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Thematic Issue on Horizontal Gene Transfer

Leaf-feeding larvae of *Manduca sexta* (Insecta, Lepidoptera) drastically reduce copy numbers of *aadA* antibiotic resistance genes from transplastomic tobacco but maintain intact *aadA* genes in their feces

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The objective of this study was to evaluate the effect of insect larval feeding on the fate and genetic transformability of recombinant DNA from a transplastomic plant. Leaves of tobacco plants with an *aadA* antibiotic resistance gene inserted into their chloroplast genome were incubated with larvae of the tobacco hornworm *Manduca sexta* (Lepidoptera). The specifically designed *Acinetobacter* strain BD413 pBAB$_2$ was chosen to analyze the functional integrity of the *aadA* transgene for natural transformation after gut passages. No gene transfer was detected after simultaneous feeding of leaves and the *Acinetobacter* BD413 pBAB$_2$ as a recipient, even though 15% of ingested *Acinetobacter* BD413 cells could be recovered as viable cells from feces 6 h after feeding. Results with real-time PCR indicated that an average of 98.2 to 99.99% of the *aadA* gene was degraded during the gut passage, but the range in the number of *aadA* genes in feces of larvae fed with transplastomic leaves was enormous, varying from 5×10^6 to 1×10^9 copies.g^{-1}. DNA extracted from feces of larvae fed with transplastomic leaves was still able to transform externally added competent *Acinetobacter* BD413 pBAB$_2$ *in vitro*. Transformation frequencies with concentrated feces DNA were in the same range as those found with leaves (10^{-4}–10^{-6} transformants per recipient) or purified plasmid DNA (10^{-3}–10^{-7}). The presence of functionally intact DNA was also qualitatively observed after incubation of 30 mg freshly shed feces directly with competent *Acinetobacter* BD413 pBAB$_2$, demonstrating that *aadA* genes in feces have a potential to undergo further horizontal gene transfer under environmental conditions.

Keywords: transplastomic plants / *aadA* gene / natural transformation / *Acinetobacter* / insect larvae

INTRODUCTION

The insertion of recombinant genes into the genome of plastids instead of the nucleus of plants is an option to increase gene expression and prevent out-crossing through pollen (Daniell et al., 2002). In contrast to nucleus-modified plants, this type of genetic modification inherently increases the copy numbers of the recombinant genes per cell and potentially also the probability of an unintended gene transfer to bacteria, the latter because of high similarities between plastid DNA and bacterial genomes. Current methods for generating transplastomic plants often use antibiotic resistance genes as selective agents, but concerns have been raised that such genes might be harmful to animals or humans (Bennett et al., 2004; Heritage, 2005; Jelenic, 2003). Especially the possibility of their uncontrolled spread into the environment followed by horizontal gene transfer from GM plants to bacteria of the gastrointestinal (GI) tract of animals fed with the modified crops is regarded as an important issue (Dröge et al., 1998).

The fate of recombinant DNA of genetically modified crops after feeding to vertebrates has received considerable attention during the last years in several experimental studies (Duggan et al., 2000; Martin-Orue et al., 2002; McAllan, 1980; Palka-Santini et al., 2003; Schubbert et al., 1997; 1998). In common, independent of the feeding organism, all studies report on a certain degradation of DNA. This ranges, however, from rapid and complete degradation, *e.g.*, after feeding transgenic maize to chicken (Chambers et al., 2002), to remaining concentrations of detectable DNA in the stomach, gut or

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feces, *e.g.*, as found with mice, rats, sheeps or humans (Duggan et al., 2003; Forsman et al., 2003; Heritage, 2004; Kharazmi et al., 2003; Mercer et al., 1999; Netherwood et al., 2004; Schubbert et al., 1997; Wilcks et al., 2004). Only two of these studies evaluated the potential of the remaining DNA to participate in gene transfer from plants to recipient bacteria by natural transformation, and none of the studies could detect this process under *in vivo* conditions (Duggan et al., 2003; Kharazmi et al., 2003). Hence, there is no experimental evidence for gene transfer from ingested food to gut bacteria and it can be expected that such processes, if they happen at all, must be very rare (Heritage, 2005). This assumption is confirmed by the fact that genome sequencing of gut bacteria, to the knowledge of the authors, never showed very recent acquisitions of foreign DNA from potential food sources.

In comparison to vertebrates, interactions between insects and genetically engineered plants have received less attention, although insects represent a large group of organisms interacting with field grown crops. Both adult insects and larvae thus inevitably mingle with genetically modified plants. Deni et al. investigated DNA transfer from transplastomic tobacco plants (*npt*II) to bacteria in the intestine of the tobacco hornworm *Manduca sexta* (Deni et al., 2005). In their study no gene transfer to the indigenous bacterial gut community or the introduced naturally competent *Acinetobacter* was detected. The lack of DNA transfer was constituted by the assumption of a high degradation of plant DNA, however, without providing quantitative data on transgene degradation.

In this study the fate of recombinant DNA from transplastomic tobacco plants after feeding to the tobacco hornworm was quantified and the quality of remaining plant DNA in feces was analyzed for its potential to participate in horizontal gene transfer from plants to microorganisms. For this purpose, transplastomic tobacco plants harboring an *aadA* antibiotic-resistance marker gene were selected. All experiments were conducted with larvae of *Manduca sexta,* as this lepidopteran insect is particularly adapted to feed on tobacco leaves (Evens, 1985). The fate of the recombinant gene as affected by larval gut passages was quantified using real-time PCR and the potential of transgenic *aadA* gene to participate in horizontal gene transfer was evaluated with the specifically designed strain *Acinetobacter* BD413 $pBAB_2$. This strain, developed by Kay et al. (2002), harbors a plasmid that carries flanking regions of the *aadA* gene in the chloroplast genome to facilitate homologous recombination. Transformed bacterial cells can be selected by growth in the presence of spectinomycin, allowing a sensitive detection of horizontal gene transfer due to the possibility of efficient counterselection of putative transformants against a background of naturally occurring bacteria.

RESULTS

No transformation of *Acinetobacter* BD413 $pBAB_2$ after feeding it together with transplastomic plants to *Manduca sexta* larvae

Experiments were carried out to detect gene transfer from transplastomic tobacco plants to the recipient *Acinetobacter* BD413 $pBAB_2$. Larvae were fed with transplastomic leaf material (0.1 g fresh weight) inoculated with the recipient strain, either by applying harvested cells onto the leave material (2×10^9 colony forming units, CFU, per 0.1 g leaf material), or direct addition of grown cells from liquid culture (1.7×10^8 CFU per 0.1 g). As a control, several specimens were fed with transplastomic or isogenic leaves, but without the recipient. No bacterial colonies were detected on LB agar amended with the antibiotics ampicillin and spectinomycin, independent of whether the larvae received leaf material inoculated with *Acinetobacter* or not. Thus, no transformation of *Acinetobacter* BD413 $pBAB_2$, which would have become detectable by an acquired spectinomycin-resistance in addition the resistance against ampicillin (see Materials and Methods), was observed. Considering the number of ingested *Acinetobacter* BD413 $pBAB_2$ cells, the threshold of detecting a transformation event was thus 1.7×10^{-8} and 2×10^{-9} transformants per recipient, respectively.

