Molecular Investigations of the Soil, Rhizosphere and Transgenic Glufosinate-Resistant Rape and Maize Plants in Combination with Herbicide (Basta®) Application under Field Conditions

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A field study was conducted during 1994 to 1998 on the Experimental Farm Roggenstein, near Fürstenfeldbruck, Bavaria, Germany to determine the effect of transgenic glufosinate-resistant rape in combination with the herbicide Basta® [glufosinate-ammonium, phosphinothricin, ammonium (2*RS*)-2-amino-4-(methylphosphinato) butyric acid] application on soil microorganisms and the behaviour of the synthetic transgenic DNA in response to normal agricultural practice. No influence of Basta® on microbial biomass could be detected. The phospholipid fatty acid analysis of soil extracts showed no difference between Basta® application and mechanical weed control, whereas conventional herbicide application revealed a different pattern. Basta® application resulted in a changed population of weeds with a selective effect for *Viola arvensis*. During senescence, transgenic rape DNA was degraded similar to endogenous control DNA. After ploughing the chopped plant material in the soil, transgenic as well as endogenous control DNA sequences could be detected for up to 4 weeks for rape and up to 7 months for maize, whereas PCR analysis of composted transgenic maize revealed the presence of the transgene over a period of 22 months.

Key words: *Brassica napus*, Glufosinate Resistance, Phosphinothricin-*N*-acetyltransferase Gene, Weed Populations

Introduction

The worldwide acreage of genetically modified plants (GMPs) is accelerating rapidly (2007: $117 \cdot 10^6$ ha; http://www.transgen.de/anbau/eu_international/531.doku.html), and cultivation of transgenic herbicide-resistant crops is accompanied by an increasing application of corresponding herbicides. Major safety concerns related to the terms of environmental impact of GMPs and herbicides are gene flow and influences on the soil microbial community.

To evaluate these environmental impacts, we conducted a 4-year field study at Roggenstein, Olching, Bavaria, Germany with GMPs and herbicide applications according to agricultural practice. As a system, we chose transgenic glufosinate-resistant rape and the corresponding herbicide (Basta®). Glufosinate inhibits glutamine synthetase, a key enzyme in the nitrogen metabolism. Glu-

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fosinate-resistant rape is transformed with a synthetic phosphinothricin-*N*-acetyl-transferase (*pat*) gene from *Streptomyces viridochromogenes*, which enables the plant to detoxify glufosinate.

Horizontal gene transfer (HGT) is a process in which an organism transfers genetic material to another cell that is not its offspring. HGT is an essential natural process in evolution and is well known among bacteria, enabling swift fast adaptation to changing environmental conditions or ecological niches. Sequence comparisons suggest recent horizontal transfer of genes among diverse species. HGT has been demonstrated by sequence comparisons and phylogenetic analysis within gene data bases, *e.g.* DNA sequence homologies and HGT of glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate isomerase and glutamine synthetase II were discussed (Carlson and Chelm, 1986; Smith *et al*., 1992; Kumada *et al*., 1993). HGT of bacterial genes of the polyglutamate biosynthesis to the nematode *Meloidogyne artiellia* was demonstrated (Veronico *et al*., 2001).

