Lack of Glyphosate Resistance Gene Transfer from Roundup Ready® Soybean to *Bradyrhizobium japonicum* under Field and Laboratory Conditions

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This paper is dedicated to the memory of the late Professor Dr. Heinrich Sandermann, former director of the Institute of Biochemical Plant Pathology, Helmholtz Zentrum München

A field study was conducted at the Russell E. Larson Agricultural Research Center to determine the effect of transgenic glyphosate-resistant soybean in combination with herbicide (Roundup) application on its endosymbiont *Bradyrhizobium japonicum*. DNA of bacteroids from isolated nodules was analysed for the presence of the transgenic 5-*enol*pyruvylshikimate-3-phosphate synthase (CP4-*EPSPS*) DNA sequence using polymerase chain reaction (PCR). To further assess the likelihood that the *EPSPS* gene may be transferred from the Roundup Ready® (RR) soybean to *B. japonicum,* we have examined the natural transformation efficiency of *B. japonicum* strain 110spc4. Analyses of nodules showed the presence of the transgenic *EPSPS* DNA sequence. In bacteroids that were isolated from nodules of transgenic soybean plants and then cultivated in the presence of glyphosate this sequence could not be detected. This indicates that no stable horizontal gene transfer (HGT) of the *EPSPS* gene had occurred under field conditions. Under laboratory conditions, no natural transformation was detected in *B. japonicum* strain 110spc4 in the presence of various amounts of recombinant plasmid DNA. Our results indicate that no natural competence state exists in *B. japonicum* 110spc4. Results from field and laboratory studies indicate the lack of functional transfer of the CP4-*EPSPS* gene from glyphosate-tolerant soybean treated with glyphosate to root-associated *B. japonicum*.

Key words: Bradyrhizobium japonicum, Glyphosate, Horizontal Gene Transfer

Introduction

The enormous success of agricultural gene technology has raised concern about the spread of novel traits into the environment. In 2010, more than 148 million hectares worldwide were planted with crops possessing biotechnologically derived traits (http://www.transgen.de/anbau/eu_international/). Herbicide tolerance is the most frequent trait found in genetically modified (GM) plants (80% of all GM crops planted worldwide; http://www.fourwinds10.com/siterun_data/science_technology/dna_gmo/news.php?q=1235154563). However, the increased use of transgenic plants in addition to herbicide selection pressure on the fields may facilitate a possible horizontal gene flow (Sandermann *et al.*, 1997; Wagner *et al.*, 2008) and cause the selection of spontaneous herbicide-resistant microorganisms.

Horizontal gene transfer (HGT) requires the bacterial cell to possess the ability to actively take

up and heritably integrate extracellular DNA into its genome, *i.e.*, to have the competence for natural transformation (Johnsborg *et al.*, 2007). More than 40 prokaryotic species, including many soil and rhizosphere bacteria, have been described that undergo natural transformation (Lorenz and Wackernagel, 1994). However, it has been demonstrated that competent bacteria are able to integrate transgenic plant DNA only if homologous bacterial DNA sequences are present in the plant genome (de Vries and Wackernagel, 2002). Laboratory studies have shown that gene transfer from plants to bacterial strains harbouring the homologous gene, inactivated by a short deletion, facilitates the selection of recombinant bacterial clones (Gebhard and Smalla, 1998; Nielsen *et al.*, 2001; de Vries and Wackernagel, 2002; Kay *et al.*, 2002). Under natural conditions, theoretical calculations predicted a very low transformation frequency ($2 \cdot 10^{-17}$, 1 transformant per $2 \cdot 10^{17}$ bacteria) (Schlüter *et al.*, 1995); nevertheless, it is presumed that selection pressure can increase the probability for a successful establishment of HGT (Nielsen *et al.*, 1998). Until now the cultivation of GM plants in the field has not revealed any evidence that transgenes have been transferred to bacteria (Gebhard and Smalla, 1998; Paget *et al.*, 1998; Badosa *et al.*, 2004; Demanèche *et al.*, 2008; Wagner *et al.*, 2008), although they were detected in the soil food web (Hart *et al.*, 2009).

Roundup Ready® (RR) soybean is currently the most widely used GM crop, which was genetically modified to tolerate glyphosate [(*N*-(phosphonomethyl)glycine]-containing herbicides, and was responsible in 2010 for about 70% of the worldwide soybean production (http://www.transgen.de/anbau/eu_international/). Furthermore, glyphosate is the most widely used herbicide in the world (Yu *et al.*, 2007), largely due to the increasing popularity of RR crops.

Glyphosate is a foliar-applied, broad-spectrum, non-selective herbicide that is symplastically translocated to the meristems of growing plants and controls a wide variety of weeds (Rubin *et al.*, 1982; Zablotowicz and Reddy, 2004). Glyphosate blocks the shikimate pathway through the inhibition of 5-*enol*pyruvylshikimate-3-phosphate synthase (EPSPS) (Amrhein *et al.*, 1980). Because the shikimate pathway is present in plants, bacteria, and fungi but absent in animals, EPSPS has been an attractive target for effective herbicide development (Coggins *et al.*, 2003; Priestman *et*

al., 2005). Inhibition of EPSPS prevents the plant from synthesizing aromatic amino acids, causes shikimate accumulation, and consequently kills plants.