As there was no growth of indigenous fecal bacteria on spectinomycin-amended agar without ampicillin, transfer of the *aadA* gene from the transplastomic leaves to other than the chosen *Acinetobacter* BD413 $pBAB_2$ was also not detected. The total number of culturable bacteria from feces in the controls detected on LB agar was 10^8 $CFU.g^{-1}$ feces (fresh weight) and, thus, thresholds of detecting transformants among fecal bacteria under the chosen experimental conditions (see Materials and Methods) were 10^{-7} transformants per cultured bacterial cells. The number of surviving *Acinetobacter* BD413 $pBAB_2$ cells could not be determined in this experiment, as the grown cells of the strain could not reliably be distinguished from other, indigenous gut bacteria forming colonies on LB agar.

A significant proportion of *Acinetobacter* survives the larval gut passage

Experiments were carried out with *Acinetobacter* BD413 pSM1890 tagged with a *gfp*-marker gene to determine the survival rates of *Acinetobacter* sp. during the gut passage of larvae. Expression of the green fluorescence protein (GFP) allowed the direct detection of this strain after colony growth on agar and differentiation from other bacterial colonies. Figure 1 shows the detection of

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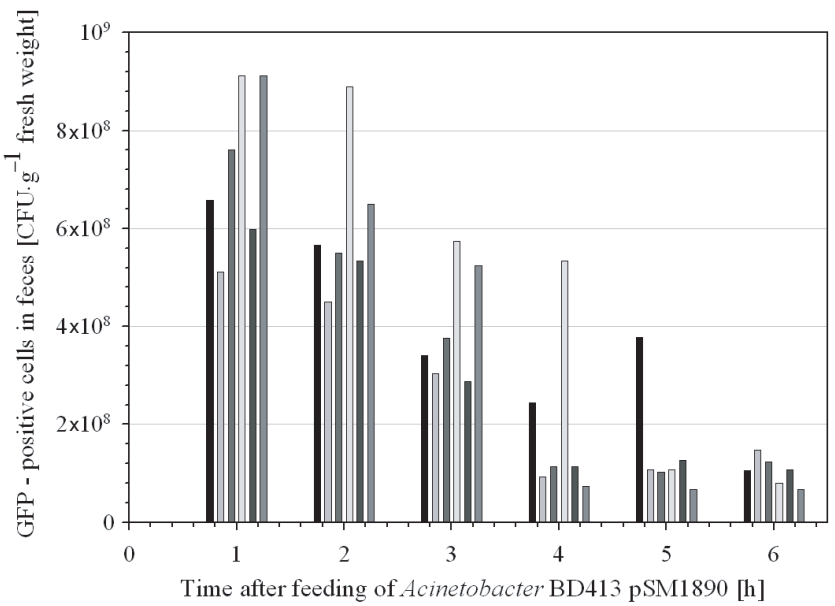

Figure 1. Detection of GFP-positive *Acinetobacter* BD413 pSM1890 cells in feces of six individual *Manduca sexta* larvae (bars) over a period of 6 h, after a pulse feeding.

Acinetobacter BD413 pSM1890 in feces of six individual *M. sexta* larvae over a period of 6 h after feeding. A decline of GFP-cells in feces by a maximum of approximately one order of magnitude, from 10^9 to 10^8 CFU.g^{-1} feces (fresh weight) was observed during this period. After 6 h, on average, a total of 15% of the food-supplied cells were recovered from the feces.

In a following experiment it was tested whether *Acinetobacter* BD413 maintained its competence to undergo natural transformation with cell-free DNA after a gut passage. Feces of larvae fed with *Acinetobacter* BD413 $pBAB_2$, were incubated with plasmid DNA carrying the *aadA* transgene (2 µL plasmid DNA (100 ng.mL^{-1}) corresponding to 4×10^7 *aadA* gene copies). No transformants were detected on LB agar amended with spectinomycin and ampicillin. Thus, in spite of the ingestion of 4×10^7 cells and the potential survival of *Acinetobacter* BD413, as demonstrated above with the GFP-tagged strain, transformation of *Acinetobacter* BD413 $pBAB_2$ after a gut passage was not detectable, suggesting that cells were not competent under the chosen conditions.

A large proportion of the *aadA* gene from transplastomic leaves is digested during the larval gut passage

A total of 90 replicates (individual larvae) were used to quantify the loss of the *aadA*-gene from tobacco leaves during digestion. DNA extracted from feces of larvae fed with transplastomic tobacco leaf material was analyzed by real-time PCR for *aadA*-gene abundance. Copy numbers detected ranged from 5×10^6 to 1×10^9 g^{-1} feces (fresh weight) (Fig. 2). The number of *aadA* genes in leaves fed to the larvae was calculated to be 6×10^{10} copies.g^{-1} fresh weight. Comparison of dry weights of leaves (10% of wet weight) and feces (20% of wet weight) was considered in the calculation of the overall loss of the transgene due to digestion, which was in the range of 98.2 to 99.99%. No transgenes were detected from the negative controls, *e.g.*, larvae fed with isogenic plant material without the *aadA* gene (10 replicates). In addition to the enormous variability of *aadA* gene copy numbers in feces between individuals, there was also a high variability in regard to the time that ingested leave material traveled through the gut. Gut passages lasted 60 to 174 min, with an average of 132 min. Under the chosen experimental conditions, the larvae needed 90 to 222 min (average 150 min) to ingest 1 g (fresh weight) of leaf material. Only a weak correlation ($r^2 = 0.21$) was observed between the time of a gut passage and the *aadA* gene copy numbers recovered from the feces (Fig. 3).

