The Probability of a Horizontal Gene Transfer from Roundup Ready® Soybean to Root Symbiotic Bacteria: A Risk Assessment Study on the GSF Lysimeter Station

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Received: 15 January 2007 / Accepted: 28 September 2007 / Published online: 13 December 2007
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Abstract The gene transfer from glyphosate tolerant soybean to *Bradyrhizobium japonicum* was evaluated in a free-air lysimeter experiment under natural conditions and increasing selection pressure, to monitor for the probability of horizontal gene transfer (HGT). A large volume lysimeter study that offers conditions comparable

to normal farming was conducted in 2004 and 2005 with Roundup Ready® (RR) soybean and Roundup® application according to agricultural practice. Analysis of nodules showed, as expected, the presence of the transgenic 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*). However, in bacteroids that were isolated from nodules and then cultivated for several rounds in the presence of high levels of glyphosate, the *EPSPS* gene could no longer be detected. This indicates no stable HGT transfer of the whole *EPSPS* gene under field conditions.

Keywords Enolpyruvylshikimate-3-phosphate synthase · Free-air lysimeter · Glyphosate · Horizontal gene transfer · Roundup Ready® soybean · Symbiotic bacteria

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1 Introduction

The worldwide acreage of genetically modified plants (GMP) is accelerating rapidly (2005: 90 mio ha). Especially the cultivation of transgenic herbicide resistant crops is accompanied by an increasing application of herbicides. Major safety concerns in terms of environmental impacts of GMPs and herbicides to the environment are gene flow, selection of resistant weed biotypes and changes of the soil quality. The vast majority of the genetically modified soybeans planted worldwide have been made resistant to herbicides. Glyphosate (Roundup®) is a broad-spectrum herbicide that kills most plants. The herbi-

cide inhibits the 5-*enol*pyruvylshikamate-3-phosphate synthase (EPSPS) in plants that is essential for the synthesis of aromatic amino acids and thus for the survival of plants (Kishore and Shah 1988). In conventional agriculture, Roundup® or glyphosate is not used on crops directly, but is typically applied as a pre-plant application or used to spray the edges of the fields and roadways (also used as pre-emergence herbicide in many crops). It is also used to spray the crop just before harvesting in order to speed up maturing of the seeds and facilitate harvest. The gene for Roundup Ready® or glyphosate tolerance was derived from *Agrobacterium* sp. strain CP4. The gene encodes for a protein, CP4-EPSPS that is not affected by glyphosate. Roundup Ready® (RR) soybeans were developed by Monsanto (<http://www.monsanto.com>).

The transfer of transgenic DNA can be caused by pollen flow resulting in hybridization with related species (vertical gene transfer) as reported for canola (Sandermann 2006). Alternatively nonsexual exchanges of genetic information between different genomes may occur (horizontal gene transfer, HGT) (Lorenz and Wackernagel 1994; Brandt 1995). HGT is an essential natural process in evolution that enables fast adaptations to changing environmental conditions or to ecological niches. HGT over evolutionary time scales has been demonstrated by sequence comparisons and phylogenetic analysis within gene data bases. Carlson and Chelm (1986); Smith et al. (1992) and Kumada et al. (1993), for instance, discuss sequence homologies and HGT of the glyceraldehyde-3-phosphate dehydrogenase, the glucose-6-phosphate isomerase and the glutamine synthetase II. Veronico et al. (2001) demonstrated a HGT of bacterial genes from the polyglutamate biosynthesis to the nematode *Meloidogyne artiellia*.

The possibilities of HGT from plants to microorganisms are frequently evaluated in risk assessment studies, although mostly in the absence of a selection pressure (Nielsen et al. 1997; Ernst et al. 1998; Gebhard and Smalla 1999). However, up to now no experimental field approaches have confirmed the occurrence of such a HGT to naturally occurring bacteria. Recently a few studies in the laboratory have shown a marker gene transfer from plants to bacteria using recipient bacterial strains harbouring deletions to facilitate homologous recombination (Gebhard and Smalla 1998; de Vries et al. 2001; Nielsen et al. 2001; de Vries and Wackernagel 2002; Kay et al.