One requirement for HGT from plants to microorganisms is the persistence and availability of free DNA in the soil. For this purpose DNA must be stable and intact outside the plant cell. It has been shown that plant DNA released into the soil during plant degradation remains stable for several months (Paget and Simonet, 1994). Specifically, DNA bound to mineral components is protected from degradation by bacterial nucleases and can persist and remain stable for natural transformation processes in the soil for several months (Paget *et al*., 1992; Lorenz and Wackernagel, 1994; Nielsen *et al*., 1997; Sandermann *et al*., 1997; Widmer *et al*., 1997; Gebhard and Smalla, 1999). Compost and litter are special microcosms harbouring soil microorganisms with high diversity. It has been shown that transgenic DNA can be detected in compost for a period of 24 months (Ernst *et al*., 1996, 1998; Sandermann *et al*., 1997; Gebhard and Smalla, 1999). In addition to other niches, compost, with its highly active microbial degradation community, represents a possible hot spot for HGT. During processing, plant DNA is released into the soil and HGT *via* transformation of competent bacteria can occur. By absorption of transgenic herbicide-resistant genes and the use of herbicides, microorganisms could get a selection advantage for stable integration and inheritance of the transgenic DNA. Recently, a few laboratory studies have shown marker gene transfer from plants to bacteria based on homologous recombination (Gebhard and Smalla, 1998; de Vries *et al*., 2001; Nielsen *et al*., 2001; de Vries and Wackernagel, 2002; Kay *et al*., 2002). However, cultivation of GMPs in the field did not detect any evidence that the transgene was transferred to bacteria (Demanèche *et al*., 2008; Wagner *et al*., 2008).

In our field study, we tested the persistence of the transgenic *pat* gene during degradation of transgenic rape in soil under normal agricultural practices. Basta® is a wide-range herbicide with antibiotic effects on several microorganisms (Dunfield and Germida, 2001; Gyamfi *et al*., 2002). The increasing application of glufosinate to agricultural areas could be followed by changes in the microbial biodiversity accompanied with effects on soil quality (Dunfield and Germida, 2001; Gyamfi *et al*., 2002). Field studies in the few last years analyzed effects of Basta® and conventional herbicides on the soil microorganisms (Ernst *et al*., 1998; Becker *et al*., 2001). To evaluate the influence of Basta® on soil microorganisms, we compared treated and untreated soil samples by measuring the microbial biomass and analyzing phospholipid fatty acids.

Materials and Methods

Field site and plant material

In three following years (1994–1997) winter rape (*Brassica napus*), maize (*Zea mays*) and winter wheat (*Triticum aestivum* L.) were cultivated on plots of 54 m^2, each in the centre of a field of 6840 m^2. In addition to transgenic rape and maize, non-transgenic parent lines were also cultivated. The field was surrounded by an area 3 m in width with non-transgenic plants (partial sterile rape and conventional hybrid maize, respectively) (Ernst *et al*., 1998).

Transgenic rape (GS 40/90 pHoe6/ac) and maize ($[(\text{LH } 82^4 \times T_{25})sf^2] \times \text{SH298}$ and $[(\text{LH } 82^4 \times T_{25}) sf^2] \times F_2$) carrying the phosphinothricin-*N*-acetyltransferase gene (*pat*) from *Streptomyces viridochromogenes* under the control of the 35S-promoter/terminator of *Cauliflower mosaic virus* (*CaMV*) were used (Donn *et al*., 1992). The bacterial *pat* sequence was adjusted to a G/C part of plant's nucleotide sequences without changing the amino acid sequence (Donn *et al*., 1992). Plots with transgenic plants were treated with Basta® (AgrEvo, Frankfurt, Germany) during the 3- to 5-leaf stage (3.0 l ha^{-1}, corresponding to 600 g a. i.

ha^{-1}, dissolved in 400 l water ha^{-1}, as recommended by the manufacturer for this field trial). Non-transgenic rape was treated with Butisan® [metazachlor, 2-chloro-*N*-(pyrazol-1-ylmethyl) acet-2′,6′-xylidide; BASF, Limburgerhof, Germany] (1.0 l ha^{-1}, corresponding to 500 g a. i. ha^{-1}) and Oleo® (94 % paraffin oil, 6 % tensides; FSG Feinchemie Schwebda GmbH, Eschwege, Germany) (0.5 l ha^{-1}). Plots without herbicide application were mechanically treated in autumn and spring to a depth of 5 cm. Weed populations were monitored after harvest of rape.