Persisting *aadA* genes in feces remain functionally intact

The *aadA* genes of the transgenic DNA extracted from feces collected in the experiment including 90 larvae fed

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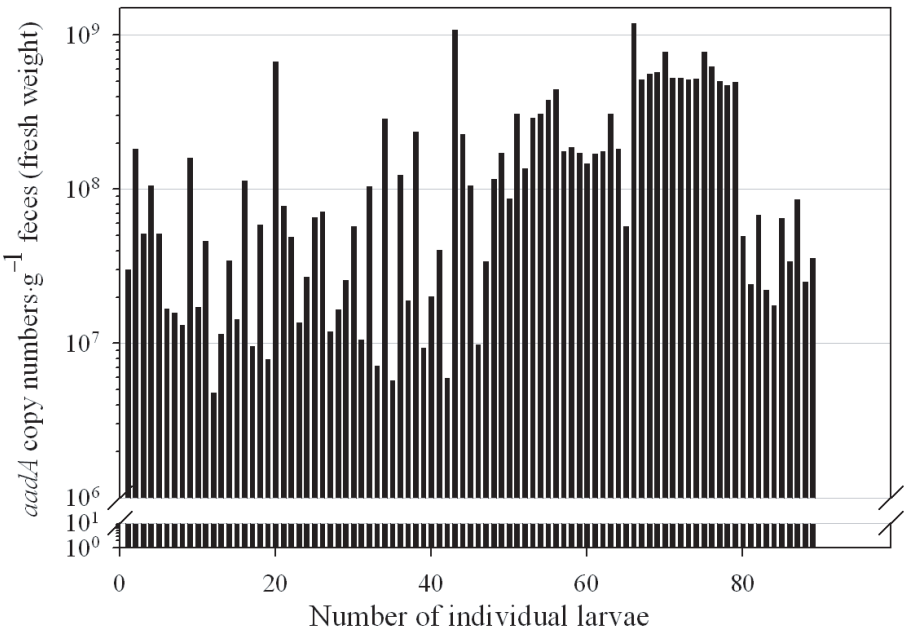

Figure 2. Transgene (*aadA*) copy numbers, determined by real-time PCR detection of a partial 176-bp sequence, found in feces of 90 individual larvae fed with *aadA* containing transplastomic tobacco leaves.

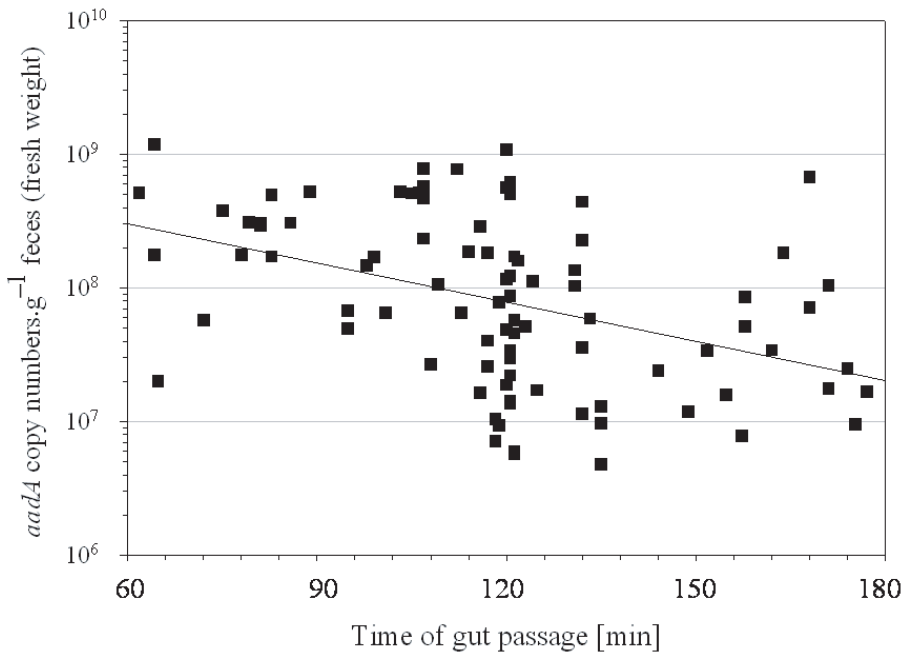

Figure 3. Correlation of transgene copy numbers (*aadA*, partial sequence of 176-bp length, determined by real-time PCR) found in feces of 90 individual *M. sexta* larvae and their corresponding times of gut passages ($r^2 = 0.21$).

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with transplastomic leaves (see above) were analyzed for their functional integrity by using the fecal DNA as a transforming substrate in transformation experiments. Concentrated transgenic feces DNA was incubated with the recipient *Acinetobacter* BD413 $pBAB_2$ and transformation frequencies were determined. As controls, total transgenic tobacco plant and plasmid DNA were also investigated for their transformation potential using the same recipient (Fig. 4A).

Transformation of *Acinetobacter* with plasmid DNA, containing the *aadA* gene, using total DNA concentrations from 0.1 pg to 1 $\mu g.mL^{-1}$, resulted in frequencies between 10^{-7} to 10^{-3} transformants per recipient, depending on the initial concentration of DNA. Frequencies increased with total DNA concentrations up to 10 $ng.mL^{-1}$ and remained at this value independent of further increasing the DNA concentration. For plant DNA, no transformants could be detected at DNA concentrations below 0.1 $ng.mL^{-1}$, but at concentrations between 0.1 ng and 1 $\mu g.mL^{-1}$ transformation frequencies ranged from 10^{-6} to 10^{-4} transformants per recipient. In comparison to plasmid DNA, frequencies with plant DNA were lower. The use of feces DNA also generated transformants, but only at total DNA concentrations of 10^0 and 10^1 $ng.mL^{-1}$ corresponding to transformation frequencies from 10^{-6} to 10^{-5}.

No further increase of transformation frequencies was achieved with higher initial DNA concentrations. As the *aadA* gene was differently diluted in total DNA of plasmids, plant DNA and feces, transformation frequencies were correlated with *aadA* gene copy numbers. Figure 4B demonstrates that, in fact, the same copy numbers generated similar transformation frequencies. Thus, there was no inhibition in the transformation assays caused by the DNA extraction procedure, neither from leaves nor from feces. Figure 4C shows PCR amplifications of *aadA* DNA sequences from total genomic DNA of transformants, as they were generated using plasmid, leaf and feces DNA, respectively. All suspected transformants, cultivated on LB agar amended with spectinomycin, were positive for the *aadA* gene, while the untransformed *Acinetobacter* BD413 $pBAB_2$ was always negative.

Effect of the initial DNA concentration on the degradation efficiency of *aadA* genes during a gut passage

The efficiency to degrade different concentrations of *aadA* genes during a gut passage was analyzed by feeding different amounts of PCR product (partial *aadA* sequence) to individual larvae and detection of *aadA* gene copy numbers in the shed feces by real-time PCR. The PCR products were supplied with colored artificial food to determine the period of the gut passages (see Materials and Methods). The results of these experiments revealed a correlation between the amounts of transgene (*aadA*) in feed and feces. However, the variability between different individual larvae was very high (Fig. 5). The correlation between supplied *aadA* genes and those found in feces suggested that the efficiency to degrade *aadA* genes decreased with increasing copy numbers, from 10^{11} to 2×10^{13} $copies.g^{-1}$ artificial food (fresh weight). The loss of *aadA* gene was 99% or above, which was similar to the loss found with transplastomic leaves. Degradation was exclusively caused by intestinal conditions: the autoclaved artificial food or the food coloring both had no influence on the stability of the added cell-free *aadA* gene, as investigated in controls (data not shown). In contrast to cell-free PCR product and transplastomic leaves, transgenic tobacco seeds fed to larvae were excreted undigested and the integrity of seeds was demonstrated by germination. Thus seed DNA was probably completely protected against the digestive forces during a larval gut passage.

