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Population structure of plant-pathogenic *Fusarium* species in overwintered stalk residues from Bt-transformed and non-transformed maize crops

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Abstract

Bt-transformed maize contains genes from *Bacillus thuringiensis* encoding for insecticidal crystal proteins. Less insect damage on Bt maize stalks can cause a reduced infection by *Fusarium* species through plant injuries. This could affect the presence of plant-pathogenic *Fusarium* species on maize residues which serve as an inoculum source for subsequent crops. We collected overwintered maize stalks of four different Bt maize hybrids and their corresponding non-Bt lines in two consecutive years in a field trial in Germany. *Fusarium* spp. were isolated from 67% of 648 collected maize stalks. Identification with new multiplex PCR assays showed that *F. graminearum*, *F. avenaceum*, and *F. proliferatum* were the most abundant *Fusarium* species, isolated from 42%, 26%, and 15% of the stalks, respectively. Species abundances varied between varieties and collection years. No consistent difference was found between Bt and non-Bt stalks. *Fusarium graminearum* isolates were subject to a population genetic structure analysis with eight newly developed microsatellites. Significant association of loci and overrepresentation of repeated multilocus haplotypes indicated a substantial asexual component of reproduction, supporting selection of haplotypes. The data suggested selection of particular *F. graminearum* haplotypes by collection years but not by maize Bt transformation. Haplotypic changes between years caused no divergence in the distribution of alleles, suggesting that gene flow beyond the field scale prevented substructuring. We present evidence for gene flow between our saprophytic *F. graminearum* population on maize residues and a wheat-pathogenic population from a field 100 km distant.

Introduction

The most frequently reported causes of stalk rot of maize (*Zea mays*) are *Fusarium graminearum*, *F. verticillioides* (syn. *F. moniliforme*), *F. proliferatum*, and *F. subglutinans* (Kommendahl and Windels, 1981). These *Fusarium* species can also cause maize ear rot (Logrieco et al., 2002) and *F. graminearum* is further known as a major causal agent of Fusarium head blight of small-grain cereals (Bottalico and Perrone, 2002). In addition to causing losses of yield, crop infestation by these *Fusarium* species is a concern for human and animal health because they are capable of producing mycotoxins such as trichothecenes, fumonisins, or moniliformin (Bennett and Klich, 2003).

Fusarium spp. can survive saprophytically on plant tissue that remains in the field after harvest and which then serves as a major inoculum source for the infection of subsequent crops (Windels

et al., 1988). Increased Fusarium head blight incidence was observed when wheat was grown after maize, particularly under reduced tillage systems for soil conservation (Champeil et al., 2004). This suggests that maize residues on the soil surface are particularly suitable for the survival of pathogenic *Fusarium* spp. Tillage can reduce the incidence of *Fusarium* diseases because the survival of *F. graminearum*, *F. verticillioides*, *F. proliferatum*, and *F. subglutinans* is lower on buried than on surface maize residues (Khonga and Sutton, 1988; Cotten and Munkvold, 1998).

Bt maize, expressing insecticidal crystal proteins from *Bacillus thuringiensis*, is one of the dominant transgenic crops, being cultivated on 11.3 million hectares worldwide in 2005 (James, 2005). Most Bt maize varieties express the Cry1Ab protein which protects the plant against the lepidopteran insect pest, particularly the European stem borer *Ostrinia nubilalis*. In our field trial, the European stem borer was detected in about 5% of the non-Bt plants in both years and completely absent in Bt plants. Less insect damage on Bt plants occasionally causes a reduced infection by *Fusarium* species through plant injuries (Munkvold et al., 1997). Gatch and Munkvold (2002) further reported a reduced proportion of *F. verticilliodes* and *F. proliferatum* in stalk rot of Bt maize, often accompanied by an increased proportion of *F. graminearum*. Such shifts in the pathogen complex of infected maize stalks could affect the species composition of *Fusarium* in overwintered maize stalks. The saprophytic survival of *Fusarium* could be further affected by unintended changes of the crop chemical composition as a result of Bt transformation. We previously reported differences in the chemical composition in two of the four Bt/non-Bt maize hybrid pairs used in this study. These differences could not be explained by the sole production of the additional Bt protein. Bioassays with a *F. graminearum* model strain on maize leaf tissue indicated that these chemical differences can affect its saprophytic growth (Naef et al., 2006b). Selection within the *Fusarium* population of species or haplotypes that can better adapt to such chemical changes could affect the *Fusarium* population structure on Bt maize residues. Such potential effects of maize Bt transformation have not yet been assessed.