Cultivation of soil bacteria

Soil suspensions were generated by treating soil (1 g fresh weight) in 9 ml extraction solution (containing 0.1 g NaCl, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$ and 5.0 g Tween 80 per l demineralized water) in a Retsch mill (type MM2; Retsch GmbH & Co, Haan, Germany) for 8 min at 70 % power. Appropriate dilutions were plated on oligotrophic, modified glutamate-free R_2A medium (DSMZ medium No. 830) agar plates (MR_2A) and incubated at 30 °C in a wet chamber for up to two weeks. Phosphinothricin (Pt) [2-amino-4-(hydroxymethyl-phosphoryl)butanoic acid] or Basta® were added to the agar at different concentrations; in the case of Basta® addition, 1/10 of the phosphate buffer concentration was used.

DNA isolation

DNA was extracted from leaves, roots, flowers and siliques according to Dellaporta (1993) as well as stems using the Genome Clean-Kit (AGS, Heidelberg, Germany). From soil samples DNA was exactly isolated according to Ernst *et al.* (1996). This procedure resulted in the isolation of DNA suitable for PCR analysis and eliminating interfering substances.

PCR amplification of selected DNA sequences

Gene specific primers for the *pat* gene resulted in the amplification of an 1135 bp and 606 bp fragment (Fig. 1A) (*pat* gene, including the 35S-*CaMV* promoter and terminator region: 5′-CATGGAG TCAAAGATTCAAATAGAGGACC-3′, 5′-GA GCTCGGTACCCACTGGATTTTGG-3′; *pat* gene, coding region: 5′-GTACCCGGGGATCCTCTA GAGTC-3′, 5′-GATTTCAGCGGCATGCCTGC AGGTC-3′). The small subunit of ribulose-bisphosphate carboxylase/oxygenase (*rbcS*) (5′-GCC

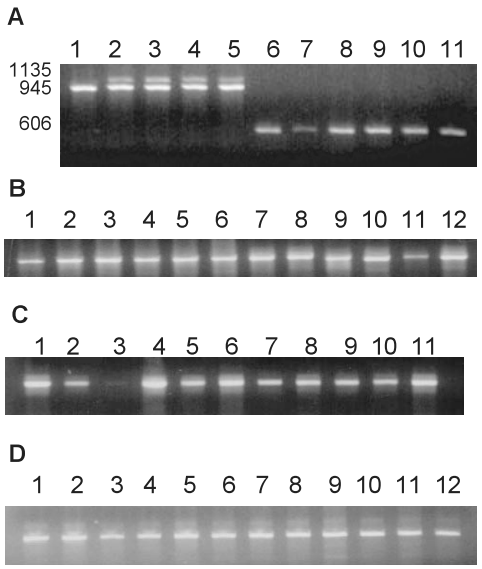

Fig. 1. Agarose gel electrophoresis of PCR-amplified DNA sequences extracted from rape leaves 29 d after the start of flowering. (A) Transgenic rape: lanes 1–5, *pat* gene including the promoter and terminator region (1135 bp); lanes 6–11, coding region of the *pat* gene (606 bp). In addition, the internal standard used for quantification of PCR products is given (945 bp). (B) Transgenic rape: lanes 1–6, ribulose-bisphosphate carboxylase/oxygenase (1183 bp); lanes 7–12: chlorophyll a/b-binding protein (1231 bp). (C) Non-transgenic rape leaves: lanes 1–11, chlorophyll a/b-binding protein. (D) Non-transgenic rape leaves: lanes 1–12, ribulose-bisphosphate carboxylase/oxygenase.

ACGTGGCCTTAACATAGTGGTCAG-3′, 5′-CG GAAAGTAACCAAGACGAAGAAGC-3′) and the chlorophyll a/b-binding protein (*cab*) (5′-GC CAATATCTGGGTATTCGAGACACCG-3′, 5′-CCCAACCATAGTCGCCGGGAAACTC-3′) were used as internal control genes. In order to get PCR fragments comparably sized to the *pat* gene, specific primers were designed that resulted in an 1183 bp and 572 bp fragment for *rbcS*, and an 1231 bp and 628 bp fragment for the *cab* gene (Figs. 1B, C, D). An external standard of a cloned 945 bp fragment of the *pat* gene was used for quantification. During PCR amplification, the standard was used at concentrations ranging from 4.5 to 0.0045 ng μl^{-1} DNA, and quantification of ethidium bromide-stained gels was carried out using a conventional scan fluorescent software program (A. Kraxenberger, GSF, Institute of Radiation Biology, Germany).