Functional *aadA* genes found in feces can transfer to *Acinetobacter* BD413 $pBAB_2$ under *in vivo* conditions

Previous experiments demonstrated that feces contain DNA which after extraction and purification in the laboratory is capable of transforming *Acinetobacter* BD413 $pBAB_2$. It was, however, an open question whether the *aadA* genes were capable to transfer without DNA extraction directly from the fecal material to the recipient *Acinetobacter* BD413 $pBAB_2$. *In vitro* transformation experiments using feces DNA had shown that at least 10^6 copies per µL of the recombinant *aadA* gene were necessary in the transformation assay to obtain transformants (see above). Under the chosen experimental conditions of this study, this copy number would correspond to 10^9 *aadA* $genes.g^{-1}$ in feces. Real-time analyses, however, had shown that only two of 90 larvae produce feces containing such high *aadA* gene copies per gram feces (see Fig. 2) and thus, it was expected that, at least on rare occasions (probability 2.2%), transfer of *aadA* to *Acinetobacter* BD413 $pBAB_2$ should become detectable, if feces itself would not inhibit the transformation assay, and the *aadA* genes would maintain their functional integrity.

In fact, with incubation of competent *Acinetobacter* BD413 $pBAB_2$ and feces from lavae fed with transplastomic leaves, it was possible to detect single transformants (transformation frequency not determined), using 30 mg of freshly shed, light green feces. The majority of feces, approx. 90% in this experiment, however, was of dark green or black color, respectively, and with this material, transformation was not detectable, possibly due to the inhibitory activity of some fecal compounds. PCR detection of the *aadA* genes in genomic DNA of the putative transformants obtained from the light green feces

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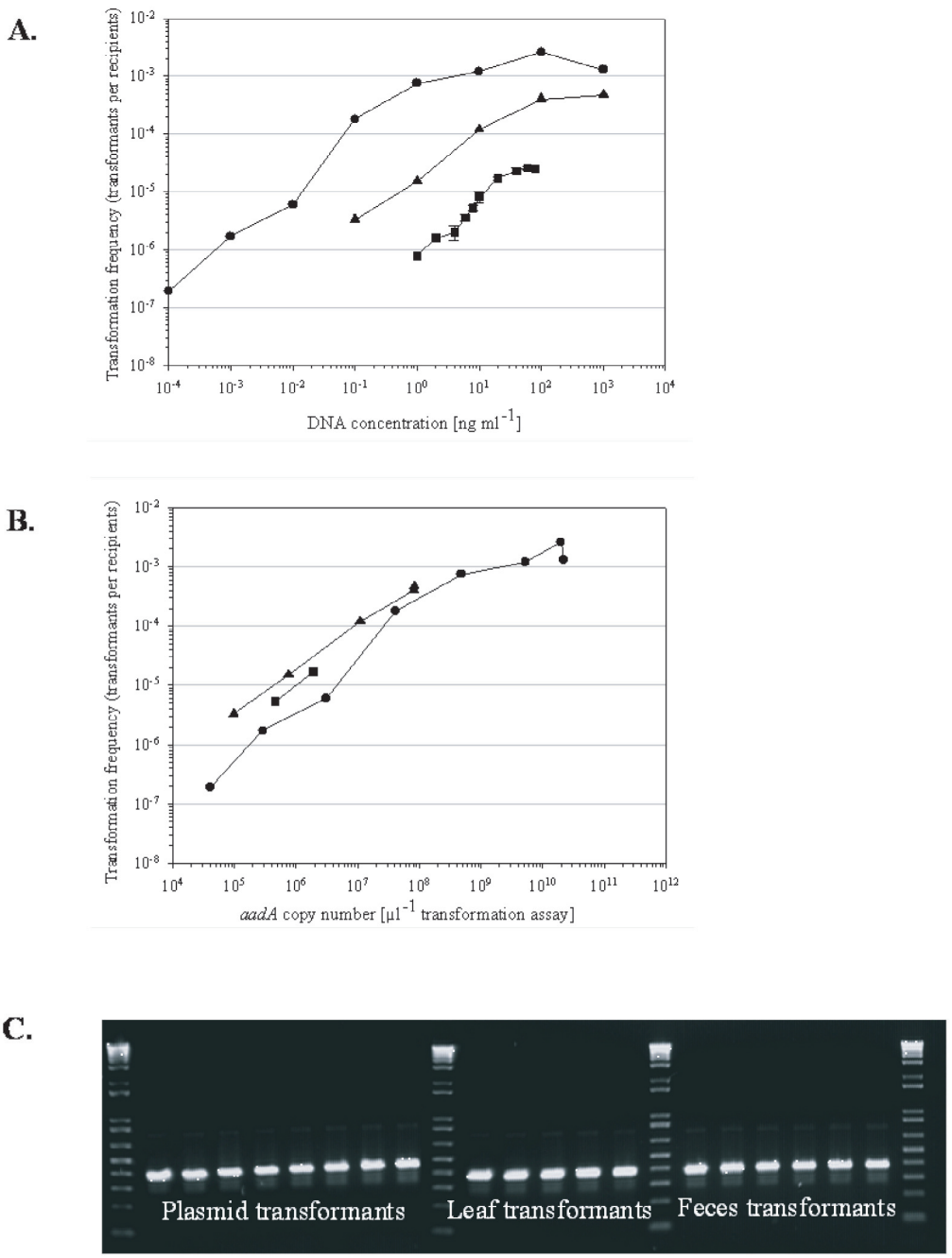

Figure 4. *In vitro* transformation frequencies of *Acinetobacter* BD413 $pBAB_2$ by *aadA* genes (complete length 785 bp) in plasmid DNA (pCEA, circle), total plant DNA extracted from transplastomic tobacco leaves (triangle) and feces of *M. sexta* larvae previously fed with transplastomic tobacco leaves (square). **A.** Correlation with DNA concentrations. **B.** Correlation with *aadA* gene copy numbers, determined by real-time PCR detection of a 176-bp partial sequence. **C.** Confirmation of transformants by PCR amplifications of partial *aadA* DNA sequence (382 bp) from total DNA of *Acinetobacter* BD413 $pBAB_2$. PCR-products evaluated on agarose gels, including a 1 kb DNA ladder as a size marker.