The basis for resistance to glyphosate in RR soybeans is the insertion of a transgene that originates from the *Agrobacterium* strain CP4 (CP4-*EPSPS*) and specifies a glyphosate-insensitive EPSPS variant (Padgette *et al.*, 1995). Molecular characterization studies determined that the genetic insertion in the RR soybean line 40-3-2 contains part of the 35S promoter of the *Cauliflower mosaic* virus (35S-CaMV), sequences of the *Petunia hybrida EPSPS* chloroplast transit peptide (CTP), the CP4-*EPSPS* gene, and an intact 3' non-translated region of the nopaline synthase gene (*NOS 3'*) (Padgette *et al.*, 1995).

The promoter from the 35S-CaMV gene is a strong constitutive promoter that is most widely used for the expression of transgenes in plants (Schnurr and Guerra, 2000). The efficient expression of an eukaryotic gene, transferred to a bacterial organism, requires a prokaryotic promoter (Jacob *et al.*, 2004). This implies that plant transgenes transferred via HGT from plants to bacteria would not be expressed in prokaryotes because plant-specific regulatory sequences are associated with the transgene. However, the 35S-CaMV promoter includes prokaryotic promoter-like recognition sequences, which allow for direct gene expression in *Escherichia coli* (Assaad and Signer, 1990) and several other bacteria (Lewin *et al.*, 1998; Jacob *et al.*, 2004).

Studies addressing the molecular basis for glyphosate resistance demonstrated that a single amino acid exchange in the active site renders CP4-EPSPS insensitive to glyphosate (Padgette *et al.*, 1991; Selvapandiyan *et al.*, 1995). The continued presence of glyphosate is likely to favour mutations that reduce glyphosate sensitivity while still maintaining catalytic efficiency. Therefore, it is not surprising that the gene coding for CP4-EPSPS was isolated from a bacterium found in an extremely glyphosate-rich environment (Funke *et al.*, 2006). This suggests that under glyphosate selection pressure, glyphosate tolerance could be achieved through spontaneous mutation or via HGT within bacterial communities.

DNA sequence alignments of *EPSPS* genes from the *Agrobacterium tumefaciens* strain CP4 and *Bradyrhizobium japonicum* revealed high homologies in many regions (Wagner *et al.*, 2008).

The probability of HGT from RR soybean to bacteria was assessed under natural field conditions, choosing the nitrogen-fixing soybean symbiont *B. japonicum* as a DNA receptor (Wagner *et al.*, 2008). Because glyphosate inhibits the growth of this symbiotic bacterium by the inhibition of aromatic acid amino biosynthesis (Jaworski, 1972; Zablotowicz and Reddy, 2004), this system provides optimal conditions for monitoring the HGT of CP4-*EPSPS* from transgenic soybeans to *B. japonicum*.

Material and Methods

Field site and plant material

In 2001, a field study of transgenic glyphosate-tolerant soybeans carrying the CP4-*EPSPS* gene was carried out at Russell E. Larson Agricultural Research Center, University Park, USA. The field size was 1276.35 m² (38.1 m x 33.5 m) with about 47,350 plants. Seeds were sown in 95 rows with a distance of 38 cm between the rows. The transgenic soybean line was Agway 364RR (Agway Farm Seeds, Tully, NY, USA). In addition, 10 rows were cultivated with a conventional soybean lacking the glyphosate-tolerant trait, Pioneer P93B-81STS (Pioneer, Johnston, IA, USA). Roundup was applied once before sowing and once during the growth period at a concentration of 560 g active material ha⁻¹. This was the third year of RR soybean monoculture on this field. The sampling scheme was as follows: from each third row, 10 nodule samples were taken. Each sample consisted of nodules from 2–3 plants. There were 320 samples in total. Samples were stored at +8 °C. In addition, 60 transgenic plants were randomly collected, and roots, including the associated nodules, were stored at +8 °C. Transgenic RR soybean (GTS 40-3-2) seeds expressing the *EPSPS* gene derived from *Agrobacterium* sp. strain CP4 were provided by Monsanto Europe (Brussels, Belgium).

Re-isolation and cultivation of B. japonicum bacteroids

Nodules were macerated in 5 ml of liquid medium nr. 0321, containing 0.2 M mannitol, and dilutions of the macerate were plated on agar medium nr. 0321, containing 0.2 M mannitol and 1 mM glyphosate (Werner, 1982). Visible colonies were detected after 9 to 12 d of growth at 28 °C, and 200 of them were further analysed.

DNA isolation

DNA was extracted from soybean leaves, nodules, and bacteroids according to Chen and Ronald (1999), and the final pellet was dissolved in 20–50 μl H₂O. For the isolation of DNA fragments of the EPSPS construct total genomic DNA was extracted from 100 mg of fresh leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

Polymerase chain reaction (PCR) amplification of selected DNA sequences

Gene-specific primers (RR01, RR02) for the *EPSPS* gene resulted in the amplification of a 509-bp fragment (Köppel *et al.*, 1997). As an internal control, soybean-specific lectin primers (GM01, GM02) were used that resulted in a fragment of 413 bp (Köppel *et al.*, 1997). To identify *B. japonicum*, specific primers for repetitive intergenic consensus sequences were used (Judd *et al.*, 1993). PCR amplifications were carried out according to standard protocols, and PCR products were run on 1.5% agarose gels, containing ethidium bromide (0.1 μg ml⁻¹).

