4.1. Effects of insect-resistant transgenic plants on solitary bees
Preliminary summary of main results

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1. Effects of transgenic plants on larvae of solitary bees

Background and methods: Larvae of many solitary bees feed almost exclusively on pollen and thus could be highly exposed to transgene products expressed in the pollen. The potential effects of pollen from oilseed rape expressing the cysteine protease inhibitor oryzacystatin-1 (OC-1) were investigated on larvae of the solitary bee *Osmia bicornis* (= *O. rufa*). Furthermore, recombinant OC-1, the Bt toxin Cry1Ab and the lectin *Galanthus nivalis* agglutinin (GNA) were evaluated for effects on the life history parameters of this important pollinator.

Results: Larval development was significantly prolonged and food conversion was significantly reduced in the larvae fed pollen with the highest level of GNA (0.1%) when compared to the control. However, no significant difference between any of the other treatments and the control was found for these two parameters. Post-emergence longevity of successfully over-wintered bees did not significantly vary among treatments. Neither did any treatment differ significantly from the control for the loss of body weight during wintering.
Of the 174 larvae tested, 5 (2.9%) died during larval development with a maximum of two within any one treatment. Neither the transgenic OC-1 expressing pollen nor any of the treatments with 'spiked' insecticidal proteins differed significantly from the control in the number of dead larvae during the course of the bioassay. Of the 169 individuals that successfully completed larval development, 17 (10.1%) died during the cocoon spinning process or subsequent wintering, with a maximum of four within anyone treatment. Also during this time span, no treatment differed significantly from the control in mortality.

Summary and significance: Pollen provisions from transgenic OC-1 oilseed rape did not affect overall development. Similarly, high doses of OC-1 (0.1%) and Cry1Ab (0.01%) as well as a low dose of GNA (0.01%) failed to cause any significant effects. However, a high dose of GNA (0.1%) in the larval diet resulted in significantly increased development time and reduced efficiency in conversion of pollen food into larval body weight.
The experimental methods developed and described in the present study are not only suitable for early tier testing in non-target risk assessments of transgenic plants, but could also be adopted for pre-release laboratory testing of agrochemicals, including systemic pesticides which may be found in the pollen. In *in vitro* toxicity tests with honey bee larvae, high levels of control mortality, probably due to grafting, can be a problem. In the present study, however, low control mortality was observed which is likely to be due to the minimal handling of eggs and larvae required when rearing solitary bees on their provisions. As a further merit of the experimental design developed in this study, not only the larval rearing but also the pollen collection and preparation were conducted under standardized conditions which is an improvement compared to earlier studies investigating potential effects of pesticides on larvae of solitary bees.

The results of the present study indicate that it is very unlikely that either OC-1 or Cry1Ab would pose a risk to larvae of *O. bicornis*. GNA, whilst hazardous to bees at high dose, was shown to not have any detrimental effect at expected expression levels. These results obtained for a single model species are expected to be relevant for a large portion of the approximately 700 solitary bee species assumed to occur in Central Europe since many of them are also polylectic, forage on agricultural crops and reproduce during the bloom of such crops.

2. Effects of transgenic plants on adults of solitary bees

Background and methods: The aims of the present study were two-fold: (i) To evaluate the impact of a transgenic crop plant expressing a protease inhibitor (PI) and of purified insecticidal proteins on longevity of a solitary bee and (ii) to characterize the profile of native digestive proteases of this solitary bee and determine possible alterations in the protease activities in response to PI ingestion.
Genes encoding PIs are known from a variety of plants, animals and microbes and in plants PIs can play a defensive role against insect herbivores by specifically binding to, and subsequently deactivating proteolytic enzymes in the insect gut. This direct inhibition of protein hydrolysis often induces the hyperproduction of proteases in the insect gut in an attempt to overcome the inhibition. The resulting depletion in essential amino acids can lead to the repressive effect of PIs on growth, fecundity or even survival of herbivorous insects observed for PI expressing transgenic plants or purified PIs administered to insect diet. PIs vary in their ability to inhibit specific proteases and an insect's susceptibility to a certain type of PI depends on the insect's profile of native digestive proteases as well as its ability to switch to insensitive enzymes in the presence of the PI. Digestive enzymes are generally classified as serine, cysteine, aspartic and metallo proteases, depending on the amino acid in the enzyme's active site.