2002). Recombinant herbicide resistance genes are derived mostly from microorganisms and correspond in their structure to microbial genes. If heterologous DNA sequences are flanked by homologous DNA sequences, transgenes from plants might be more easily transferred to microorganisms via homologous recombination, and the efficiency of such a recombination will increase with increasing common sequences (de Vries and Wackernagel 2002). Model experiments indicated that a HGT from plants to microorganisms is in principle possible and occurred during evolution (Syvanen 1994). The main barriers of HGT from plant DNA to microorganisms seem to be the lack of sequence homology and the non-competent status of recipient cells (Nielsen et al. 1998).

Recent studies and theoretical calculations resulted in a transformation frequency of 2×10^{-17} under idealized natural conditions (Schlüter et al. 1995). This indicates that transfer frequencies under natural conditions are extremely low. Therefore a selection pressure seems to be very important for the successful establishment of a HGT (Nielsen et al. 1997, 1998). Only under a selection pressure novel genes are expected to become stable. This has been well demonstrated for various antibiotic resistance genes. Initially Jaworsky (1972) and subsequent available data for Roundup® and rhizobia bacteria (Zablotowicz and Reddy 2004) revealed growth inhibition at a concentration of 0.01 to 1.0 mM. Further calculations revealed an expected selection pressure under field conditions at a concentration of 50 µM (Sandermann et al. 1997). Using RR-soybean a strong selection pressure is given for the associated rhizobia bacteria, as glyphosate accumulated in nodules of field grown glyphosate-resistant soybeans (Zablotowicz and Reddy 2004). This might be further increased by the known root exudation of glyphosate (Grossbard and Atkinson 1985).

While some laboratory investigations have indicated that glyphosate may inhibit pure cultures of nitrogen-fixing bacteria (Moorman et al. 1992; Santos and Flores 1995), effects were only observed at glyphosate concentrations above normal field application rates. Several researchers (Hoagland et al. 1999; King et al. 2001; Goos et al. 2002; Zablotowicz and Reddy 2004) have investigated potential effects of glyphosate herbicides on nitrogen-fixing bacteria associated with glyphosate-tolerant soybeans. In general, any effects observed on nodulation or

nitrogen fixation were not observed uniformly and were noted to be transient in nature. Hoagland et al. (1999) reported some reduction in nodulation in RR soybean, but noted that effects were of minimal consequence due to the soybean's ability to compensate for short durations of stress caused by environmental factors such as high or low temperature, water availability, or nutrient status. King et al. (2001) reported that application of Roundup Ultra® herbicide delayed nitrogen fixation and decreased nitrogen accumulation in some glyphosate-tolerant soybean cultivars. However, effects were only observed under drought conditions and at rates of glyphosate above the recommended label rates. The soybean yield was not affected. In a study that included four soybean varieties and four sites, Goos et al. (2002) reported that, apart from a small reduction in ureide content in one soybean variety at one site, there was no indication that the Roundup Ultra® herbicide inhibited nitrogen fixation. At the recommended rates of application the exposure to glyphosate-based herbicides in the glyphosate-tolerant soybean production system is not expected to negatively affect soil fertility, nodulation nor nitrogen fixation. Therefore this system may be a good model for the evaluation of a possible HGT in field studies. On the other hand, certain rhizobial strains can actively degrade glyphosate, so that it is difficult to predict what happens in actual field situations (Liu et al. 1991). Due to the fact, that part of the soil and rhizosphere microbes are sensitive to glyphosate (Dunfield and Germida 2004), the occurrence of transfer of the glyphosate resistance gene from the transgenic plant to rhizosphere microbes seems probable. Of particular interest are bacteria from the *Rhizobiaceae* family, because the transgene originated from *Agrobacterium sp.* and may move back using mechanisms of homologues recombination. *Bradyrhizobium japonicum* are colonising the nodules of soybean; if glyphosate-sensitive *Bradyrhizobia* are chosen as nodulating bacterium, the gene transfer may be stimulated due to the smooth contact between the plant and microbe. In addition *Ochrobactrum* spp., also members of the *Rhizobiaceae*, are found colonising the rhizosphere and could be the target of gene transfer too. In the present lysimeter study we analyzed the probability of a HGT from transgenic RR soybean to *B. japonicum* and *Ochrobactrum* spp. under natural field conditions according to agricultural practice.

2 Materials and Methods

2.1 Bacterial Growth Conditions

B. japonicum strains isolated from root nodules and *Ochrobactrum anthropi* DSMZ 14396 strain 1a were grown in liquid yeast-mannitol media and yeast-mannitol petri dishes (Werner 1982) and Roundup was added at concentrations up to 30 mM.