PCR amplification of the EPSPS construct from RR soybean

DNA fragments of the *EPSPS* construct for cloning and sequencing were generated by PCR (AccuPrime™ *Taq* DNA Polymerase High Fidelity Kit; Invitrogen, Darmstadt, Germany). Gene-specific primers were as follows: forward, TGGAAAAGGAAGGTGGCTC; reverse, GGGATCGATCCCCGATCT (AccNr. AB209952). The optimized PCR program for the *EPSPS* construct was performed as follows: 94 °C for 3 min, 33 cycles at 94 °C for 30 s, 58 °C for 45 s, 68 °C for 2 min 20 s; final elongation at 68 °C for 10 min.

Cultivation of E. coli and of B. japonicum

One Shot® TOP10F' electrocompetent *E. coli* (Table I) (Invitrogen) was used as a host in the standard cloning procedures, and *E. coli* S17-1 (Simon *et al.*, 1983) served as a donor in the conjugative plasmid transfer. *E. coli* was cultivated at 37 °C on LB agar plates or in liquid cultures according to standard methods (Sambrook *et al.*, 1989).

The *B. japonicum* strain 110spc4 was used in this study (Table I). PSY medium (Regensburger

Table I. Bacterial strains and plasmids used.

Strain or plasmid	Relevant genotype or description	Source or reference
E. coli K12 strain One Shot® TOP10F' electrocompetent *E. coli*	Strain used for general cloning F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
E. coli K12 strain S17-1	Mobilizing donor strain, *thi pro recA hsdR hsdM* RP4-2-Tc::Mu-Km::Tn7	Simon *et al.* (1983)
B. japonicum 110*spc*4	110 derivate, *Spc*^{r}	Acuña *et al.* (1987)
pCR®2.1-TOPO®	Cloning vector, *Amp*^{r}, *Kan*^{r}	Invitrogen
pT-35S-EPSPS	*EPSPS* from transgenic soybean (GTS 40-3-2) in pCR®2.1.-TOPO®	This work
pRJ1042	*Tc*^{r} *Km*^{r} *Bj-(D-lacZ)* in prJ1035	Acuña *et al.* (1987)
pRJ-35S-EPSPS	*EPSPS* from transgenic soybean (GTS 40-3-2) in pRJ1042	This work

and Hennecke, 1983) supplemented with 0.1% (w/v) arabinose (Narberhaus *et al.*, 1998) was used for natural transformation experiments. YEX medium (Adams *et al.*, 1984) was employed for electroporation experiments. For selection of the *B. japonicum* strain 110spc4 spectinomycin (100 μg ml^{-1}) and chloramphenicol (20 μg ml^{-1}) were added to the media (Loh *et al.*, 2002).

B. japonicum electrocompetent cells

B. japonicum 110spc4 cells were prepared for electroporation using modifications of two protocols (Guerinot *et al.*, 1990; Hattermann and Stacey, 1990). Cell cultures (500 ml) were grown to an optical density (OD$_{600}$) of 0.4 to 0.6, then placed on ice, and harvested by centrifugation. The resulting pellet was washed with 500 ml distilled water, 250 ml distilled water, and 10 ml of sterile 10% glycerol. Cells were resuspended in 1 ml of 10% glycerol, frozen in liquid nitrogen, and stored at –80 °C.

E. coli transformation

Bacterial transformation was carried out by electroporation using a Gene Pulser (Bio Rad, München, Germany). For electroporation, *E. coli* DH5α electrocompetent cells (Invitrogen) and *E. coli* S17-1 (Simon *et al.*, 1983) were thawed on ice and mixed with plasmid DNA (10–100 ng).

Electroporation of *B. japonicum*

For electroporation, a Gene Pulser (Bio Rad) was used. The plasmid pRJ1042 was used for the *B. japonicum* 110spc4 electroporation. Following DNA concentrations were tested: 10 ng ml^{-1}, 125 ng ml^{-1}, 250 ng ml^{-1}, 500 ng ml^{-1}, 1 μg ml^{-1}.

Electrocompetent cells were thawed on ice, and 1–2 μl plasmid DNA were then mixed thoroughly with 40 μl of a cell suspension, placed on ice for 1 min, and transferred to a chilled cuvette. A 2.5-kV pulse with a capacity of 25 μF and resistance of either 200 Ω or 400 Ω was applied. Cells were then suspended in 1 ml of YEX or PSY medium and incubated on a horizontal shaker at 30 °C for 20 h. Dilutions were plated on selective and non-selective media. Controls consisted of cells mixed with plasmid DNA, which were not subjected to a pulse prior to incubation in the liquid medium.

Cloning EPSPS constructs into TA Cloning® system vectors in *E. coli* and into the *B. japonicum* integration vector pRJ1042

Amplified *35S-EPSPS* products were ligated, according to the manufacturer's instructions, to the pCR® 2.1.-TOPO® vector (Invitrogen). The ligation products were transformed into One Shot® TOP10F' electrocompetent *E. coli* cells (Table I).

The *35S-EPSPS* cassette, including the 35S-CaMV promoter, was excised from the vector pT-35S-EPSPS by digestion with *Eco*RI and integrated into the same restriction site of the vector pRJ1042 (Acuña *et al.*, 1987).