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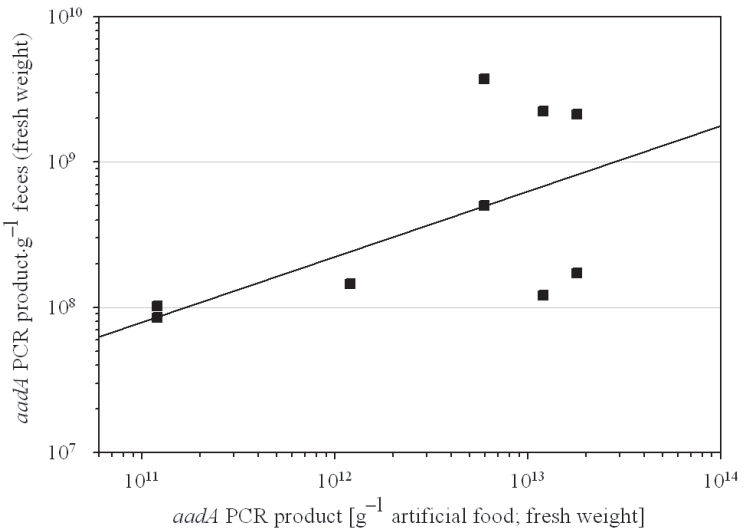

Figure 5. Correlation of transgene copy numbers (*aadA* PCR product, lengths 176 bp) found in feces to numbers supplied in food of *M. sexta* ($r^2 = 0.35$).

confirmed that functionally intact *aadA* gene transferred from feces to *Acinetobacter* BD413 $pBAB_2$ (Fig. 6). As expected, PCR of *Acinetobacter* BD413 $pBAB_2$ not incubated with feces from larvae fed with transplastomic leaves was negative.

DISCUSSION

The main objective of this study was to characterize the probability of horizontal gene transfer from a transplastomic plant to gut bacteria of a leaf-feeding insect by detecting transformation events and measuring their frequencies. The transplastomic plants of this study were tobacco, and therefore the choice of suitable insects was relatively small, since the nicotine produced by tobacco is toxic for most leaf-feeding species. *Manduca sexta*, however, is insensitive. Different studies demonstrated that *M. sexta* passes nicotine through the digestive tract and excrete it chemically unchanged (Evans, 1985; Snyder et al., 1994; Wink et al., 2002). In this study, larvae of *M. sexta*, reared under laboratory conditions and fed with artificial diet, were used in feeding experiments under defined conditions so that the rates of food uptake and times of gut passages could be quantified. We found that an individual larva needed on average 150 min for taking up 1 g leaf material (fresh weight) under the chosen conditions, while a gut passage, from ingestion to defecation, lasted on average 132 min.

The persistence of recombinant genes, as affected by the gut passage, was determined by real-time PCR of the recombinant *aadA* gene from the tobacco plant material. A comparison between gene copy numbers in tobacco leaves fed to larvae and larval feces indicated a loss of 98 to 99.99% by digestion. A decline of recombinant DNA from plant material during digestion is also typically found with vertebrates after passages through the stomach or gastrointestinal tract (Chambers et al., 2002). The pH in those systems is normally low, but the lepidopteran gut shows a remarkable specialization of the alimentary canal by generation of the highest pH values known in a biological system (Dow, 1992; Giordana et al., 1999). The pH values vary among species, but typically range from 8 to 11 (Berenbaum, 1980; Coddington and Chamberlin, 1999), in some species up to pH 12 (Dow, 1984; Moffett and Cummings, 1994; Waterhouse, 1949). *In vitro*, DNA at higher pH values (8 to 12) is more stable than at lower, *e.g.*, 5.0, as acid conditions mediate extensive depurination of nucleic acids (Raw, 1983). Despite these higher pH values, only a maximum of 2% of the ingested *aadA* genes were retrieved in the feces, indicating the existence of DNases with high activities under those conditions. Thus, both the plant cells and the plastids only provided a very limited protection against the digestive activity, and it is likely that the DNA itself serves as a nutrient source, either for the gut microorganisms or for the hosts themselves.

Interestingly, there was no striking difference between the degradation rates of *aadA* genes provided as intact transplastomic leaves or *aadA* DNA sequences supplied cell-free as a PCR product. This suggested that neither the cell nor the plastid membranes protected the DNA against the digestive processes. In contrast, seeds provided complete protection against DNA degradation, as demonstrated by the fact that they maintained their capacity to germinate and generate intact plants.

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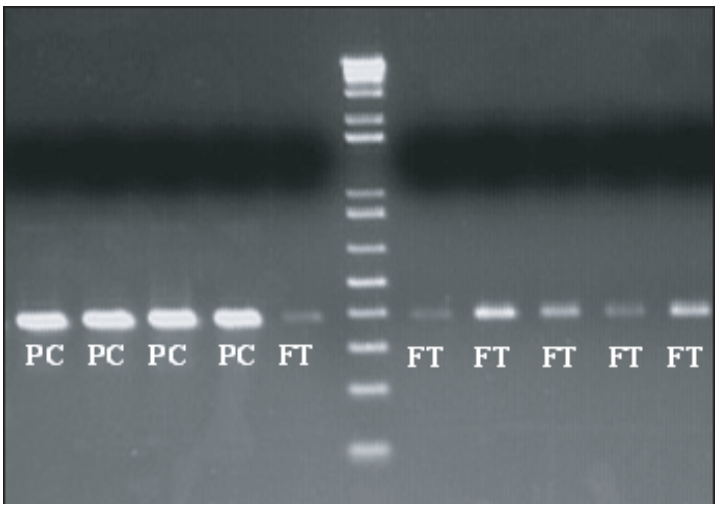

Figure 6. Detection of transgene (*aadA*, partial sequence, 382 bp) in *Acinetobacter* BD413 pBAB$_2$ after incubation with freshly shed light green larval feces collected from *M. sexta* larvae fed with transplastomic tobacco leaves. PC, positive controls (transformants obtained with plasmid DNA, pCEA); FT, transformants obtained from feces.

