began to fly and also died about three days sooner than control bees (Malone et al., 2001).

The impact of exposure to sub-lethal doses of PIs on adult honey bees or bumblebees is not yet known, but some studies of one component of foraging behaviour, olfactory learning, have been carried out with honey bees that have consumed PIs. Addition of cowpea trypsin inhibitor (CpTI) at 1, 5 or 10 μg/ml to the reward syrup offered in a conditioned proboscis extension assay significantly reduced the ability of bees to learn this response (Picard-Nizou et al., 1997). In contrast, addition of BBI or cystatin at the same concentrations did not affect short- or long-term learning ability in 15-day-old bees (Girard et al., 1998). Furthermore, the learning performances of bees that had been fed ad lib with syrup containing 26 μg/ml of either OC-I or BBI for about 13 days prior to the proboscis extension assay were unaltered by this treatment (Girard et al., 1998). When bees were fed with SBTI, OC-I, BBI or a OC-I/BBI mixture

at 1, 0.1 or 0.01 mg/ml for 15 days prior to testing, their learning ability was significantly impaired only with the 1 mg/ml BBI treatment (Jouanin et al., 1998; Pham-Delègue et al., 2000).

The effects of four PIs, BPTI, SBTI, POT-1 and POT-2, on bumblebee survival and gut protease activities have also been determined (Malone et al., 2000). Unlike honey bees, bumblebees consume pollen throughout adult life so that one might expect protease inhibition to have a different impact on them. Trials with the four PIs mixed into pollen-based food at a range of concentrations and fed continuously to adult bumblebees (between 0 and 8 weeks old) showed that those fed with SBTI (10 mg/g of pollen-based food) and POT-1 (10 and 5 mg/g) had significantly reduced survival. Bumblebees fed POT-2 (10 mg/g) had poorer survival than those fed 0.1 or 0.01 mg/g POT-2. BPTI had no effect. Untreated bumblebee midguts had elastase-like (283.0 ± 9.6 nmol/min/gut), chymotrypsin (148.5 ± 8.4), trypsin (27.2 ± 2.8) and LAP (258.6 ± 9.6) activities. Elastase-like and chymotrypsin activities were inhibited by SBTI, POT-1 and POT-2, but not BPTI. Trypsin activity was reduced by each inhibitor and LAP activity was unaffected. Thus, like honey bees, bumblebees are affected by high doses of some PIs. The relatively low levels of trypsin activity in bumblebee midguts, compared with those in honey bees, may explain why BPTI did not affect bumblebee survival.

2.2.2. Tests with transgenic plants

The interactions between foraging honey bees and flowering transgenic oilseed rape plants expressing OC-I have been investigated (Grallien et al., 1995). Experiments were conducted under confinement in a climatised flight room. Foragers from a one-comb observation hive were given a choice between five genetically modified oilseed rape and five control plants at the same flowering stage. Plant material was one spring "00" line and its transformed derivative with a gene encoding the cysteine PI, OC-I. The number of bees visiting each line and the number of flowers visited were counted. No significant differences between lines were found. Additionally, video recordings were made of the flower scapes. Individual foraging sequences (location on the plant, behavioural events such as searching, foraging, cleaning) were analysed. There were no significant differences between the two lines with respect to the total duration of visits and the mean time spent on each flower. Only the frequency of searching events appeared to be higher on the transformed plants.

Research with PIs and bees so far suggests that adult bee gut protease activities may be reduced, with a resultant impact on bee longevity, when bees ingest these proteins. However, the effects will depend on the specificity of the particular inhibitor and the concentration to which the bee is exposed.

2.3. Chitinase genes

Genes encoding chitin-degrading enzymes have been isolated from a number of sources, including plants, insects and entomopathogenic micro-organisms (e.g. Bogo et al., 1998; Gatehouse et al., 1997; Girard et al., 1998; Kang et al., 1998; Kim et al., 1998; Kramer and Muthukrishnan, 1997). As chitin is an important structural component in fungi and insects, chitinase genes have been engineered into plants in order to protect them from fungal infection and pest attack (e.g. Wang et al., 1996; Gatehouse et al., 1996, 1997; Ding et al., 1998). As with other insects, chitin is an important component of the cuticle of honey bees and bumblebees. Thus bees might be affected by ingesting chitinases expressed in transgenic plants.