B. japonicum natural transformation on agar plates and in liquid cultures

The development of a natural competence state in *B. japonicum* 110spc4 was tested on agar plates according to the method used by Lorenz and Wackernagel (1991) to monitor natural transformation, as well as under liquid medium conditions (Demanèche *et al.*, 2001). Experiments were performed on PSY medium. Purified plasmid

DNA (pRJ-35S-EPSPS) was added to exponentially grown *B. japonicum* cultures (OD_{600} = 1) at different concentrations (10 ng ml^{-1}, 100 ng ml^{-1}, 250 ng ml^{-1}, 500 ng ml^{-1}, and 1 μg ml^{-1}). Samples with plasmid DNA at equivalent concentrations were mixed with cultures for plate and liquid transformations, respectively. For plate transformations, 15 μl of *B. japonicum* suspensions mixed with plasmid DNA were spotted on PSY agar. In parallel, 10-fold concentrated mixtures were also spotted. After incubation for 48 h or 62 h at 30 °C, spots were re-suspended in 1 ml of PSY medium and plated on PSY medium containing 100 μg ml^{-1} kanamycin for the selection of clones harbouring the pRJ-35S-EPSPS vector. For the *B. japonicum* transformations, cells were grown in liquid cultures (200 rpm, 30 °C) at different stages of the exponential growth phase (OD_{600} = 0.5, 0.65, and 0.8). Aliquots of 100 μl were plated at 12-h intervals onto selective plates, which were then incubated at 30 °C. All experiments were done in triplicate.

Results

Field studies

PCR amplification of DNA from *B. japonicum* revealed discrete amplified DNA sequences indicating the presence of *B. japonicum* in nodules isolated from this field trial (Fig. 1a). The same probes were used for the amplification of the soybean *lectin* gene and the transgenic *EPSPS* gene. As shown in Fig. 1b and c, PCR products were of the expected sizes; 413 bp for *lectin* DNA and 509 bp for the *EPSPS* sequence. The presence of these genes was expected because the nodule structure contains both plant and bacterial genes. Isolated bacteroids were then cultivated over two passages in the presence of 1 mM glyphosate. This herbicide concentration resulted in a selection pressure, still allowing bacterial growth (Wagner *et al.*, 2008). *B. japonicum*-specific sequences could be confirmed (Fig. 1 d). However, no *EPSPS* PCR amplification product could be found in any of the 200 cultivated bacteroid samples analysed.

Cloning the 35S-EPSPS construct into the B. japonicum integration vector pRJ1042

The *35S-EPSPS* cassette was excised from the vector pT-35S-EPSPS by digestion with *Eco*RI and integrated into the vector pRJ1042. The *35S-EPSPS* construct possesses an *Eco*RI site between the *EPSPS* gene and the *NOS* terminator. This resulted in loss of the *NOS* terminator sequence (296 bp). The NOS terminator sequence
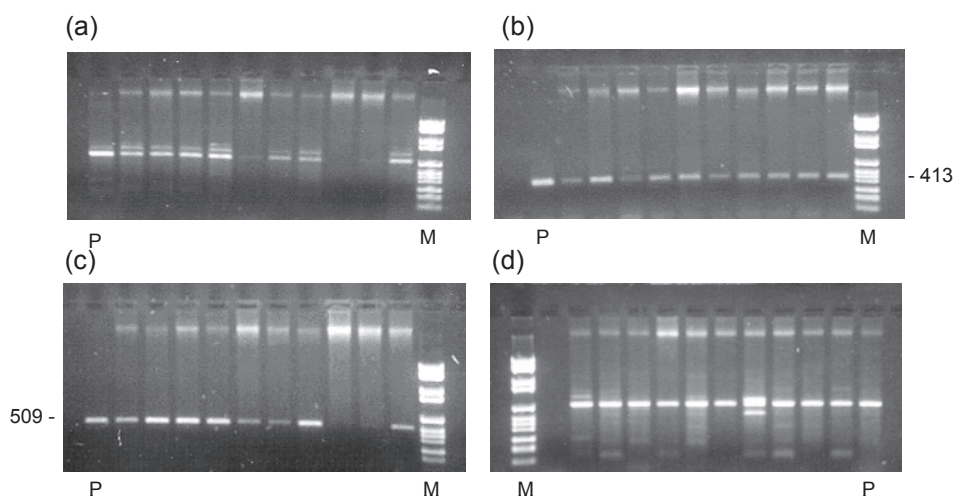

Fig. 1. (a) Identification of *B. japonicum* in nodules of transgenic soybean; P, positive control (cultivated *B. japonicum*); M, bp marker. (b) Detection of the lectin gene in nodules of transgenic soybean. PCR was carried out with lectin-specific primers that resulted in a 413-bp product; P, positive control (soybean leaf DNA); M, bp marker. (c) Detection of the CP4-*EPSPS* gene in nodules of transgenic soybean. PCR was carried out with *EPSPS* and 35S-CaMV promoter-specific primers that resulted in a 509-bp product; P, positive control (RR soybean leaf DNA); M, bp marker. (d) Identification of *B. japonicum* in cultivated bacteroids. P, positive control (*B. japonicum* strain); M, bp marker.

was therefore absent in the cloning procedures. Translation of the *35S-EPSPS* construct lacking the *NOS* terminator region was expected to finish at two stop codons at the 3'-end of the *EPSPS* gene, allowing for the correct protein expression in bacteria without the requirement of an additional terminator sequence.