In the context of an unintended spread of recombinant genes from genetically engineered plants, transformation is the most likely horizontal gene transfer process considering bacteria as recipients (Bertolla and Simonet, 1999; Bertolla et al., 1999; Gebhard and Smalla, 1998; Nielsen et al., 1998). PCR-based detections in this study demonstrated, as described above, that the larval feces in fact contained *aadA* gene sequences at a considerable concentration (up to 10^9 copies per g of feces, fresh weight). PCR, however, only detects a partial sequence of this gene and could give little information about its functional integrity and availability for bacterial transformation. Therefore, marker-rescue based detection using a naturally transformable *Acinetobacter* strain was included in this study. Such systems have already been used in several instances to demonstrate the presence of transformable DNA from transgenic plants in plant residues and soil (de Vries et al., 2003; Nielsen et al., 2000; Paget et al., 1998). Transformants of *Acinetobacter* strain BD413 pBAB$_2$ were selected by growth on spectinomycin-amended growth agar (Kay et al., 2002). In other studies, it was possible with this particular strain to detect *aadA* gene acquisition and recombination from plants cells in tissues wounded by another pathogenic bacterium (Kay et al., 2002). Using *Acinetobacter* strain BD413 pBAB$_2$ in our study, we could detect functional *aadA* genes from DNA extracted from larval feces. It should be noted that gene transfer of functional *aadA* genes to other bacteria than the chosen marker-rescue strain would also have become detectable, as there was no natural background resistance of the gut bacteria against spectinomycin. The fact that spectinomycin-resistant bacterial cells were never detected in experiments with feces of larvae fed with transplastomic plants demonstrates the lack of successful gene transfer (which includes gene expression) to the gut bacteria culturable on the chosen media.

Direct inoculation of *Acinetobacter* BD413 pBAB$_2$ onto leaf material that was subsequently fed to larvae did not result in the detection of transformants. Surviving *Acinetobacter* cells from feces could not be transformed with *aadA* genes. This lack of detection indicated a loss of compentence during the gut passage, but this negative result may have been also influenced by limiting conditions in the transformation assay, which included incubation for 90 min in saline. Acquiring competence is dependent on environmental and genetic factors (Lorenz and Wackernagel, 1994). In our study, *Acinetobacter* sp. was not able to take up the transgenic plant DNA even in the nutrient rich gut environment. Possible reasons might be the high pH value inside the gut or toxic effects of the nicotine from tobacco plants (Yuan et al., 2005), damaging *Acinetobacter* and leading to a loss of competence.

With the use of the recipient strain *Acinetobacter* BD413 pBAB$_2$ it was possible to detect functionally intact *aadA* genes from DNA extracted from leaf material (positive control) and from feces with transformation rates highly similar, based on the number of total *aadA* genes. This suggested that all *aadA* genes detected as partial sequences by real-time PCR were also complete and functionally intact. Even more, it was possible to detect functional *aadA* genes directly from freshly shed feces of larvae fed with transplastomic leave material when this feces was incubated with competent *Acinetobacter*

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BD413 $pBAB_2$. However, such transfer was only possible in feces with a light green color, and not in feces of dark green to black color. It is unlikely that dark colored feces did not contain functional *aadA* genes, and thus it is tempting to attribute these differences between positive detection in light green and negative detection in dark green feces to phenolic compounds that may have inhibited the transformation reactions. In fact, transformation efficiencies of *Escherichia coli* decrease in the presence of humic acids (Tebbe and Vahjen, 1993), and humic acids also contain considerable amount of phenolic compounds. Despite the detection of single transformation events with feces, the data of this study do not allow to directly calculate the proportion of intact functional *aadA* sequences in feces in comparison to the total number of partial *aadA* genes quantified by real-time PCR.

In conclusion, our study failed to detect horizontal gene transfer from transplastomic plants to bacteria as a consequence of feeding of larvae on transplastomic plants under laboratory conditions. Neither indigenous gut bacteria of larvae nor competent food supplied recipients (*Acinetobacter* BD413 $pBAB_2$) were genetically transformed by the selectable antibiotic resistance gene *aadA*. However, a significant proportion of the *aadA* genes, *i.e.*, up to 1.8% of the copy numbers found in the originally ingested plant material, survived the gut passage, and a detectable proportion of them maintained its functional integrity to serve as a substrate for natural transformation. Thus, for an ecological risk assessment it should be considered that some remaining functionally intact transgenes may change their immediate environment, *e.g.*, from a cytosol of plant cell to an organic surface in feces, and thereby potentially participate in further ecological interactions.

MATERIALS AND METHODS

Plants, insects and microorganisms

The tobacco plants used in this study were plastid transformants generated by particle bombardment of *Nicotiana tabacum* cv. PBD6 (Kay et al., 2002). The transplastomic tobacco plants express the *aadA*-marker gene, which confers resistance to the aminoglycosides spectinomycin and streptomycin, and naturally occurs in Gram-negative organisms, *e.g.*, *Escherichia coli* (Magrini et al., 1998; Sandvang, 1999). The marker gene is located in the chloroplast genome between the *rbcL* gene, encoding the large subunit of ribulose-1,5-bisphosphate carboxylase, and the *accD* gene, encoding acetyl CoA carboxylase. Transplastomic tobacco seeds, seeds of isogenic control plants and plasmid pCEA, containing the *aadA* marker gene and used to transform *Nicotiana tabacum* cv. PBD6, were kindly provided by P. Simonet and coworkers from the Institute Microbial Ecology, Université Claude Bernard (Lyon, France). Tobacco plants were grown in pots with a diameter of 30 cm filled with potting soil (Einheitserde Typ ED 73, Einheitserdewerke, Hameln, Germany) at 28 °C and under a light-dark cycle of 12 h each per day. The tobacco plants were fertilized with 0.2% (vol./vol.) Manna Wuxal Super (Haug, Ammerbuch-Pfäffigen, Germany) as recommended.

A breeding stock of *Manduca sexta* L. (Lepidoptera: Sphingidae) was established in the greenhouse. The starting material, eggs and pupae, were kindly provided by the Institute of Animal Physiology of the Philipps-University of Marburg and the Institute for Chemical Ecology of the Max Planck Institute in Jena. The adult insects were kept in a flight cage containing a tobacco plant for egg deposition at 28 °C under a 16 h : 8 h (light : dark) photoperiod. Eggs were collected and transferred in transparent 250 mL plastic cups closed with a lid, in which the hatched larvae then were fed until pupation with an artificial diet consisting of wheat, agar, vitamin solution (Bell and Joachim, 1976). For maturation, the pupae were transferred into wooden boxes protected from light. Mature pupae were then transferred to the flight cage. All feeding experiments reported in this study were conducted with larvae of the 5th larval stage (L5). In feeding experiments, each larva was put into a separate plastic cup (see above), where it was fed with transplastomic leaf material or leaves from isogenic, non-engineered controls. Excreted feces of each larva was collected and cooled on ice before DNA extraction. The feeding time and time of first appearance of "leaf-feces", *i.e.*, green colored instead of yellowish feces, were recorded.

The strain *Acinetobacter* BD413 (DSM586), modified with plasmid $pBAB_2$ was also kindly provided by P. Simonet. Details of the strain construction and utilization of the marker-rescue system for the detection of natural transformation have been described elsewhere. (Kay et al., 2002). In addition, the strain *Acinetobacter* BD413 pSM1890, which constitutively expresses the green fluorescent protein, was used for survival studies. This strain was obtained from Søren Molin (Kopenhagen).