Acute toxicity tests with 10-day-old adult honey bees fed sugar solution containing a chitinase purified from tomato (11 μg per

bee) showed that this transgene product had no significant impact on bee survival after 24 or 48 hours (Picard-Nizou et al., 1997). Bees injected with 1.69 μg of chitinase were similarly unaffected.

Using a standard conditioned proboscis extension assay in individual restrained bees, it was demonstrated that concentrations of 1, 5 or 10 μg/ml chitinase added to the sugar reward delivered during the training period, did not affect olfactory learning performance (Picard-Nizou et al., 1997). Complementary studies were conducted at the colony level in a flight room, using an artificial flower device (Picard et al., 1991). Sucrose solutions, either pure or combined with 1.3 mg/ml chitinase diluted either 100 or 1000 times, were presented in a choice situation. There was no evidence of discrimination in the weights of solution collected. However the number of visits was lower by a factor of four on the protein-added sources, compared to the control solution.

The foraging behaviour of honey bees on transgenic oilseed rape plants engineered with a bean chitinase gene under the control of CaMV 35S promoter has been examined under indoor and outdoor conditions (Picard-Nizou et al., 1995). Two different lines each of control and transgenic oilseed rape plants were used. The transgenic plants had 20-fold higher chitinase levels in leaves and cotyledons than the controls. The mean number of visits per five flowers did not differ significantly between the transgenic plants and their respective controls, for both pairs of lines and both experimental conditions. Thus the transformation events did not seem to have affected foragers' choices, although significant differences were found between the two different breeding lines under the same conditions (indoor), or for the same pair tested under the two experimental conditions. Individual behavioural sequences recorded by video consistently indicated that the transformation of the plant did not induce a significant effect on bee foraging behaviour, in terms of the number of flowers visited per bee, of the time spent on the plant, or of the number of nectar collection trials per bee.

Results so far suggest that bees will not be directly affected by the chitinases tested, although the effects of a range of doses have not yet been ascertained.

2.4. β-1,3 glucanase genes

Glucanase genes have been isolated from a number of different plants, where they form an important part of the plant's response to attack from fungal pathogens (e.g. Neuhaus et al., 1992; Chang et al., 1992; Gottschalk et al., 1998). They have also been isolated from micro-organisms (e.g. Haapalainen et al., 1998; Okada et al., 1998). Transgenic plants expressing β-1,3 glucanase have demonstrated enhanced resistance to fungal pathogens (Jongedijk et al., 1995). This protein is highly unlikely to be harmful to bees, since its substrate, β-1,3 glucan, has not been found in insects.

Purified β-1,3 glucanase had no effect on the 24- or 48-hour survival of adult bees fed with 11 μg per bee or injected with 0.3 μg of this transgene product (Picard-Nizou et al., 1997).

As with chitinase, the effects of 1, 5 or 10 μg/ml β-1,3 glucanase on bee olfactory learning abilities has also been tested using the conditioned proboscis extension assay (Picard-Nizou et al., 1997). With this protein, a lower resistance to the extinction of the conditioned response was found, i.e. after training, bees stopped exhibiting the proboscis extension response to unrewarded presentations of the olfactory stimulus more rapidly than in the control group. At the colony level, the level of visits to feeders of an artificial flower device set in a flight room, filled with sucrose solution added with 110 μg/ml β-1,3 glucanase diluted between 100 and 10000 times was weaker as the concentration increased. However,

there were no differences in the amounts of solution collected that could be attributed to the type of feeder solution presented (Picard et al., 1991).

2.5. Biotin-binding proteins

Proteins that bind to vitamins, such as biotin, represent another category of potential pest-resistance transgene products (e.g. Morgan et al., 1993). Genes encoding two such proteins have been isolated from chickens (avidin) (Keinanen et al., 1994) and a bacterium (streptavidin) (Argarana et al., 1986). Pollen contains between 0.16 and 2.4 μM biotin (Schmidt and Buchmann, 1992 and personal communication, Christeller) and bee bread 1.83 μM biotin (personal communication, Christeller). However, the role of biotin in honey bee or bumblebee nutrition is unknown. Preliminary toxicity tests with newly-emerged adult honey bees fed with pollen-based food containing either 6.7 or 20 μM avidin showed that this protein had no significant impacts on the rate at which bees consumed their food or on their longevity (Christeller et al., 1999).