Ligation of the *35S-EPSPS* cassette in the vector pRJ1042 (14.7 kb) yielded the vector pRJ-35S-EPSPS, which was transformed into *E. coli* donor strain S17-1. Plasmids from 20 transformants were isolated, and the integration of the *35S-EPSPS* (1,920 bp) construct into pRJ1042 (14.7 kb) was verified by enzymatic digestion, PCR amplification of the insert, and DNA sequencing.

B. japonicum transformation by electroporation

Attempts to transform *B. japonicum* 110spc4 cells by electroporation were carried out, including modifications of the described procedures (Guerinot *et al.*, 1990; Hattermann and Stacey, 1990). However, under the tested conditions, no positive transformants carrying the pRJ-35S-EPSPS plasmid insertion were obtained.

B. japonicum natural transformation

For plate transformation, mixtures of a bacterial culture and plasmid DNA were spotted on non-selective agar plates and incubated for 48 h or 62 h under standard conditions. After resuspension of the growing colonies and further incubation under kanamycin-selective pressure, 100 colonies from each experiment were analysed by PCR. However, no *35S-EPSPS* insert was found. The development of a natural competence state under liquid conditions was assessed by adding plasmid DNA to *B. japonicum* 110spc4 shaking cultures at different time points of the exponential growth phase. Aliquots of the bacterial suspension were then plated on kanamycin-selective plates. Antibiotic-resistant colonies were screened by PCR for the *35S-EPSPS* insertion. However, no PCR amplification product was found. All evaluated colonies were spontaneously resistant to kanamycin. Under the tested agar and liquid conditions, no natural transformation was detected in *B. japonicum* strain 110spc4 in the presence of various excess amounts of recombinant plasmid DNA.

Discussion

Assessment of HGT under field conditions

Bacteria that have developed intricate symbiotic or pathogenic relationships with plants are theoretically exposed to the best conditions for gene transfer in nature (Kay *et al.*, 2002). Thus, *B. japonicum*, as a symbiont of soybean and carrier of an EPSPS enzyme sensitive to glyphosate, could be an ideal candidate to monitor the uptake of the *EPSPS* gene from RR soybean DNA under glyphosate selection pressure in the nodule (Zablotowicz and Reddy, 2004). The mechanism by which such HGT could occur is likely to be related to the natural transformation of bacteria by DNA released from plants (Lorenz and Wackernagel, 1994).

The gene transfer from RR soybean to *B. japonicum* was evaluated under natural conditions and selection pressure to monitor for the likelihood of HGT. Roundup was applied according to agricultural practice over a period of three years. In a lysimeter study, the glyphosate content in the nodules reached about 63 μg g^{-1} dry weight under normal glyphosate application doses (Grundmann *et al.*, 2008), whereas in another study 39 – 147 ng g^{-1} dry weight were detected (Reddy and Zablotowicz, 2003).

The nodule analysis documented, as expected, the presence of the transgenic CP4-*EPSPS* sequence. However, in bacteroids that were isolated from nodules and cultivated in the presence of glyphosate, this *EPSPS* gene could not be detected. This argues against the HGT transfer of the whole *EPSPS* gene under field conditions from transgenic soybeans to *B. japonicum* bacteria. These results are in agreement with a previous lysimeter study, also using glyphosate-tolerant soybeans and *B. japonicum* (Wagner *et al.*, 2008), and with other reports on the field cultivation of GM plants lacking evidence of transgene transfer to bacteria (Paget *et al.*, 1998; Gebhard and Smalla, 1998; Badosa *et al.*, 2004; Demanèche *et al.*, 2008; EFSA Statement, 2009). However, the methods used for the detection of the CP4-*EPSPS* gene might have been not sensitive enough, as about 1,000 copies of the gene were necessary to obtain an amplification product.

Assessment of HGT under laboratory conditions

The two main barriers of HGT from plant to microorganisms are the non-competent status of recipient cells and lack of sequence homology required for homologous recombination (Nielsen *et al.*, 1998). Accordingly, in this study, attempts to enhance HGT to *B. japonicum* 110spc4 under controlled laboratory conditions included the optimization of sequence homology between DNA molecules to be recombined and evaluation of optimal conditions for natural transformation.

Among the enormous diversity in the bacterial kingdom, more than 40 species from different environments are known so far to be naturally transformable (Lorenz and Wackernagel, 1994; Nielsen *et al.*, 1998; de Vries *et al.*, 2001). Because nearly every transformable organism has its own specific set of conditions that induces competence (Lorenz and Wackernagel, 1994), several parameters were varied to find the optimal conditions for the potential natural transformation of *B. japonicum* 110spc4. The plasmid used in this study for *B. japonicum* transformations carried a non-essential DNA region of the *B. japonicum* genome that constituted the recombination site of the plasmid into the chromosome (Acuña *et al.*, 1987). Previous natural transformation experiments were performed with engineered plasmids in the highly natural transformable bacterium *Acinetobacter* sp. BD413, containing a partially deleted antibiotic resistance gene that was completed after a successful transformation (Chamier *et al.*, 1993; Nielsen *et al.*, 1997; Gebhard and Smalla, 1998; de Vries and Wackernagel, 2002). This system has been already extended to other soil bacterial species like *Erwinia chrysanthemi* (Schlüter *et al.*, 1995) and *Pseudomonas stutzeri* (de Vries *et al.*, 2001). Additionally, *Acinetobacter baylyi* has been transformed with DNA from transgenic plants, using the same antibiotic recombination system under controlled laboratory conditions, although transformation frequencies with plant DNA have been shown to be drastically reduced compared to transformation with recombinant plasmid DNA (Gebhard and Smalla, 1998; de Vries and Wackernagel, 2002).