PCR protocol: 0.5 μg DNA, 0.2 μM primers, 2.5 units Taq polymerase, 2 mM dNTP's, 1 × PCR

buffer [100 mM Tris-(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01 % (w/v) gelatine]. Denaturation at 92 °C for 2 min, then 56 °C for 2 min, and finally 72 °C for 2 min. 30 cycles were used for plant material and 35 cycles were used for soil and compost material. PCR products were separated on 2.0 % agarose gels containing $5 \cdot 10^{-3}$ % ethidium bromide. Quantification of ethidium bromide-stained gels was carried out using a conventional scan fluorescent software program (A. Kraxenberger). To confirm the correct amplification of DNA sequences, PCR products were sequenced using the thermo sequenase kit from Amersham Pharmacia (Freiburg, Germany).

Microbial biomass

The microbial biomass of the soil was measured using the glucose-induced microcalorimetry method (Sparling, 1983). Soil samples were preincubated at 22 °C for 24 h. 10 g were mixed with 80 mg of a 1:1 D-(+) glucose/talcum mixture. A 1 g dry soil equivalent was weighed into a glass ampoule, closed with a rubber stopper, sealed with aluminium and measured in a calorimeter (BioActivity Monitor, LKB, Bromma, Sweden).

Phospholipid fatty acid determination

Extraction of the lipids, hydrolysis of the fatty acids, gas chromatographic separation and qualitative as well as quantitative analyses by mass spectrometry of phospholipid fatty acids (PLFAs) from soil organisms were carried out following the instructions of Zelles and Bai (1993). To compare the PLFA composition from soil samples of the three different herbicide treatment variations, PLFA patterns were subjected to cluster analysis using the SPSS program and to principal component analysis using the Sigma Plot program (Jandel Scientific, San Rafael, USA).

Results and Discussion

Microbial biomass

The microbial biomass (C_{mic}) of surface soil and rhizosphere soil was estimated by substrate-induced microcalorimetry (Fig. 2). In autumn 1994, about 300 mg C_{mic} kg^{-1} dry weight (dw) was measured in soil samples of Basta®-treated plots. In spring 1995, just before herbicide application, this value increased to 500 mg C_{mic} kg^{-1} dw, indicating a seasonal fluctuation of soil microbial biomass. There were no significant differences in the microbial biomass values between comparable samples of the three treatments (Basta®, conventional herbicide Butisan®, and mechanical tillage). Moreover, one day after herbicide application, the microbial biomass was about the same in all three treatments and comparable to the values obtained from samples of the day before (Fig. 2). Additionally, these values did not significantly change after one week. This is in agreement with a 2-year study of transgenic herbicide-resistant oilseed rape and Basta® or Butisan® application (Becker *et al.*, 2001).

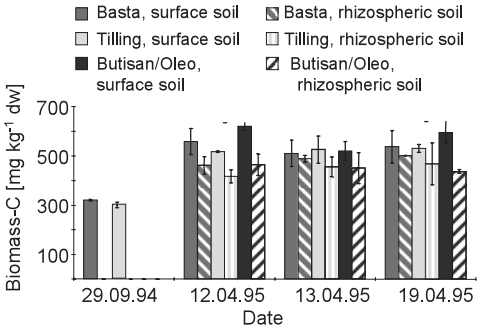

Fig. 2. Microbial biomass of soil samples from rape plots in 1994/95.