The traceability of haplotype selection within a particular *Fusarium* species depends on the relative dominance of sexual versus asexual reproduction. Sexual reproduction could mask selection of haplotypes because the ascospores produced are wind-dispersed beyond fields (de Luna et al., 2002) and because recombination creates new haplotypes. On the other hand, asexual reproduction produces a large number of genetically identical conidia which are splash-dispersed over short distances only (Sutton, 1982). Therefore, in a mainly asexually reproducing *Fusarium* population, selection of particular haplotypes on Bt maize residues could be detected as changes in haplotype frequencies (McDonald et al., 1996).

The objective of this work was to assess potential effects of transgenic Bt maize residues on the population structure of pathogenic *Fusarium* species in a field trial in southern Germany. We investigated the abundance of eight agronomically important maize- and wheat-pathogenic *Fusarium* species on naturally overwintered maize stalks of four transgenic Bt varieties and their near-isogenic non-transgenic lines in two consecutive years. Multiplex PCR assays with established PCR primers were developed for efficient identification of *Fusarium* isolates. Genetic data were generated for *F. graminearum*, *F. culmorum*, and *F. crookwellense* isolates with seven newly developed and one previously published microsatellite marker to assess the mode of reproduction and to detect potential selection of haplotypes on Bt maize residues. Furthermore, we assessed the extent of genetic exchange between our saprophytic *F. graminearum* population and a pathogenic population from a German wheat field (Miedaner et al., 2001).

Materials and methods

Fungal reference strains and populations

Reference isolates of *F. acuminatum* (3 isolates), *F. avenaceum* (3 isolates), *F. culmorum* (20 isolates), *F. crookwellense* (5 isolates), *F. graminearum* (20 isolates), *F. poae* (2 isolates), *F. oxysporum* (2 isolates), *F. proliferatum* (1 isolate), *F. subglutinans* (1 isolate), and *F. verticillioides* (3 isolates) were obtained from Swiss and Italian culture collections (Agroscopes FAL Reckenholz and RAC Changins, Switzerland; Sezione di patologia vegetale, University Federico II, Naples, Italy). For

comparison of our saprophytic *F. graminearum* isolates with plant-pathogenic isolates, we used a German *F. graminearum* population from a wheat field (Miedaner et al., 2001). This population consisted of 70 strains from 35 wheat heads with head blight symptoms collected at nine different sampling points within the field. For each head, two strains were isolated from two different spikelets.

Field trial design and Fusarium *isolations*

Fungi were isolated from overwintered maize stalks which were collected after the fifth (2002) and the sixth (2003) season of a field trial with six year continuous maize cropping (1998–2003) in the German Rhine valley. Transgenic Bt maize hybrids and their near-isogenic non-transgenic lines were planted on adjacent strip plots of about 0.5 ha (20×250 m). The Bt lines were grown on the same plots in each year, but cultivars were changed between years. In 2002, the planted Bt/non-Bt hybrid pairs were Valmont/Prelude (Syngenta Seeds, Basel, Switzerland), X0920 RT/Benicia (Pioneer Hi-Bred International, Des Moines, IA, USA), and Novelis/Nobilis (Euralis, Lescar, France) and in 2003, they were Valmont/Prelude, X0920 RT/Benicia, and TXP 138/DKC 3420 (Dekalb % Monsanto, St. Louis, MO, USA). Valmont carries the Bt transformation event Bt176 (Syngenta Seeds) causing expression of native Cry1Ab protein in pollen and green tissue only, whereas X0920 RT, Novelis, and TXP 138 carry the Bt transformation event MON810 (Monsanto) causing expression of a truncated version of Cry1Ab protein in the whole plant (Biotech Crop Database available online at *http://www.agbios.com* from AGBIOS, Merrickville, Canada). For easier presentation, we call the Bt hybrids by the name of their near-isogenic non-transgenic lines followed by the suffix Bt in this study.

In April 2003 and April 2004, about 50 maize stalks from the previous maize crop were collected randomly from the soil surface along a transect running the length of each plot. The distance between collected stalks within plots was at least 1 m. The stalks were air-dried at room temperature for five days prior to isolations. From each stalk, two pieces of about 1 cm^2 from the first above-crown internode were surface-sterilized by washing sequentially in 98% ethanol, 1% sodium hypochlorite, and twice in sterile water. Subsequently, the two pieces were placed on a maltagar plate containing rifampicin [15 g malt extract (Oxoid, Hampshire, United Kingdom), 12 g agar (Oxoid), 100 mg rifampicin (Sigma-Aldrich, Steinheim, Germany), pH 6.5]. The plates were incubated for five days at 24 °C in the dark and from each plate mycelium tips of up to six fungal colonies with morphology resembling a *Fusarium* species were transferred to new agar plates. The isolated fungi were grown in flasks containing 40 ml of 1.5% malt extract (Oxoid) solution for 5–7 days at 24 °C on a shaker at 100 rpm. Mycelium was harvested by filtration through paper discs, lyophilized, and ground with glass balls in a FastPrep FP120 machine (Savant Holbrook, NY, USA). DNA was extracted from 20 mg pulverized mycelium of each isolate with the NucleoSpin 96 Plant kit (Macherey-Nagel, Düren, Germany) using a Tecan Genesis RSP 150 robotic sample processor (Tecan, Männedorf, Switzerland). DNA concentrations were estimated by gel electrophoresis.