HGT requires the genomic integration of the transferred gene to achieve high genetic stability without the need for selective pressure (de Vries *et al.*, 2003). Chromosomal integration can then be monitored by antibiotic selection. Therefore, we used an integration vector designed for the chromosomal homologous recombination of foreign DNA into the *B. japonicum* genome (Acuña *et al.*, 1987). However, under all conditions tested, no natural transformation events were observed in *B. japonicum* strain 110spc4, indicating lack of functional transfer of the gene. This is in contrast to the transformation of *B. japonicum* strain 211 and might be traced back to a different competence of various strains of *B. japonicum* (Marečková, 1969).

Naturally transformable bacteria are able to integrate homologous DNA. However, when they were transformed with plant DNA, the transformation frequency dropped below the detection limit. One of those bacteria is *A. baylyi*, which is a model organism for gene-transfer studies (Metzgar *et al.*, 2004) and has the highest natural transformation frequency found *in vitro* with homologous chromosomal DNA (10^{-2} transformants per recipient) (Nielsen *et al.*, 1997; Palmen and Hellingwerf, 1997). The second most transformable bacterium is *Ralstonia solanacearum*, a bacterium able to develop natural competence *in vitro*, which was transformed with recombinant plasmid DNA but at a much lower transformation rate (10^{-7}) (Bertolla *et al.*, 1997). In both cases, when *Acinetobacter* or *Ralstonia* were incubated with non-homologous DNA, the transformation rate was much lower than with recombinant plasmid DNA and at times was even undetectable (Gebhard and Smalla, 1998; Bertolla *et al.*, 2000; de Vries and Wackernagel, 2002). Since *B. japonicum* strain 110spc4 is not able to undergo natural transformation with homologous DNA as found in this study, then the likelihood of the integration of heterologous DNA is certainly even lower. However, one has to bear in mind that there are many problems in demonstrating HGT from transgenic plants into bacteria (Heinemann and Traavik, 2004; Nielsen and Townsend, 2004), and thus the number of bacteroids analysed in this study might have be too small to detect a HGT. In addition, if naturally occurring resistances are present in the field, such as point mutations in the *EPSPS* gene, no selection pressure and specific selective advantage for these bacteria would exist, as reported recently for antibiotic-resistant soil bacteria in transgenic plant fields (Demanèche *et al.*, 2008)

Acuña G., Alvarez-Morales A., Hahn M., and Hennecke H. (1987), A vector for the site-directed, genomic integration of foreign DNA into soybean root-nodule bacteria. Plant Mol. Biol. **9**, 41–50.

Adams T. H., McClung C. R., and Chelm B. K. (1984), Physical organization of the *Bradyrhizobium japonicum* nitrogenase gene region. J. Bacteriol. **159**, 857–862.

Amrhein N., Deus B., Gehrke P., and Seinrücken H. C. (1980), The site of the inhibition of the shikimate pathway by glyphosate. II. Interference of glyphosate with chorismate formation *in vivo* and *in vitro*. Plant Physiol. **66**, 830–834.

Assaad F. F. and Signer E. R. (1990), Cauliflower mosaic virus P35S promoter activity in *Escherichia coli*. Mol. Gen. Genet. **223**, 517–520.

Badosa E., Moreno C., and Montesinos E. (2004), Lack of detection of ampicillin resistance gene transfer from Bt176 transgenic corn to culturable bacteria under field conditions. FEMS Microbiol. Ecol. **48**, 169–178.

Bertolla F., Van Gijsegem F., Nesme X., and Simonet P. (1997), Conditions for natural transformation of *Ralstonia solanacearum*. Appl. Environ. Microbiol. **63**, 4965–4968.

Bertolla F., Pepin R., Passelegue-Robe E., Paget E., Simkin A., Nesme X., and Simonet P. (2000), Plant genome complexity may be a factor limiting *in situ* the transfer of transgenic plant genes to the phytopathogen *Ralstonia solanacearum*. Appl. Environ. Microbiol. **66**, 4161–4167.

Chamier B., Lorenz M. G., and Wackernagel W. (1993), Natural transformation of *Acinetobacter calcoaceticus* by plasmid DNA adsorbed on sand and groundwater aquifer material. Appl. Environ. Microbiol. **59**, 1662–1667.

Chen D.-H. and Ronald P. C. (1999), A rapid DNA minipreparation method suitable for AFLP and other PCR applications. Plant Mol. Biol. Rep. **17**, 53–57.

Coggins J. R., Abell C., Evans L. B., Frederickson M., Robinson D. A., Roszak A. W., and Lapthorn A. P. (2003), Experiences with the shikimate-pathway enzymes as targets for rational drug design. Biochem. Soc. Trans. **31**, 548–552.

Demanèche S., Kay E., Gourbière F., and Simonet P. (2001), Natural transformation of *Pseudomonas fluorescens* and *Agrobacterium tumefaciens* in soil. Appl. Environ. Microbiol. **67**, 2617–2621.