Detection of gene transfer from transplastomic leaves to *Acinetobacter* BD413 $pBAB_2$ during a passage through the larval guts

The availability of plant DNA for natural transformation was tested with *Acinetobacter* BD413 $pBAB_2$. The strain was cultured in 50 mL Luria Bertani (LB) medium (in 1000 mL distilled water: Bactotryptone, 5 g, yeast extract, 5 g, and NaCl, 5 g), amended with 50 $mg.L^{-1}$ ampicillin, at 28 °C, 120 rpm overnight (approx. 17 h). To reach the competent state, an aliquot of this culture was diluted 25-fold into fresh LB liquid medium and cultured

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for another 2 h (Palmen et al., 1993). Competent *Acinetobacter* cells were then fed to the larvae, depending on the experiment, as liquid culture or cell pellet, the latter harvested by centrifugation for 40 min at 8000× *g*. Colony forming units (CFU) of liquid cultures and pellets were determined before feeding to larvae. DNA for the transformation assays was obtained from transplastomic tobacco leaves.

In feeding experiments, twelve larvae from the breeding stock were incubated with tobacco leaves for 24 h. A total of 4 larvae were fed with 0.1 g transplastomic leaf material and 0.1 g bacterial cell pellet (*Acinetobacter* BD413 $pBAB_2$). Another 4 larvae received 0.1 g transplastomic leaves and 1 mL liquid culture. For initial cell numbers see Results. During the 24 h of incubation, the feed was completely ingested by the larvae. The larvae were then transferred into clean plastic cups and the feces produced during the following 6 h and after 12 h and 24 h were collected. Each fecal deposition, which corresponded to approx. 0.05 g of fresh weight, was directly suspended in 100 μL saline (0.85% wt/vol NaCl in distilled water). The solid fecal components were then separated by centrifugation for 10 s at 2000 rpm in a table centrifuge (Centrifuge 5417C) and the supernatants were plated as 50 μL aliquots onto LB agar containing ampicillin and spectinomycin (both 50 $\mu g.mL^{-1}$, respectively) for selection of transformants.

In the same experiment the total number of bacterial cells (CFU per g feces) of larvae fed with transplastomic and isogenic tobacco plants, was also determined. Furthermore, the occurrence of natural spectinomycin-resistant bacteria in larval feces was analyzed. Therefore, another four larvae were fed with transplastomic and isogenic tobacco leaves. Feces from those larvae were suspended and plated on LB agar and on LB agar with spectinomycin (50 $\mu g.mL^{-1}$).

Test for *Acinetobacter* BD413 cells surviving a gut passage

Six larvae were fed with the GFP (green fluorescent protein)-tagged strain *Acinetobacter* BD413 pSM1890, which was also resistant to streptomycin, rifampicin and gentamycin, to determine the survival rate of *Acinetobacter* sp. during the gut passage of larvae. The *Acinetobacter* strain was cultivated as described above. Each larva was fed with 0.08 g bacterial cell pellet (fresh weight), corresponding approx. to 1.5×10^9 CFU, smeared onto 1 g isogenic tobacco leaf material. Freshly excreted feces was collected at 1 h intervals over a period of 6 h and suspended in saline. Colony forming units were determined by plating dilutions onto LB containing 50 μg streptomycin.mL^{-1}, 50 μg rifampicin.mL^{-1} and 10 $\mu g.mL^{-1}$ gentamycin, respectively. GFP-tagged colonies were scored under UV light at a wavelength of 360 nm.

Competence of *Acinetobacter* BD413 after a gut passage

Two larvae were each fed with 0.5 g tobacco leaves with 0.1 g (6×10^9 CFU) *Acinetobacter* BD413 $pBAB_2$ cell pellet onto the surface, while two other larvae each received 0.5 g tobacco leaves inoculated with 1 mL (4×10^7 CFU) of an *Acinetobacter* BD413 $pBAB_2$ liquid culture. The excreted green feces (approx. 0.15 g) of each larva were suspended in 500 μL saline. After spinning down the solid fecal particles (10 s at 2000 rpm, Centrifuge 5417C), 100 μL of each supernatant were transferred in a 1.5 mL tube (Eppendorf, Hamburg, Germany) and were 2 μL pCEA plasmid DNA (100 $ng.mL^{-1}$) were added to transform *Acineobacter* BD413 $pBAB_2$ *in vitro*. After incubation for 90 min at 28 °C and 120 rpm, transformants were selected on LB with ampicillin and spectinomycin, both 50 $\mu g.mL^{-1}$. Inoculated plates were incubated for 48 h at 28 °C in the dark.

Extraction of plasmid, plant and fecal DNA

DNA from tobacco leaves was extracted using the Bio101 Fast DNA kit (Qbiogene, Heidelberg, Germany) according to the recommended protocol. *Escherichia coli* DM52, harboring the pCEA plasmid with the *aadA* marker gene and chloroplast sequences, was cultured overnight in LB with 50 μg of spectinomycin.mL^{-1}, before plasmid DNA was extracted by using the Plasmid DNA Purification Kit (Macherey-Nagel, Düren, Germany).

DNA extraction from feces was carried out using the Plant DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions except for one modification: an additional DNA washing step with ethanol (96% vol./vol.) was included. In transformation experiments concentrated feces DNA was used to transform *Acinetobacter* BD413 $pBAB_2$ *in vitro*. Feces was collected from 90 individual larvae. A total of 100 mg feces of each larva was used for DNA extraction as described above and eluted in 50 μL elution buffer (EB) each. Feces DNA from 90 larvae were combined and precipitated. For the precipitation process, the total volume of 4.5 mL DNA solution was divided into eight aliquots. To each aliquot two volumes of polyethylene glycol 6000 (Roth, Karlsruhe, Germany, 30% vol./vol.) in 1.6 M NaCl were added. After a centrifugation step of 14 000 rpm for 30 min at room temperature (Centrifuge 5417C), the supernatants were removed, and 500 μL 4 °C ethanol (70% vol./vol.) were added to each approach. Ethanol was removed after centrifugation (14 000 rpm, 4 min, room temperature) and the precipitate was eluted in 100 μL EB. Solutions of eight precipitates were combined to one sample.

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Leaf, plasmid and feces DNA was quantified with PicoGreen according to the instructions of the manufacturer (PicoGreen dsDNA Quantification Kit, Molecular Probes, Karlsruhe, Germany) and used in the following concentration ranges for *in vitro* transformation experiments: total leaf DNA 0.1 pg to 1000 ng.mL^{-1}, plasmid DNA 0.1 pg to 1000 ng.mL^{-1} and feces DNA 0.1 pg to 10 ng.mL^{-1}. Copy numbers of *aadA* genes of each DNA were determined by real-time PCR (see below). Transformation tests were conducted as mentioned below.