Results Longevity: Longevity of bees foraging on transgenic OC-1 expressing oilseed rape did not significantly differ from longevity of bees foraging on control plants. Mean bee longevity was 45.9 days (± 5.01) on the OC-1 line and 52.1 days (± 7.10) on the isoline with both values being considerably higher than what is known for *Osmia* from the field.
Longevity was found to be significantly different among the experimental groups with purified insecticidal protein dissolved in sugar solution. Bees that received GNA (0.01% or 0.1%) or a high dose of SBTI (0.1%) in their diet suffered from severely reduced longevity, but also bees that received a low dose of SBTI (0.01%) or a high dose of OC-1 (0.1%) lived significantly shorter than the control. Only longevity of bees fed Cry1Ab (0.01%) or a low dose of OC-1 (0.01%) were not significantly different from the control.

Results digestive enzymes: Susceptibility of an insect species to a certain protease inhibitors depends (i) on the range of native proteases present in the insect's digestive system and (ii) on its ability to adapt to ingested inhibitors by overproduction of the sensitive native proteases or by switching to the expression of insensitive proteases.
In microplate enzyme assays with synthetic class specific substrates and diagnostic protease inhibitors the protease profile of midgut extracts from *O. bicornis* adults was examined. We found strong evidence for the presence of serine proteases like chymotrypsin and trypsin (approximately 60% of total digestive proteolytic activity) in midgut extract. Cysteine and metallo proteases were found to play a less dominant role in protein digestion of *O. bicornis* (each approx. 20% of total activity), whereas no activity of aspartic proteases was detected.
In order to characterize the compensatory response of *O. bicornis* to ingestion of two PIs targeting different classes of proteases and to relate adverse effects on bee longevity to this metabolic stressor, we analyzed the proteolytic activity in the midgut of bees that had received

either SBTI or OC-1 over seven consecutive days. Consumption of OC-1 led to a 30% increase in general hydrolysis when compared to the activity level in gut extract from control bees. Characterization of this increased activity revealed a moderate up-regulation of aspartic and possibly serine and cysteine proteases indicating a rather complex compensation mechanism that included both overproduction of native proteases as well as induction of a new insensitive protease. Consumption of SBTI led to an increase in general hydrolysis of at least 60% which suggests a strong and effective compensation for SBTI inhibited trypsin as apparently high levels of unbound proteases could be achieved. Characterization of this compensatory response to SBTI revealed changes in the protease profile which are similar to, but more pronounced than in OC-1 fed bees, which is probably due to the more dominant role of serine proteases than cysteine proteases in the native protease profile of *O. bicornis*.

Summary and significance: The results of the present study indicate that it is very unlikely that either the Bt toxin Cry1Ab or the cysteine protease inhibitor OC-1 at an expected expression level would pose a risk to adult bees of *O. bicornis*. The lectin GNA, the serine protease inhibitor SBTI as well as an unrealistically high concentration of OC-1 significantly reduced bee longevity. The GNA results are in agreement with the well documented insecticidal activity of this protein for a wide range of insect species. Effects on bee longevity by the two PIs are best interpreted in the light of the in vitro enzyme studies, which suggested a rather complex digestive system of serine (trypsin and chymotrypsin) and cysteine proteases and possibly also metallo proteases in the midgut of *O. bicornis*. Whereas the dominant role of serine proteases in protein digestion is in agreement with what is known for the honey bee *Apis mellifera*, our results on the presence of cysteine activity in the midgut of *O. bicornis* contrast with most other studies on the protease activity in bees, which did not report on cysteine proteases in the digestive tract of bees. However, serine activity is most likely essential for effective digestion of dietary protein in *O. bicornis*, as seen by the stronger physiological response to SBTI ingestion compared to OC-1 and the stronger effect of SBTI on bee longevity. These findings highlight the importance of taking into account the native protease profile as well as the enzyme complement of key non-target organisms in an environmental risk assessment of PI expressing transgenic plants.