2.6. Glufosinate resistance genes

Herbicide resistance is one of the most commonly-used traits in commercial cultivars of transgenic crop plants (Anon, 1997). Since this resistance operates via the production of an enzyme to break down the herbicide and bees lack such substrates, they are extremely unlikely to be harmed by these plants.

The impacts on honey bees of transgenic herbicide (glufosinate)-resistant oilseed rape have been assessed under semi-field conditions (Chaline et al., 1999). Bee colonies were introduced into tunnel greenhouses containing either transgenic plants, control plants, or a mixture of the two. Both cultivars produced similar numbers of flowers and there were no consistent differences in nectar volume or sugar concentration between the two types of plant. There were no significant differences that could be attributed to plant type in worker bee mortality, foraging activity, foraging preferences or colony health (bee population, brood area, presence of diseases or hive food stores).

2.7. Other genes

There are as yet no published reports of the effects on honey bees or bumblebees of other transgenic plants or their protein products. Of particular interest in this respect are genetic modifications aimed at protecting plants against pest insect attack, e.g. incorporation of genes encoding lectins (Rao et al., 1998) or spider venom (Penaforte et al., 2000).

3. DISCUSSION

Results from tests with bees and transgene products so far suggest that direct effects of transgenic plants on honey bees and bumblebees will depend largely upon the type of transgene and the biological activity of the protein it encodes. Thus proteins such as lepidopteran-specific Bt toxins and glucan-degrading enzymes are extremely unlikely to affect bees. Proteins that target more general aspects of insect biology, such as protease inhibitors or chitinases, are more likely to have effects on bees. In these cases, the dosage of transgene product ingested by the bee is very likely to determine the extent of such effects, if any.

Obviously, the concentration of expressed protein in the pollen, nectar or resin of the transgenic plant will influence the extent of its impact on bees. Although some pollen and nectar expression data are available for genes driven by CaMV 35S promoter (Greenplate, 1993; Anon, 2000), it is clear that even for this well-known promoter a

better understanding of transgene expression in these plant tissues and secretions is urgently required. Further data will also be required for new promoters under development, such as wound-inducible or phloem-specific promoters (e.g. Keinonen et al., 1998; Rao et al., 1998). In the first instance, reporter genes such as glucuronidase (GUS) of green fluorescent protein (GFP) could be used to gain an indication of likely patterns of gene expression. If new gene constructs or expression strategies that minimise the presence of transgene products in the parts of plants that are ingested by honey bees and bumblebees can be developed, then any risks to bees from transgenic plants will be largely circumvented.

Standard tests for determining the impacts of transgene products on bees need to be developed and refined. These must have a sound scientific basis and also fulfil the various biosafety legislative requirements of the country in which the plants will be grown. It is generally accepted that toxicity testing methods developed for chemical insecticides are not entirely appropriate for testing pest-resistant transgenic plants. Unlike a chemical spray, a transgenic plant with pollen expression may present the bee with a continuous source of insecticidal protein during the flowering period. To balance this however, the insecticidal proteins produced by these plants (e.g. Bt toxins, PIs) tend to have lower toxicity to bees, fish and mammals than many registered chemical insecticides, particularly those that act as neurotoxins (e.g. some synthetic pyrethroids and organophosphates) (Walton, 2000).

Interestingly, while tests with transgene products and transgenic plants and adult bees predominate in the published scientific literature (see above), the EPA required only larval toxicity tests for honey bees before registering Bt-cotton plants (Anon, 2000). The dosage level of Bt toxin used in these larval tests was 1700 times that expressed in Bt-cotton pollen and 10000 times that found in Bt-cotton nectar. Not surprisingly, since the toxin was already known to be specific for Lepidoptera, there were no significant negative effects on bee larvae. While this methodology may be more than adequate for assessing the safety of a Bt toxin, the appropriateness of such a high-dose method for other testing other gene products, which may not be so specific but may still present only an extremely low ecological risk, must be questioned.