2.2 Plant Material and Lysimeters

Non transgenic and transgenic soybeans (GTS 40-3-2) expressing the *EPSPS* gene derived from *Agrobacterium sp.* strain CP4 were provided by Monsanto Europe (Brussels, Belgium). In 2002, agricultural soil characterized as haplic arenosol from Weichselstein near Neumarkt (Bavaria) was filled into four lysimeters with a base of 1 m² and a length of 2 m, retaining the natural horizons and compaction. In 2003 non transgenic soybean (*Glycine max*) line Merlin was sown for testing germination and growth under the climatic conditions of Bavaria. In 2004 transgenic RR soybean line GTS 40-3-2 was sown into the four lysimeters that were surrounded in the interspaces by non transgenic soybeans. For an inoculation with *B. japonicum* seeds were treated with Histick (Becker Underwood, Littlehampton, UK). Seven weeks after sowing a mixture of Roundup Ready® and ¹⁴C-glyphosate was applied on two lysimeters at a concentration of 1 kg active ingredient ha⁻¹ (specific radioactivity: 1.92 MBq mg⁻¹). One, 3, 7, and 8 weeks after glyphosate application, up to 4 plants including the nodules were harvested per lysimeter. Nodules were air-dried; that resulted in a residual water content of 21%. In 2005 glyphosate was applied 8 weeks before and again 7 weeks after sowing on two lysimeters at a radioactivity concentration mentioned above. All plants, including the nodules were harvested 13 weeks after sowing and nodules were dried as mentioned above.

2.3 Redifferentiation and Cultivation of *B. japonicum* Bacteroids

After surface sterilization nodules were macerated and bacteroids were isolated using a self-generating Percoll gradient (Reibach et al. 1981). Redifferentiation and cultivation of bacteroids was carried out in a yeast-mannitol medium in the presence of 4 mM (year

Fig. 1 Phylogenetic comparison of the *EPSPS* gene from different α -proteobacteria. Cluster analysis was carried out of *EPSPS* DNA sequences from the NCBI database

2004) and 15 mM (year 2005) glyphosate according to Werner (1982). Visible colonies were detected after growth of 5–6 days at 30°C.

2.4 PCR Amplification

DNA from bacteria was isolated using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Quantitative real-time PCR (qPCR; TaqMan PCR) was carried out using an *EPSPS*01R primer (5'-GGAAAAGCCA GAGGATTG-3'), a *CTP*01 primer (5'-GCAAATTC TATGTTGGTTTGA-3') and a TaqManTM probe (5'-TTCAGCATCAGTGGCTACAGCCT-3'). The PCR product had a length of 147 bp. For standardization the PCR products of the *EPSPS* specific primers RR01 and RR02 (509 bp) (Köppel et al. 1997) were cloned into *E. coli*, the plasmid was isolated and the number of copies was quantified for each run. Quantitative PCR was performed with the

ABI-PRISM 770 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). To identify *B. japonicum*, partial 16S rDNA was amplified with primers 8F and 1522R (~1,500 bp). PCR amplifications were conducted according to standard protocols. Briefly, for amplification of the *EPSPS* gene 30 cycles with denaturation 1 min at 94°C, annealing and extension 1 min at 65°C were run, specific *B. japonicum* sequences were verified with 35 cycles, denaturation 1 min at 94°C, annealing 45 s at 53°C and extension 1 min 30 s at 72°C. The 1,500 bp PCR product was digested with *Hin*6I. PCR products were run on 1.5% and restriction analysis products on 2.5% agarose gels containing ethidium bromide (4 μ g/50 ml gel).

The *EPSPS* gene of *O. anthropi* was amplified using degenerated *EPSPS* primers 198f (5'-YTBCTB-GARGGCGARGACGT-3') and 1272r (5'-SWCATS GCGATGCGRTGRTC-3') and the PCR product (~1050 bp) was then sequenced (Eurofins Medigenomix, Martinsried, Germany).

For the phylogenetic comparison the sequences were automatically aligned using the CLUSTAL X (version 1.64b) program (Thompson et al. 1997). The neighbor-joining tree was calculated with the in CLUSTAL X integrated components of the PHYLIP 3.5c package (Felsenstein 1995).