PCR detection of *aadA*-DNA sequences

DNA was extracted as described above from leaf, plasmid and feces DNA and partial *aadA* genes were PCR-amplified using primers FGPaad1172 (5'-ATTCCGTGGCGTTAT-3') and FGPaad1554 (5'-TGACGGGCTGATACT-3') (Kay et al., 2002). The size of the expected PCR product was 382 base pairs (bp) (Bertolla et al., 1999). The PCR master mix contained 0.5 μM of each primer (MWG Biotech, Ebersberg, Germany), each nucleotide as the respective triphosphate at a concentration of 0.2 mM (Qbiogene, Heidelberg, Germany), and 1.25 U of *Taq* polymerase (Hot star *Taq*, Qiagen, Hilden, Germany) with the corresponding 1 X PCR buffer containing 1.25 mM $MgCl_2$. Template DNA was added to a final volume of 25 μL for each PCR. Amplifications were conducted in a Primus96 thermocycler (MWG Biotech). An initial denaturation step at 95 °C for 15 min was followed by 35 cycles of 45 s at 94 °C, 45 s at 58 °C, 1 min at 72 °C and a final extension of 5 min at 72 °C. The PCR products were analyzed for size and yield by electrophoresis in a 1% agarose gel stained with ethidium bromide.

PCR quantification of *aadA* genes using real-time PCR

Transgene quantification in leaves and feces was performed by real-time PCR using the RotorGene 3000 (Corbett Research, LTF, Wasserburg, Germany). For amplification of a 176-bp-long DNA fragment of the *aadA* gene by real-time PCR, primers *aadA*f (5'-TATCCAGCTAAGCGCGAACT-3'), *aadA*r (5'-TCAGGAACCGGATCAAAGAG-3') and a TaqMan probe *aadA*176 (5'-CTTGCAGGTATCTTCGAGCC-3'), FAM (5') and TAMRA (3') labeled, were selected using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

The PCR master mix contained 1.5 μM of the forward primer (*aadA*f), 4.5 μM of the reverse primer (*aadA*r) and 6.25 μM of the probe (*aadA*176). Primers and probe were synthesized by MWG Biotech. Each nucleotide was added at a concentration of 0.2 mM (Qbiogene), and also 1.25 U of *Taq* polymerase (Hot star *Taq*, Qiagen) with the corresponding 1 X PCR buffer containing 1.25 mM $MgCl_2$. Template DNA was added to a final volume of 25 μL for each PCR. The 2-step-PCR started with the initial denaturation at 95 °C for 7 min followed by 45 cycles of 20 s at 95 °C (step 1) and 50 s at 60 °C (step 2). An elongation step of 6 min at 72 °C finished the PCR. Calibration curves were generated by preparing a dilution series of *aadA* PCR product (382-bp) using ultrapure water (Fluka, Sigma Aldrich, Taufkirchen, Germany) in the range of 7.5×10^{-1}–7.5×10^{-6} ng.μL^{-1}. Analysis of data was performed with the RotorGene software (Version 4.6), the number of amplifiable genes from leaf- and feces-derived samples was calculated (Concentration [ng.μL^{-1}] $\times 6.023 \times 10^{23}$ [copies.moL^{-1}] / MW [g.moL^{-1}]). For each analysis, negative controls (no *aadA* genes) were included.

In vitro transformation of *Acinetobacter* BD413 pBAB$_2$ using plasmid, leaf and feces DNA

In vitro transformation experiments were carried out in 1.5 mL tubes (Eppendorf) filled with 100 μL competent cell culture of *Acinetobacter* BD413 pBAB$_2$, corresponding to 4.5×10^7 cells (CFU).mL^{-1}. DNA was added to a final concentration ranging from 0.1 pg to 1000 ng.mL^{-1} depending on the experiment. Tubes were closed with a special lid (Lid$_{BAC}$, Eppendorf) allowing aeration during incubation for 90 min at 30 °C and 120 rpm. After incubation, mixtures were plated on LB agar with 50 μg of ampicillin.mL^{-1} for recipient quantification and on LB agar containing ampicillin and spectinomycin, both 50 μg.mL^{-1}, for the detection of transformants. Colonies were counted after incubation for 48 h at 30 °C. Transformants were additionally controlled for the *aadA* uptake by PCR. DNA was extracted from clones by fast lysis. Each clone was resuspended in 50 μL lysis buffer (0.05 M sodium hydroxide, 0.025% SDS) and incubated for 15 min at 95 °C. After addition of 450 μL dH_2O the sample was mixed, centrifuged for 4 min at 10 000 rpm (Centrifuge 5417C) and used as template for PCR. PCR with *Acinetobacter* BD413 pBAB$_2$ cells not incubated with *aadA*-containing DNA was always negative.

Stability of cell-free transgenic DNA (*aadA*-PCR product) during a gut passage

The stability of cell-free *aadA* marker gene during digestion was investigated by feeding five different transgene concentrations, ranging from 0.01 to 1.5 μg, to ten individual larvae. The transgene was fed as an *aadA*-PCR product in combination with 200 mg artificial food (composition, see above), colored with 1 mL green food coloring (McCormick) before DNA was extracted from feces.

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Transgene amplification, extraction of DNA from feces and transgene detection in feces DNA were conducted as described above.

Transformation of *Acinetobacter* using fresh larval feces

Cultivation of *Acinetobacter* BD413 $pBAB_2$ was conducted as described above. Feces used for transformation originated from *M. sexta* larvae grown up on transplastomic *N. tabacum* until the 5th larval stage. Up to 100 larvae were directly reared on transplastomic tobacco plants. Released feces was divided into three color groups (light green, dark green and black, respectively) and directly stored at 4 °C to a maximum of 3 h before using for transformation experiments. Fecal samples (0.03–0.3 g) of the same color were transferred to 1.5 mL tubes containing 1 mL competent *Acinetobacter* BD413 $pBAB_2$ cells (10^8.mL^{-1}) each. For positive control, 2 μL plasmid DNA (100 ng.mL^{-1}) were added to 100 μL competent cells. Each tube was closed as described above and incubated for 90 min at 28 °C. A centrifugation step of 10 s at 2000 rpm (Centrifuge 5417C) was conducted to collect fecal particles. Aliquots (50 μL) of supernatant were plated onto transformant agar as described above. Plates were incubated at 28 °C for 2 days. Colonies were transferred onto fresh solid media and sub-cultured twice before DNA was extracted by fast lysis. This DNA was used as a template to examine the presence of the *aadA* genes by PCR.

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