Furthermore, the relative pollen exposure levels likely to be experienced by bee larvae, compared with adults, are yet to be comprehensively studied. Young adult bees have a clearly established dietary requirement for pollen (Crailsheim and Stolberg, 1989; Crailsheim, 1990) and realistic dosage levels for adult bee toxicity tests can be determined with some knowledge of pollen expression levels in transgenic plants. However, there is a paucity of published quantitative data on the amounts of pollen ingested by bee larvae, which are known to receive mixtures of worker bee glandular secretions and pollen (Crailsheim, 1990). Planta (1888, quoted in Haydak, 1943, 1957) reported that the food of a four-day-old drone larva contained pollen at a concentration of 15000 grains per mg of food, while the food of worker larvae of the same age was almost pollen-free. Further estimations of this type are required, as is some knowledge of how this varies among the different agro-ecosystems where transgenic crops may be grown. With these data, more realistic dose levels for bee larval toxicity tests can be established.

Legislative requirements for registration of transgenic crop plants will vary from country to country and we would hope that the debate on the scientific merits of the various methodologies proposed continues. As a starting point for discussion, we suggest that any bee-safety testing schedule should include the following:

1. Determination of gene expression levels in pollen, nectar and resin.

2. Estimation of the highest potential exposure levels for bee adults (workers and

reproductives) and larvae, given the levels of expression determined above and the bees' potential for gathering and ingesting the pollen, nectar and resin of the transgenic plants in question.

3. Toxicity and sub-lethal effects tests conducted with purified proteins and caged bees in the laboratory (or in the field if permitted), which should include:

• Determination of effects of a dose of purified protein corresponding to the highest potential exposure level on bee larval survival and growth.

• Determination of effects of the above dose on adult worker bee survival, development (particularly development of the hypopharyngeal glands, which produce secretions for feeding larvae) and behaviour, particularly that connected with foraging.

• Determination of effects of the above dose on queen bee survival, fertility and possibly pheromone production.

• Determination of effects of the above dose on drone survival and possibly sperm production.

The toxicity tests should be conducted first. If there is significant toxicity at the highest dose, then the effects of lower, realistic doses should also be determined.

4. Determination of flower attractiveness (e.g. nectar volumes, nectar sugar concentrations, flower structure) as part of the selection of transgenic plant lines for release.

5. Confirmation of results obtained in laboratory tests via field tests, preferably with transgenic plants rather than purified proteins.

Finally, the information obtained above should be used to assess the risks posed by the transgenic plant in question in relation to the risks to bees of continuing with the current agricultural practice that the plant is designed to supplant (e.g. chemical or biological insecticides, fungicides or herbicides).

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Résumé – Effets des produits de transgènes sur les abeilles domestiques (*Apis mellifera*) et les bourdons (*Bombus* sp.). Au fur et à mesure qu'un nombre croissant de plantes transgéniques cultivées sont commercialisées, il y a un besoin grandissant d'informations concernant leurs impacts sur les abeilles domestiques (*Apis mellifera* L.) et les bourdons (*Bombus* sp.). Des effets directs sur les insectes peuvent provenir de l'ingestion de protéines codées par les transgènes, s'ils s'expriment dans le pollen, le nectar ou les résines. Des effets indirects peuvent arriver si la transformation de la plante modifie par mégarde le phénotype de la fleur. Cette mise au point résume les connaissances actuelles sur les effets de l'ingestion d'un produit de transgène sur les effets de la physiologie de l'intestin moyen des abeilles adultes, sur la consommation alimentaire, le comportement d'apprentissage olfactif et la longévité.

Les tests des toxines de Bt purifié, Cry1Ac, Cry1Ab, Cry1Ba, Cry3A et Cry3B, n'ont pas montré de toxicité pour les larves d'abeilles domestiques et les adultes. Un test en champ avec des colonies d'abeilles domestiques dans un champ de maïs transgénique n'a montré aucuns effets négatifs sur les performances de la colonie. Les tests avec les inhibiteurs de protéase (IP) purifiés ont montré que les IP à sérine peuvent inhiber les protéases de l'intestin moyen des abeilles domestiques et des bourdons et, à concentrations élevées, cela peut causer une réduction de la longévité des insectes adultes. Les PI à cystéines, telles que

l'oryzacystatine, sont moins susceptibles d'affecter les abeilles. Lors d'expériences avec des abeilles domestiques et des plantes transgéniques à IP en fleurs, il n'y a pas eu de différence dans le comportement de butinage sur les plantes transgéniques et sur les témoins non transgéniques.