2.5 HPLC Analyses

Dried nodules were broken up in a ball mill and then extracted with 0.1 M KH_2PO_4 , pH 1.9, at 100°C and

Fig. 2 Growth of *B. japonicum* strain isolated from root nodules in dependence on glyphosate concentrations in liquid nutrient medium, monitored as optical density. Bacteria were grown in the presence of the indicated concentrations of glyphosate and harvested at different time

Fig. 3 Identification of *B. japonicum* with 16S rDNA restriction analysis (year 2004). DNA was isolated from bacteroids and partial 16S rDNA was amplified. The resulting 1,500 bp PCR product was digested with *Hin6I* and the

products were separated by gel electrophoresis. Molecular weight markers are given on the left, middle and right site, and the size of typical restriction products in bp is indicated

100 bar in an accelerated solvent extractor (ASE-200, Dionex, Idstein, Germany). The extract was reduced and then filtered through a 0.2 μm Whatman filter (Whatman, Dassel, Germany). HPLC separation for determination of glyphosate and aminomethyl phosphonic acid (AMPA) was carried out on a PRP-X400 column (Hamilton, Martinsried, Germany). Samples were eluted from the column by 5 mM KH_2PO_4 , pH 1.9 at a rate of 0.5 ml min^{-1} . Radiodetection was carried out with a Radioflow Detector LB509 (Berthold, Bad Wildbad, Germany).

3 Results and Discussion

3.1 Sequence Alignments

DNA sequence alignments of the *EPSPS* gene from *B. japonicum* (NCBI Accession no.: AP005937) and *Agrobacterium* strain CP4 resulted in great homologies in many regions. In addition a phylogenetic comparison including *EPSPS* sequences of several α -proteobacteria resulted in a close relationship (Fig. 1).

EPSPS sequence alignments of *O. anthropi* and *Agrobacterium* CP4 resulted in a much higher homology (>90%) and a phylogenetic comparison showed a very close relationship (Fig. 1).

3.2 Effect of Glyphosate on Growth of *B. japonicum* and *O. anthropi*

Cultivation of a *B. japonicum* strain isolated from root nodules on a defined yeast-mannitol media showed no inhibition of growth up to a concentration of 20 μM glyphosate (Fig. 2). At a concentration of 200 μM the growth rate was slightly reduced, inhibited by 50% at a concentration of 2 mM and completely inhibited at a concentration of 20 mM (Fig. 2). Therefore concentrations higher than 200 μM (34 $\mu\text{g ml}^{-1}$) will result in a selection pressure, the basis for HGT and a successful and stable integration of foreign DNA via homologous recombination. Initial studies by Jaworsky (1972) showed that glyphosate inhibited growth of *B. japonicum* strain USDA 71 at a concentration of 10 μM and three other strains were completely inhibited at concentrations of 5 mM (Moorman et al.

Fig. 4 Detection of the *EPSPS* gene. **a** Ethidium bromide stained agarose gel. Qualitative PCR was carried out with *EPSPS* specific primers that resulted in a 147 bp product.

Molecular weight markers are given on the left and right site. **b** Quantitative real-time PCR using an ABI-PRISM 770 Sequence Detection System

Fig. 5 TaqMan PCR of isolated *B. japonicum* bacteroids from nodules of transgenic soybean (year 2004)

1992). A recent review (Zablotowicz and Reddy 2004) indicated that the level of glyphosate inhibition of bacteroids nitrogenase activity was related to *in vitro* glyphosate sensitivity of *B. japonicum* strains; however no yield reductions due to glyphosate application to glyphosate-resistant soybean have been observed in extensive field trials (Zablotowicz and Reddy 2004).

In contrast to *B. japonicum* liquid cultures of *O. anthropi* showed no growth inhibition upon application of glyphosate (data not shown). Even up to a concentration of 30 mM these bacteria grew well. Considering the great homology of the *EPSPS* gene from *O. anthropi* and *Agrobacterium* CP4 this indicates that the *EPSPS* enzyme of *O. anthropi* is insensitive towards glyphosate. Therefore no selection pressure is evident for *O. anthropi*, and a HGT is not likely and even more, might be excluded.