To analyze a possible herbicide effect on the cultivable rhizosphere microflora, soil samples of all three treatments were incubated in culture medium in the absence and presence of different amounts of phosphinothricin as well as Basta®. The number of living bacteria showed no difference between the three treatments over a sampling period of one week (Fig. 3). With increasing phosphinothricin amounts in the culture medium (up to 200 mg l^{-1}) and at a Basta® amount of 20 mg l^{-1} the number of living bacteria decreased slightly within all the treatments. At a Basta® concentration amount of 100 mg l^{-1}, the number of living bacteria dropped dramatically and was close to zero with 200 mg Basta® in the medium (Fig. 3). These results were confirmed in the following year (Fig. 4). These results indicate that the resistance of the bacterial flora towards Basta® following Basta® application in the field did not increase.

Taken together, the data of microbial biomass and number of culturable bacteria demonstrate that no ecotoxicological effects of Basta® applica-

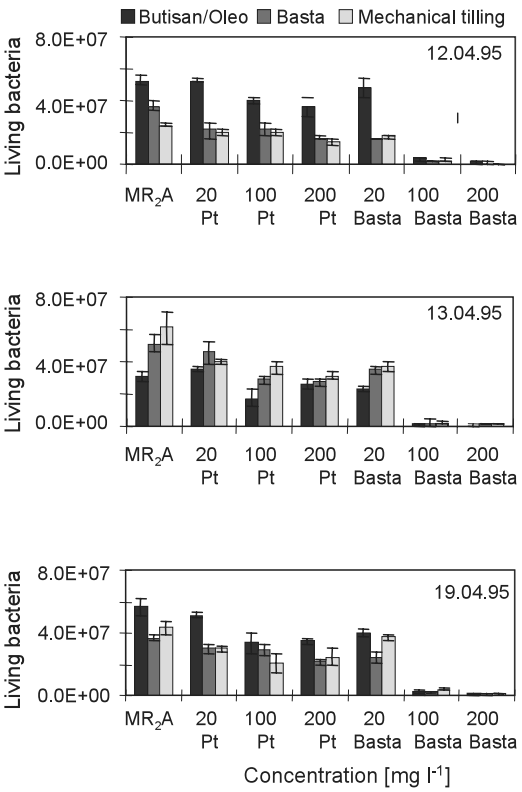

Fig. 3. Number of cultivable bacteria (colony-forming units) in soil samples (year 1995). Cultivation was carried out using modified oligotrophic glutamate-free R_2A medium agar plates (MR_2A) in the absence and presence of phosphinothricin (Pt) or Basta®, respectively, as indicated in mg l^{-1}.

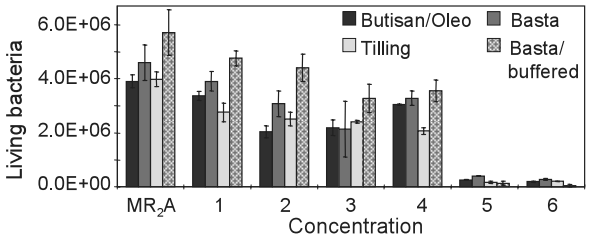

Fig. 4. Number of culturable bacteria (colony-forming units) in soil samples (year 1996). MR_2A, modified oligotrophic glutamate-free R_2A medium agar plate; 1, 20 mg l^{-1} phosphinothricin (Pt); 2, 100 mg l^{-1} Pt; 3, 200 mg l^{-1} Pt; 4, 20 mg l^{-1} Basta®; 5, 100 mg l^{-1} Basta®; 6, 200 mg l^{-1} Basta®. In the Basta®-supplemented plates, 1/10 phosphate buffer concentration was used.

tion in transgenic rape were detectable under field application conditions in our experimental design, which was similar to the manufacturer's recommended agronomical practice (Hoechst, 1982).

Phospholipid fatty acid determination

The population structure of the microbial flora phospholipid fatty acid pattern of all three agricultural treatments was analyzed in 1995 according to Zelles and Bai (1993). Comparing variants Basta® and Butisan® with the mechanically tilled control, a difference was detected in the fraction of multiple unsaturated 18:*n* fatty acids. The amount of fatty acid 18:2,9,12, typical of fungal cells, was decreased in the herbicide-treated compared to mechanically tilled samples. This indicates that herbicide treatments might have a general influence on fungal flora. The amount of minor omega-hydroxy fatty acids, typical of eukaryotic cells, was increased in the herbicide-treated compared to mechanically tilled control samples. These higher values could be caused by an increased algae growth in the herbicide-treated plots.