Il n'y a pas encore de données publiées concernant les tests de toxicité des IP pour les larves d'abeilles. La toxicité et les tests d'apprentissage olfactif avec des abeilles domestiques adultes ont montré qu'une chitinase purifiée n'a aucun effet, ce qui a été confirmé par un test avec des colzas transgéniques à chitinase. La toxicité et les tests de comportement d'apprentissage olfactif effectués avec une glucanase purifiée ont montré la même absence de toxicité pour les abeilles domestiques adultes. Une protéine fixant la biotine purifiée n'a eu aucun effet sur les abeilles adultes. Des colzas résistants à l'herbicide glufosinate n'ont eu aucun impact sur la mortalité des abeilles domestiques, ni sur la santé de la colonie, ni sur le comportement de butinage en champ. Jusqu'à présent les résultats suggèrent que l'évaluation de l'impact des plantes transgéniques sur les pollinisateurs nécessitera une analyse au cas par cas du gène concerné et dépendra de son expression dans les parties de la plante ingérée par les abeilles /insectes.

***Apis mellifera* / *Bombus terrestris* / plante transgénique / *Bacillus thuringiensis* / inhibiteur de protéase**

Zusammenfassung – Auswirkungen transgener Erzeugnisse auf Honigbienen und Hummeln. Durch die vermehrte Nutzung transgener Pflanzen besteht ein zunehmender Informationsbedarf über ihre Auswirkungen auf Honigbienen und Hummeln. Direkte Wirkungen auf Bienen können durch die Aufnahme von transgen codierten Eiweiβen entstehen, wenn diese im Pollen, Nektar oder Harzen exprimiert werden. Indirekte Auswirkungen könnten durch unbeabsichtigte Veränderungen des Erscheinungsbild der Blüten durch die Transformation der Pflanzen entstehen. Hier wird eine Übersicht über derzeitige Befunde zu den Auswirkungen der Aufnahme gereinigter transgener Produkte auf die Physiologie des Darmes adulter Bienen, auf die Menge der Futteraufnahme, auf das Erlernen der Duftstoffe und auf die Lebensdauer gegeben.

Untersuchungen mit den gereinigten Bt Giften Cra1Ac, Cry1Ab, Cry1Ba, Cry3A und Cry3B zeigten keine Giftigkeit für Honigbienenlarven und adulte Bienen. Ein Freilandtest mit Honigbienenvölkern in einem transgenen Cry1Ab Maisfeld zeigte keine negativen Auswirkungen auf die Volksleistungen. Versuche mit gereinigten Protease-Inhibitoren (PI) haben gezeigt, dass serine PIs Darmproteasen bei Honigbienen und Hummeln hemmen können. In hohen Konzentrationen kann dies zu einer Verminderung der Lebensdauer führen. Bei Cystein PIs wie Oryzastatin ist eine Auswirkung auf Bienen weniger wahrscheinlich. In Experimenten mit Honigbienen in blühenden PI-transgenen Pflanzen traten im Vergleich zu nicht-transgenen Pflanzen keine Unterschiede im Sammelverhalten auf. Publizierte Daten über Toxizitätstests mit PIs stehen noch nicht zur Verfügung. Versuche über Toxizität und dem geruchlichen Lernverhalten mit adulten Bienen haben gezeigt, dass gereinigte Chitinasen keine Auswirkungen haben, dies wurde mit einem Test mit transgenen Chitinase-Ölrapssamenpflanzen bestätigt. Teste zur Toxizität und dem olfaktorischen Lernverhalten mit gereinigten Glukanasen zeigen eine ähnliche Ungiftigkeit auf adulte Bienen. Ein gereinigtes Biotin-bindendes Protein hatte keine Auswirkungen auf adulte Honigbienen. Herbizid (Glufosinat) resistente Rapspflanzen hatten keine Auswirkungen auf die Honigbienensterblichkeit, die Gesundheit der Völker oder das Sammelverhalten im Freiland. Die bisher vorliegenden Ergebnisse legen nahe, dass die Auswirkungen transgener Pflanzen auf Bestäuber Einzelfallanalyse

der betroffenen Gene und ihrer Expression in den von den Bienen aufgenommenen Pflanzenteilen bedarf.

***Apis mellifera* / *Bombus terrestris* / transgene Pflanzen / *Bacillus thuringiensis* / Proteinhemmer**

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