3.3 Glyphosate Concentration in Nodules of RR Soybean

For leaching analysis of ^{14}C -labeled glyphosate and AMPA this study must be carried out in lysimeters,

according to the National Board for Radiation Protection. Nodule glyphosate concentration was determined using ^{14}C -labeled glyphosate and HPLC separation. Glyphosate concentration in nodules of treated plants in the two lysimeters was $63.6 \mu\text{g g}^{-1}$ dry weight and $155.6 \mu\text{g g}^{-1}$ dry weight, respectively. The concentration of AMPA, the first metabolite ranged up to $25.8 \mu\text{g g}^{-1}$ dry weight. Zablotowicz and Reddy (2004) reported nodule glyphosate concentrations from 39 to 147 ng g^{-1} dry weight and a reduced nodule mass per plant. Therefore the higher glyphosate concentration found in our study is expected to result in a selection pressure.

3.4 Analysis of *B. japonicum* Isolated from the Lysimeters

In this study we used bacteroids of *B. japonicum* that were isolated from lysimeter soybean plants to test the probability of a HGT from plants to microorganisms. The transgenic *EPSPS* sequence showed great homologies to the *EPSPS* sequence of *B. japonicum*, important for HGT (de Vries et al. 2001; Nielsen et al. 2001; Kay et al. 2002). The herbicide concentration applied resulted in a selective selection pressure giving an advantage to the host (Sandermann et al. 1997; Nielsen et al. 1998, 2001). In 2004 four samplings were taken after application of Roundup Ready® and bacteroids were isolated from nodules. After redifferentiation of bacteroids and cultivation on a yeast mannitol medium, containing 4 mM glyphosate, total DNA was isolated. After amplification of partial 16S rDNA from *B. japonicum*, restriction analysis of the 1,500 bp fragment resulted in a specific DNA pattern (Fig. 3). All 60 isolates tested could be verified as *B. japonicum* (Fig. 3). In a

Fig. 6 Restriction analysis of partial 16S rDNA (year 2005). DNA was isolated from bacteroids and partial 16S rDNA was amplified. The resulting 1,500 bp PCR product was digested with *Hin6I* and the products were separated by gel electrophoresis. Molecular weight markers are given on the left and right site

preliminary study we showed the presence of the *EPSPS* gene in transgenic soybean leaves and also in the nodules using RR01/RR02 primers and qualitative PCR (data not shown). However, at least 1000 copies of the gene were necessary to obtain an amplification product (Fig. 4a). We developed therefore a more sensitive qPCR technique (Taqman assay) that allowed the detection of a single copy gene (Fig. 4b). Using this technique we analyzed all 60 isolates but could not detect the transgenic DNA (Fig. 5). Natural transformation of *Acinetobacter* with transgenic plant DNA in soil microcosms, based on homologous recombination, resulted in a transformation frequency of 1.4×10^{-8} (Nielsen et al. 2000). Therefore, we harvested only once in 2005 to increase the number of *B. japonicum* bacteroids. Bacteroids were isolated from 12 g of nodules using a Percoll gradient. This resulted in about 5×10^9 bacteroids g⁻¹ fresh weight of nodules. Redifferentiation of bacteroids and selection on yeast-mannitol media plates was carried out at a concentration of 10^5 bacteria per plate. To increase the selective detection method, the concentration of glyphosate was increased to 15 mM glyphosate. 600 plates, corresponding to 6×10^7 bacteria, were analyzed. After 24 h fast growing glyphosate resistant bacteria (about 100 colonies per plate) were detected and after 3 weeks a visual control for *B. japonicum* colonies was carried out. However, no typical *B. japonicum* colonies could be found. Fifty randomly picked colonies from different plates were further cultivated. After isolation of DNA and restriction analysis two different restriction patterns were observed (Fig. 6). However, these patterns reflected not the typical *B. japonicum* restriction pattern (compare Fig. 3 and 6). Problems in monitoring horizontal gene transfer to soil bacteria have been discussed recently (Heinemann and Traavik 2004; Nielsen and Townsend 2004) and thus the number of bacteroids analyzed in our study might have been too small to detect a HGT. However, it has to be mentioned that these studies had a focus on all soil bacteria and not on nodules colonizing bacteroids.

The results of this lysimeter study under real free-air conditions have so far not shown evidence of a HGT of the glyphosate-tolerance *EPSPS* gene from soybeans to symbiotic, nodules forming *B. japonicum* bacteria. As yet undetected competent bacteria might lead to a different result under selection pressure. However, little is known about the abundance of

naturally competent bacteria in the environment and triggering environmental factors, thus impairing predictions of a HGT.