3. Honeydew as potential route of exposure to transgene products

Background and Methods: In areas dominated by intensive agriculture pollen and nectar sources can be scarce and honeydew is likely to be the primary exogenous carbohydrate source available. Whereas honeydew foraging is well known for honey bees, there are only anecdotal reports for bumble bees and virtually no quantitative information regarding the use of honeydew is available for solitary bees. Insight into the contribution of honeydew feeding to the diet of solitary bees is of importance to the risk assessment of genetically modified plants, as honeydew has been found to be a potential route of exposure to insecticidal transgene products.
To test whether *O. bicornis* feeds on honeydew young females were released to field-cages filled with (i) just aphid infested oilseed rape plants (no-choice honeydew situation), or (ii) just flowering non-infested oilseed rape plants providing nectar (no-choice nectar situation) or (iii) both infested AND flowering plants (choice situation). Two aphid species (green peach aphid *Myzus persicae* and the cabbage aphid *Brevicoryne brassicae*) were tested and high performance liquid chromatography (HPLC) was used to analyse the sugar profile of the crop content. Honeydew feeding was assessed by direct behavioural observations and by the presence of honeydew-specific "signature sugars", i.e. di- and oligosaccharids (normally erlose and melezitose) which are typically synthesized by phloem-feeding insects and excreted in the honeydew.

Results: None of the signature sugars were detected in crops of unfed, newly emerged bees and no indication was found that *O. bicornis* is capable of synthesising the honeydew-specific sugars. The olisaccharide erlose was not only found to be present in honeydew both from *M. persicae* and *B. brassicae*, although at higher levels in the first (6.4 – 14.5% vs. 0.5 – 3.5% of total sugars), but was also detected in crops of bees that were fed with hand-collected honeydew. Thus erlose was identified as appropriate signature sugar for the detection of honeydew feeding in *O. bicornis*, although only for relatively recent feeding as the sugar level in the crop was found to be significantly reduced 24 h after feeding and reaching the level of unfed bees 96 h after feeding.
Bees from cages with flowering oilseed rape plants (i.e. the no-choice nectar situation and the two choice situations) showed very high total sugar levels in their crops and a sugar profile very similar to oilseed rape nectar (i.e. strongly dominated by glucose and fructose). Bees that had only *Brevicoryne* honeydew available showed extremely low total sugar levels. (29 of 33 bees lower than 1 µg total sugar in the crop) and the indicator sugar erlose was found in none of these bees. Bees that had only *Myzus* honeydew available had also relatively low total sugar levels although clear indication for honeydew consumption was found among these bee as erlose was detected in approximately 50% of the bees. These HPLC results are in agreement with the direct behavioural observations; feeding on *Myzus* honeydew was observed on few occasions whereas feeding on *Brevicoryne* honeydew was never observed and mortality was considerably higher among bees that had only carbohydrate source available.

Summary and significance: The results of our semi-field experiment showed that *O. bicornis* clearly prefers floral nectar over honeydew and forages on nectar as carbohydrate source if flowers are available. If these findings can be transferred to the field situation, they would indicate that in periods and areas with abundant bloom, honeydew is highly unlikely to be exploited as food source and would play a minor role as potential route of exposure to transgene products. However, under certain conditions, i.e. if other carbohydrate sources are scarce, honeydew consumption can not be completely ruled out. We here have clearly shown that *O. bicornis* is able to detect aphid honeydew and exploit this carbohydrate source depending on aphid species and environmental conditions. Thus we conclude that exposure of bees to the transgene product via honeydew should be considered in an environmental risk assessment of an insect-resistant transgenic plant, especially when the trait is present in the phloem sap.