Demanèche S., Sanguin H., Poté J., Navarro E., Bernillon D., Mavingui P., Wildi W., Vogel T. M., and Simonet P. (2008), Antibiotic-resistant soil bacteria in transgenic plant fields. Proc. Natl. Acad. Sci. USA **105**, 3957–3962.

de Vries J. and Wackernagel W. (2002), Integration of foreign DNA during natural transformation of *Acinetobacter* sp. by homology-facilitated illegitimate recombination. Proc. Natl. Acad. Sci. USA **99**, 2094–2099.

de Vries J., Meier P., and Wackernagel W. (2001), The natural transformation of the soil bacteria *Pseudomonas stutzeri* and *Acinetobacter* sp. by transgenic plant DNA strictly depends on homologous sequences in the recipient cells. FEMS Microbiol. Lett. **195**, 211–215.

de Vries J., Heine M., Harms K., and Wackernagel W. (2003), Spread of recombinant DNA by roots and pollen of transgenic potato plants, identified by highly specific biomonitoring using natural transformation of an *Acinetobacter* sp. Appl. Environ. Microbiol. **69**, 4455–4462.

EFSA Statement (2009), Use of antibiotic resistance genes as marker genes in GM plants – EFSA-Q-2009–00589. EFSA J. **1034**, 1–82.

Funke T., Han H., Healy-Fried M. L., Fischer M., and Schönbrunn E. (2006), Molecular basis for the herbicide resistance of Roundup Ready crops. Proc. Natl. Acad. Sci. USA **103**, 13010–13015.

Gebhard F. and Smalla K. (1998), Transformation of *Acinetobacter* sp. strain BD413 by transgenic sugar beet DNA. Appl. Environ. Microbiol. **64**, 1550–1554.

Grundmann S., Dörfler U., Ruth B., Loos C., Wagner T., Karl H., Munch J. C., and Schroll R. (2008), Mineralization and transfer processes of ^{14}C-labeled pesticides in outdoor lysimeters. Water Air Soil Pollut. Focus **8**, 177–185.

Guerinot M. L., Morisseau B. A., and Klapatch T. (1990), Electroporation of *Bradyrhizobium japonicum*. Mol. Gen. Genet. **221**, 287–290.

Hart M. M., Powell J. R., Gulden R. H., Levy-Booth D. J., Dunfield K. I., Pauls K. P., Swanton C. J., Klironomos J. N., and Trevors J. T. (2009), Detection of transgenic cp4 epsps genes in the soil web. Agron. Sustain. Dev. **29**, 497–501.

Hattermann D. R. and Stacey G. (1990), Efficient DNA transformation of *Bradyrhizobium japonicum* by electroporation. Appl. Environ. Microbiol. **56**, 833–836.

Heinemann J. A. and Traavik T. (2004), Problems in monitoring horizontal gene transfer in field trials of transgenic plants. Nat. Biotechnol. **22**, 1105–1109.

Jaworski E. G. (1972), Mode of action of *N*-phosphonomethylglycine: Inhibition of aromatic amino acid biosynthesis. J. Agric. Food Chem. **20**, 1195–1198.

Jacob D., Lewin A., Meister B., and Appel B. (2004), Plant-specific promoter sequences carry elements that are recognised by the eubacterial transcription machinery. Transgenic Res. **11**, 291–303.

Johnsborg O., Eldholm V., and Håvarstein L. S. (2007), Natural genetic transformation: prevalence, mechanisms and function. Res. Microbiol. **158**, 767–778.

Judd A. K., Schneider M., Sadowsky M. J., and de Bruijn F. J. (1993), Use of repetitive sequences and polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. Appl. Environ. Microbiol. **59**, 1702–1708.

Kay E., Vogel T., Bertolla F., Nalin R., and Simonet P. (2002), *In situ* transfer of antibiotic resistance genes from transgenic (transplastomic) tobacco plants to bacteria. Appl. Environ. Microbiol. **68**, 3345–3351.

Köppel E., Stadler M., Lüthy J., and Hübner P. (1997), Sensitive Nachweismethode für die gentechnisch veränderte Sojabohne <Roundup Ready™>. Mitt. Geb. Lebensmittelunters. Hyg. **88**, 164–175.

Lewin A., Jacob D., Freytag B., and Appel B. (1998), Gene expression in bacteria directed by plant-specific sequences. Transgenic Res. **7**, 403–411.

Loh J., Lohar D. P., Andersen B., and Stacey G. (2002), A two-component regulator mediates population-density-dependent expression of *Bradyrhizobium japonicum* nodulation genes. J. Bacteriol. **184**, 1759–1766.

Lorenz M. G. and Wackernagel W. (1991), High frequency of natural genetic transformation of *Pseudomonas stutzeri* in soil extract supplemented with a carbon/energy and phosphorus source. Appl. Environ. Microbiol. **57**, 1246–1251.

Lorenz M. G. and Wackernagel W. (1994), Bacterial gene transfer by natural genetic transformation in the environment. Microbiol. Mol. Biol. Rev. **58**, 563–602.

Marečková H. (1969), Transformation of *Rhizobium japonicum*. Arch. Microbiol. **68**, 113–115.

Metzgar D., Bacher J. M., Pezo V., Reader J., Döring V., Schimmel P., Marlière P., and de Crécy-Lagard V. (2004), *Acinetobacter* sp. ADP1: an ideal model organism for genetic analysis and genome engineering. Nucleic Acids Res. **32**, 5780–5790.

Narberhaus F., Kowarik M., Beck C., and Hennecke H. (1998), Promoter selectivity of the *Bradyrhizobium japonicum* RpoH transcription factors *in vivo* and *in vitro*. J. Bacteriol. **180**, 2395–2401.