To verify these observations, fatty acid spectra were analyzed in 1997. Interestingly, samples treated with the conventional herbicide Butisan® were significantly different compared to the samples from other treatments; the total extractable amount of fatty acids was much lower. Cluster and principal component analysis showed a different fatty acid composition for samples treated with Butisan® (Figs. 5A, B). As shown in 1995, fatty acid 18:2,9,12 decreased in response to Butisan® treatment. However, this was not observed for Basta® treatment. In addition, the non-ester bound non-substituted hydroxy fatty acid fraction differed in Butisan®-treated soil samples compared to the samples from the other two treatments (Fig. 5C). This fatty acid fraction indicated the presence of anaerobic microrganisms. For Basta® and mechanically tilled plots this difference was less clear. The amount of omega-hydroxy fatty acids, indicative of eukaryotic cells, did not differ between samples obtained from all three treatments at the two sampling times. In conclusion, application of Butisan®, a conventional herbicide, had a stronger impact on the structure of the soil microbial community compared to Basta® treatment or mechanical tilling.

Weed populations

Classification of weeds was carried out after rape harvest. Basta® application resulted in a changed weed composition compared to Butisan® or mechanical treatment (Table I). *Viola arvensis* dominated after Basta® application, whereas *Stel-*

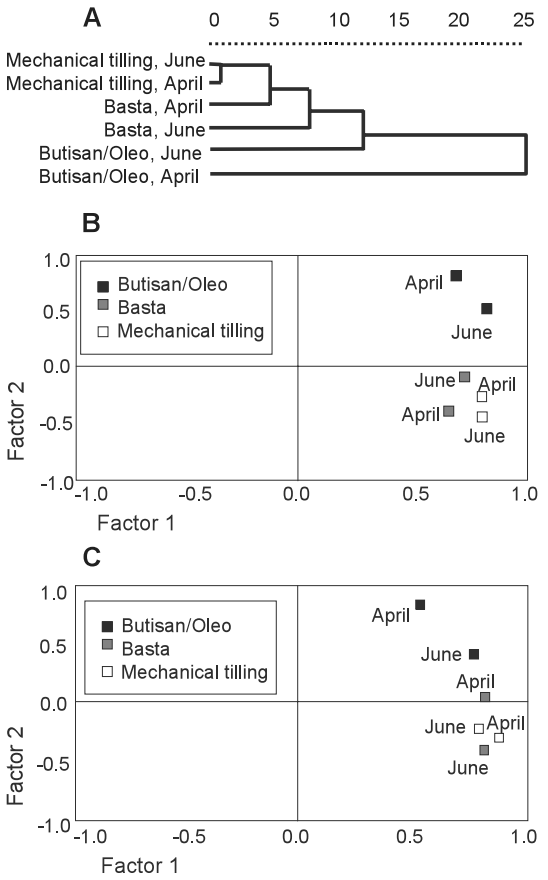

Fig. 5. Phospholipid fatty acid analysis from soil samples in 1997. (A) Cluster analysis. (B) Principal component analysis of all fatty acid fractions. (C) Principal component analysis of non-ester bound non-substituted hydroxy fatty acids.

laria media was most prominent in mechanically tilled plots. Similarly, Basta® application did not decrease the *Viola arvensis* extent in a previous field study (Becker *et al.*, 2001). Violet was also a problematic weed in canola management systems and glufosinate contents of 500 g a. i. ha^{-1} were insufficient for control (Degenhardt *et al.*, 2005). Thus, continuous Basta® application can result in selection for insensitive weed biotypes as has been reported for the herbicide glyphosate (Sandermann, 2006). It should be noted that the manufacturer recommends amounts of 1.5–2.0 kg a. i. ha^{-1} for a number of problem weeds (Hoechst, 1982).