Acknowledgements We wish to thank Silvia Fernandez (Monsanto) for critically reading the manuscript. Soybean seeds were kindly provided by Monsanto Europe.

References

- Brandt, P. (1995). *Transgene Pflanzen. Herstellung, Anwendung, Risiken und Richtlinien*. Basel: Birkhäuser Verlag.
- Carlson, T. A., & Chelm, B. K. (1986). Apparent eucaryotic origin of glutamine synthetase II from the bacterium *Bradyrhizobium japonicum*. *Nature*, 322, 568–570.
- de Vries, J., Meier, P., & 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 Microbiology Letters*, 195, 211–215.
- de Vries, J., & Wackernagel, W. (2002). Integration of foreign DNA during natural transformation of *Acinetobacter* sp. by homology-facilitated illegitimate recombination. *Proceedings of the National Academy of Sciences of the USA*, 99, 2094–2099.
- Dunfield, K. E., & Germida, J. J. (2004). Impact of genetically modified crops on soil and plant-associated microbial communities. *Journal of Environmental Quality*, 33, 806–815.
- Ernst, D., Rosenbrock, H., Hartmann, A., Kirchhof, G., Bauer, S., Ludwig, W., et al. (1998). Sicherheitsforschung zu Freisetzungsversuchen in Roggenstein (Bayern). *Bundesgesundheitsblatt*, 12, 523–530.
- Felsenstein, J. (1995). *PHYLIP (Phylogeny Inference Package) version 3.5c*. Distributed by the author. Department of Genetics, University of Washington, Seattle.
- Gebhard, F., & Smalla, K. (1998). Transformation of *Acinetobacter* sp. BD413 by transgenic sugar beet DNA. *Applied and Environmental Microbiology*, 64, 1550–1554.
- Gebhard, F., & Smalla, K. (1999). Monitoring field releases of genetically modified sugar beets for persistence of transgenic plant DNA and horizontal gene transfer. *FEMS Microbiology Ecology*, 28, 261–272.
- Goos, R. J., Johnson, B., Reinert, M., Helms, T., Henson, R., & Martin, L. (2002). Effect of roundup on nitrogen fixation by roundup-ready soybeans. *Proceedings of the Great Plains Soil Fertility Conference*.
- Grossbard, E., & Atkinson, D. (Eds.) (1985). *The herbicide glyphosate*. London: Butterworths.
- Heinemann, J. A., & Traavik, T. (2004). Problems in monitoring horizontal gene transfer in field trials of transgenic plants. *Nature Biotechnology*, 22, 1105–1109.
- Hoagland, R. E., Reddy, K. N., & Zablotowicz, R. M. (1999). Effects of glyphosate on *Bradyrhizobium japonicum* interactions in roundup-ready soybeans. *Annual Meeting of the Weed Science Society of America*, Vol 39.
- Jaworsky, E. G. (1972). Mode of action of *N*-phosphinomethylglycine: inhibition of aromatic amino acid biosynthesis. *Journal of Agricultural and Food Chemistry*, 20, 1195–1198.