Nielsen K. M. and Townsend J. P. (2004), Monitoring and modelling horizontal gene transfer. Nat. Biotechnol. **22**, 1110–1114.

Nielsen K. M., Gebhard F., Smalla K., Bones A. M., and van Elsas J. D. (1997), Evaluation of possible horizontal gene transfer from transgenic plants to the soil bacterium *Acinetobacter calcoaceticus* BD413. Theor. Appl. Genet. **95**, 815–821.

Nielsen K. M., Bones A. M., Smalla K., and van Elsas J. D. (1998), Horizontal gene transfer from transgenic plants to terrestrial bacteria – a rare event? FEMS Microbiol. Rev. **22**, 79–103.

Nielsen K. M., van Elsas J. D., and Smalla K. (2001), Dynamics, horizontal transfer and selection of novel DNA in bacterial populations in the phytosphere of transgenic plants. Ann. Microbiol. **51**, 79–94.

Padgette S. R., Re D. B., Gasser C. S., Eichholtz D. A., Frazier R. B., Hironaka C. M., Levine E. B., Shah D. M., Fraley R. T., and Kishore G. M. (1991), Site-directed mutagenesis of a conserved region of the 5-enolpyruvylshikimate-3-phosphate synthase active site. J. Biol. Chem. **266**, 22364–22369.

Padgette S. R., Kolacz K. H., Delannay X., Re D. B., La Valle B. J., Tinius C. N., Rhodes W. K., Otero Y. I., Barry G. F., Eichholtz D. A., Peschke V. M., Nida D. L., Taylor N. B., and Kishore G. M. (1995), Development, identification, and characterization of a glyphosate-tolerant soybean line. Crop Sci. **35**, 1451–1461.

Paget E., Lebrun M., Freyssinet G., and Simonet P. (1998), The fate if recombinant plant DNA in soil. Eur. J. Soil Biol. **34**, 81–88.

Palmen R. and Hellingwerf K. J. (1997), Uptake and processing of DNA by *Acinetobacter calcoaceticus* – a review. Gene **192**, 179–190.

Priestman M. A., Healy M. L., Becker A., Alberg D. G., Bartlett P. A., Lushington G. H., and Schönbrunn E. (2005), Interaction of phosphonate analogues of the tetrahedral reaction intermediate with 5-enolpyruvylshikimate-3-phosphate synthase in atomic detail. Biochemistry **44**, 3241–3248.

Reddy K. N. and Zablotowicz R. M. (2003), Glyphosate-resistant soybean response to various salts of glyphosate and glyphosate accumulation in soybean nodules. Weed Sci. **51**, 496–502.

Regensburger B. and Hennecke H. (1983), RNA polymerase from *Rhizobium japonicum*. Arch. Microbiol. **135**, 103–109.

Rubin J. L., Gaines C. G., and Jensen R. A. (1982), Enzymological basis for herbicidal action of glyphosate. Plant Physiol. **70**, 833–839.

Sambrook J., Fritsch E. F., and Maniatis T. (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sandermann H., Rosenbrock H., and Ernst D. (1997), Horizontaler Gentransfer bei Herbizidresistenz? Der Einfluß von Genstabilität und Selektionsdruck. In: Zukunft der Gentechnik (Brandt P., ed.). Birkhäuser Verlag, Basel, pp. 209–220.

Schlüter K., Fütterer J., and Potrykus I. (1995), "Horizontal" gene transfer from a transgenic potato line to a bacterial pathogen (*Erwinia chrysanthemi*) occurs – if at all – at an extremely low frequency. Nat. Biotechnol. **13**, 1094–1098.

Schnurr J. A. and Guerra D. J. (2000), The CaMV-35S promoter is sensitive to shortened photoperiod in transgenic tobacco. Plant Cell Rep. **19**, 279–282.

Selvapandiyan A., Majumder K., Fattah F. A., Ahmad S., Arora N., and Bhatnagar R. K. (1995), Point mutation of a conserved arginine (104) to lysine introduces hypersensitivity to inhibition by glyphosate in the 5-enolpyruvylshikimate-3-phosphate synthase of *Bacillus subtilis*. FEBS Lett. **374**, 253–256.

Simon R., Priefer U., and Pühler A. (1983), A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in Gram negative bacteria. Nat. Biotechnol. **1**, 784–791.

Wagner T., Arango Isaza L. M., Grundmann S., Dörfler U., Schroll R., Schloter M., Hartmann A., Sandermann H., and Ernst D. (2008), The probability of a horizontal gene transfer from Roundup Ready® soybean to root symbiotic bacteria: A risk assessment study on the GSF lysimeter station. Water Air Soil Pollut. Focus **8**, 155–162.

Werner D. (1982), Biologische Versuchsobjekte. Kultivierung und Wachstum ausgewählter Versuchsorganismen in definierten Medien. Gustav Fischer Verlag, Stuttgart.

Yu Q., Cairns A., and Powles S. (2007), Glyphosate, paraquat and ACCase multiple herbicide resistance evolved in a *Lolium rigidum* biotype. Planta **225**, 499–513.

Zablotowicz R. M. and Reddy K. N. (2004), Impact of glyphosate on the *Bradyrhizobium japonicum* symbiosis with glyphosate-resistant transgenic soybean: A minireview. J. Environ. Qual. **33**, 825–831.