Analysis of the pat gene during plant senescence

Senescence of living organisms results in degradation of biological material and is easily recognized in plants by loss of the green photosynthetic pigment chlorophyll. In addition to visible symptoms, other processes such as degradation of proteins, nucleic acids and secondary compounds take place. Stable integrated synthetic transgenic DNA that encodes a protein should undergo the same degradation processes compared to endogenous non-transgenic DNA as a result of the universality of the chemical structure of DNA. PCR analysis showed that the *pat* gene excluding (606 bp) and including (1335 bp) the flanking promoter and terminator region of the 35S-*CaMV* was present in leaves of rape during senescence (Fig. 1A). As an example of endogenous control DNA sequences, PCR products of the *rbcS* and *cab* gene from transgenic and non-transgenic rape are shown

	Butisan®+ Oleo	Basta®	Mechanical treatment
Weed covering extent	27	73	62
Viola arvensis	4	53	3
Stellaria media	12	10	48
Galeopsis tetrahit	1	1	1
Matricaria inodora	2	5	1
Polygonum lapathifolium	–	1	–
Polygonum convolvulus	1	1	1
Polygonum aviculare	–	1	–
Myosotis arvensis	–	1	1
Lapsana communis	–	1	–
Chenopodium album	–	1	1
Veronica persica	–	1	1

Table I. Weed covering extent in 1994/95 after rape harvest. The weed covering extent and the mean covering extent of individual weeds are given in %.

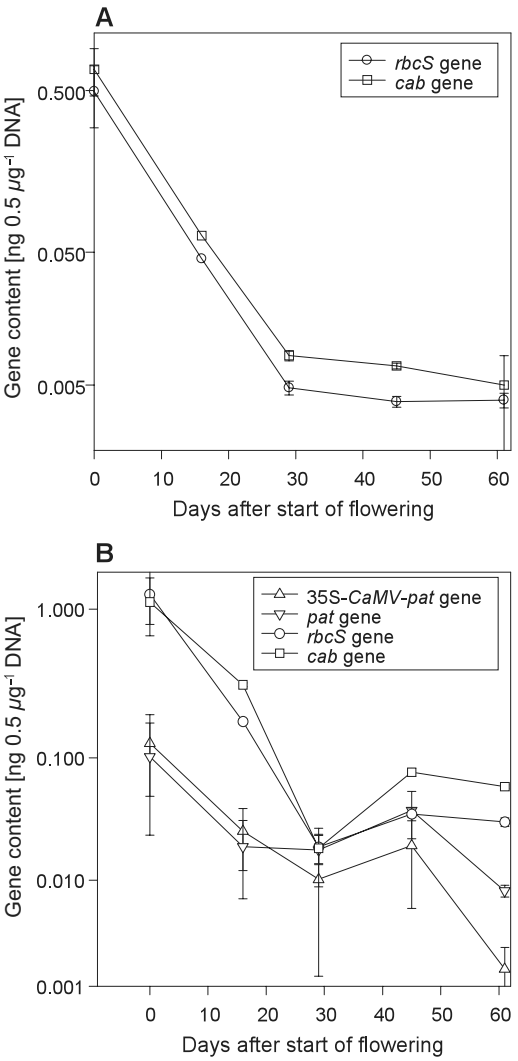

Fig. 6. Degradation of DNA in leaves of rape after the start of flowering. Specific DNA sequences were detected by PCR. Time points of harvest and analysis of soil samples are indicated. (A) Non-transgenic rape, $n = 11–12$. (B) Transgenic rape, $n = 4–6$.