- Kay, E., Vogel, T. M., Bertolla, F., Nalin, R., & Simonet, P. (2002). In situ transfer of antibiotic resistance genes from transgenic (transplastomic) tobacco plants to bacteria. *Applied and Environmental Microbiology*, 68, 3345–3351.
- King, C. A., Purcel, L. C., & Vories, E. D. (2001). Plant growth nitrogenase activity of glyphosate-tolerant soybean in response to foliar glyphosate applications. *Agronomy Journal*, 93, 179–186.
- Kishore, G. M., & Shah, D. M. (1988). Amino acid biosynthesis inhibitors as herbicides. *Annual Review of Biochemistry*, 57, 627–663.
- Köppel, E., Stadler, M., Lüthy, J., & Hübner, P. (1997). Sensitive Nachweismethode für gentechnisch veränderte Sojabohne <Roundup Ready™>. *Mitteilungen Gebiete der Lebensmittel Hygiene*, 88, 164–175.
- Kumada, Y., Benson, D. R., Hillemann, D., Hosted, T. J., Rochefort, D. A., Thompson, C. J., et al. (1993). Evolution of the glutamine synthetase gene, one of the oldest existing and functioning genes. *Proceedings of the National Academy of Sciences of the USA*, 90, 3009–3013.
- Liu, C.-M., McLean, P. A., Sookdeo, C. C., & Cannon, F. C. (1991). Degradation of the herbicide glyphosate by members of the family Rhizobiaceae. *Applied and Environmental Microbiology*, 57, 1799–1804.
- Lorenz, M. G., & Wackernagel, W. (1994). Bacterial gene transfer by natural genetic transformation in the environment. *Microbiology and Molecular Biology Reviews*, 58, 563–602.
- Moorman, T. B., Becerril, J. M., Lydon, J., & Duke, S. O. (1992). Production of hydroxybenzoic acids by *Bradyrhizobium japonicum* strains after treatment with glyphosate. *Journal of Agricultural Food Chemistry*, 40, 289–293.
- Nielsen, K. M., Bones, A. M., Smalla, K., & van Elsas, J. D. (1998). Horizontal gene transfer from transgenic plants to terrestrial bacteria – a rare event? *FEMS Microbiology Reviews*, 22, 79–103.
- Nielsen, K. M., Gebhard, F., Smalla, K., Bones, A. M., & van Elsas, J. D. (1997). Evaluation of possible horizontal gene transfer from transgenic plants to the soil bacterium *Acinetobacter calcoaceticus* BD413. *Theoretical and Applied Genetics*, 95, 815–821.
- Nielsen, K. M., van Elsas, J. D., & Smalla, K. (2000). Transformation of *Acinetobacter* sp. strain BD413 (pFG4ΔnptII) with transgenic plant DNA in soil microcosms and effects of kanamycin on selection of transformants. *Applied and Environmental Microbiology*, 66, 1237–1242.
- Nielsen, K. M., van Elsas, J. D., & Smalla, K. (2001). Dynamics, horizontal transfer and selection of novel DNA in bacterial populations in the phytosphere of transgenic plants. *Annals of Microbiology*, 51, 79–94.
- Nielsen, K. M., & Townsend, J. P. (2004). Monitoring and modelling horizontal gene transfer. *Nature Biotechnology*, 22, 1110–1114.
- Reibach, P. H., Mask, P. L., & Streeter, J. G. (1981). A rapid one-step method for the isolation of bacteroids from root nodules of soybean plants, utilizing self-generating Percoll gradients. *Canadian Journal of Microbiology*, 27, 491–495.
- Sandermann, H. (2006). Plant biotechnology: Ecological case studies on herbicide resistance. *Trends in Plant Science*, 11, 324–328.
- Sandermann, H., Rosenbrock, H., & Ernst, D. (1997). Horizontaler Gentransfer bei Herbizidresistenz? Der Einfluss von Genstabilität und Selektionsdruck. In P. Brandt (Ed.) *Zukunft der Gentechnik* (pp. 209–220). Basel: Birkhäuser Verlag.
- Santos, A., & Flores, M. (1995). Effects of glyphosate on nitrogen fixation of free-living heterotrophic bacteria. *Letters of Applied Microbiology*, 20, 349–352.
- Schlüter, K., Fütterer, J., & 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. *Biotechnology*, 13, 1094–1098.
- Smith, M. W., Feng, D.-F., & Doolittle, R. F. (1992). Evolution by acquisition: The case for horizontal gene transfers. *Trends in Biochemical Sciences*, 17, 489–493.
- Syvanen, M. (1994). Horizontal gene transfer: evidence and possible consequences. *Annual Review of Genetics*, 28, 237–261.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The ClustalX windows interfaces: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 24, 4876–4882.
- Veronico, P., Jones, J., Di Vito, M., & De Giorgi, C. (2001). Horizontal transfer of a bacterial gene involved in polyglutamate biosynthesis to the plant-parasitic nematode *Meloidogyne artiellia*. *FEBS Letters*, 508, 470–474.
- Werner, D. (1982). *Biologische Versuchsobjekte. Kultivierung und Wachstum ausgewählter Versuchsorganismen in definierten Medien* (p. 65). Stuttgart: G. Fischer Verlag.
- Zablotowicz, R. M., & Reddy, K. N. (2004). Impact of glyphosate on the *Bradyrhizobium japonicum* symbiosis with glyphosate-resistant transgenic soybean: A minireview. *Journal of Environmental Quality*, 33, 825–831.