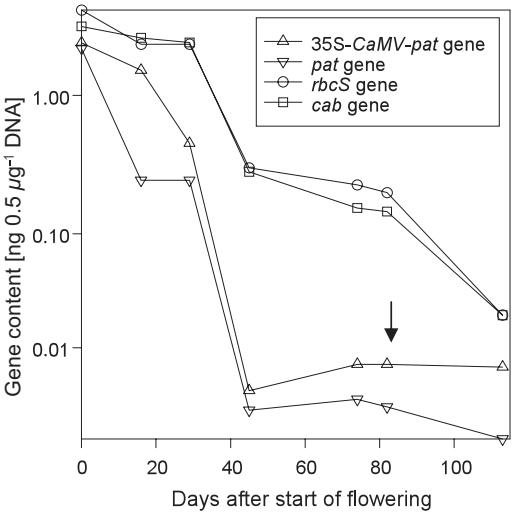

Fig. 7. Degradation of DNA in stems of transgenic rape after the start of flowering and in soil samples after ploughing the chopped straw. Specific DNA sequences were detected by PCR. The time of ploughing is indicated by an arrow. Transgenic plants, $n = 4–6$; 6 soil samples were taken from the transgenic lots of land up to a depth of 15 cm.

(Figs. 1B, C, D). Quantification of all PCR products, using an external standard of a cloned 945-kb PCR product of the *pat* gene, was carried out at different times after the onset of flowering (Figs. 6, 7). As shown in Fig. 6B, the amount of *pat* gene decreased in the leaves of transgenic rape during senescence to values below 0.01 ng 0.5 μg^{-1} DNA. Additionally the amount of *rbcS* and *cab* in leaves of transgenic rape also decreased during senescence (Fig. 6B). In addition, there was no significant difference in the *rbcS* and *cab* degradation process when comparing transgenic and non-transgenic rape (Figs. 6A, B). Similarly, degradation processes for all three genes analyzed took place in stems (Fig. 7) as well as flowers and siliques (data not shown). During the 6 weeks after flowering, about 90 % of the four DNA sequences were degraded. This indicates that synthetic DNA encoding a protein undergoes the same senescence processes under normal agricultural practices as non-transgenic DNA. DNA sequence analysis confirmed the flanking 35S-*CaMV*, as well as the synthetic *pat* gene sequence.

Persistence of transgenic DNA in soil

Free DNA can effectively bind to soil particles and might therefore be available for the transformation of microorganisms (Lorenz and Wackernagel, 1994). PCR amplification of DNA in soil samples can be inhibited by co-extracted humic acids. However, addition of $CaCl_2$ (Ernst *et al.*, 1996) or $NH_4Al(SO_4)_2$ (Lerat *et al.*, 2005) to the DNA extraction buffer resulted in a successful amplification of transgenic DNA sequences. *pat* DNA sequences could be verified by PCR for up to 4 weeks after ploughing chopped rape plants into the soil (Fig. 7). PCR was also positive for the *pat*

gene of transgenic maize for up to 7 months (data not shown). As the synthetic *pat* gene sequence has been adapted to the codon usage of plants it can be distinguished from the naturally occurring *pat* DNA sequence, and DNA sequence analysis of the PCR products again confirmed the existence of the synthetic *pat* gene. During the same period of time, endogenous control sequences of *rbcS* and *cab* could also be found (Fig. 7). Again, no difference in soil degradation processes between synthetic transgenic and non-transgenic DNA was found. The persistence of transgenic DNA in soil samples was in agreement with literature reports, ranging from weeks to months (Paget *et al*., 1992; Paget and Simonet, 1994; Lorenz and Wackernagel, 1994; Nielsen *et al*., 1997; Sandermann *et al*., 1997; Widmer *et al*., 1997; Gebhard and Smalla, 1999). During litter degradation, microbial activity is very high, representing a possible hot spot for horizontal gene transfer (van Elsas *et al*., 2003). Similarly, compost can be regarded as a specialized microcosm, harbouring a large number of active microorganisms. In composted transgenic maize the *pat* sequence remained present after 22 months (Sandermann *et al*., 1997), representing another possible hot spot for HGT processes (van Elsas *et al*., 2003). However, little is known about the abundance of naturally competent bacteria in the environment. This impairs our ability to predict the occurrence of HGT.

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