PCR identification of Fusarium *isolates*

In order to develop a multiplex PCR assay for efficient identification of *Fusarium* isolates from overwintered maize stalks, group- and species-specific PCR primers documented in the literature were tested with various PCR protocols on a collection of reference isolates. A PCR protocol with stepwise decreased annealing temperature allowed the combination of 10 primer pairs in two multiplex reactions. The multiplex PCR 1 contained three primer pairs (Table 1), one for the detection of the genus *Fusarium*-specific ITS sequence, one for the detection of the trichodiene synthase gene *tri5* required for trichothecene biosynthesis, and one for the detection of the polyketide synthase gene *fum5* required for fumonisin biosynthesis. The multiplex PCR 2 contained seven primer pairs (Table 1), each amplifying a specific fragment for an agronomically important *Fusarium* species. The primer pair VER for identification of *F. verticillioides* and the primer pairs JIA and FAC for improved discrimination between *F. avenaceum* and *F. acuminatum* (Demeke et al., 2004) did not amplify under multiplex PCR conditions and were used in individual PCR (Table 1). All PCRs were

Table 1. Primers used for identification of *Fusarium* isolates in multiplex and single primer PCRs

Primer specificity	Primer name	Primer sequence (5′ → 3′)	Fragment size (bp)	PCR No.	Test isolate	Reference
Fusarium spp.	ItsF	AACTCCCAAACCCCTGTGAACATA	431	1	all below	(Bluhm et al., 2002)
	ItsR	TTTAACGGCGTGGCCGC				
tri5 gene	Tox5F	GCTGCTCATCACTTTGCTCAG	658	1	Fg9701	(Niessen and Vogel, 1998)
	Tox5R	CTGATCTGGTCACGCTCATC				
fum5 gene	Fum5F	GTCGAGTTGTTGACCACTGCG	845	1	FmoMS2	(Bluhm et al., 2002)
	Fum5R	CGTATCGTCAGCATGATGTAGC				
F. poae	Fp82F	CAAGCAAACAGGCTCTTCACC	250	2	Fpo696	(Parry and Nicholson, 1996)
	Fp82R	TGTTCCACCTCAGTGACAGGTT				
F. graminearum	Fg16NF	ACAGATGACAAGATTCAGGCACA	280	2	Fg9701	(Nicholson et al., 1998)
	Fg16NR	TTCTTTGACATCTGTTCAACCCA				
F. proliferatum	TH5F	GATAACGTCCAAGGCTACG	330	2	FproM827	(Waalwijk et al., 2003)
	TH5R	GGGGTCGTTCAGCTCAAGG				
F. subglutinans	61-2F	GGCCACTCAAGAGGCGAAAG	445	2	Fsu685	(Möller et al., 1999)
	61-2R	GTCAGACCAGAGCAATGGGC				
F. culmorum	Fc01F	ATGGTGAACTCGTCGTGGC	570	2	Fc9711	(Nicholson et al., 1998)
	Fc01R	CCCTTCTTACGCCAATCTCG				
F. crookwellense	CroAF	CTCAGTGTCCACCGCGTTGCGTAG	842	2	Fcr677	(Yoder and Christianson, 1998)
	CroAR	CTCAGTGTCCCAATCAAATAGTCC				
F. avenaceum	FaF	CAAGCATTGTCGCCACTCTC	920	2	FavM928	(Doohan et al., 1998)
(*F. acuminatum*)	FaR	GTTTGGCTCTACCGGGACTG				
F. avenaceum	JIAf	GCTAATTCTTAACTTACTAGGGGCC	220	3	FavM928	(Turner et al., 1998)
	JIAr	CGTTAATAGGTTATTTACATGGGCG				
F. acuminatum	FAC-F	GGGATATCGGGCCTCA	602	4	Fac1397	(Williams et al., 2002)
	FAC-R	GGGATATCGGCAAGATCG				
F. verticillioides	VER1	CTTCCTGCGATGTTTCTCC	578	5	FmoMS2	(Mule et al., 2004)
	VER2	AATTGGCCATTGGTATTATATATCTA				

done in a volume of 10 µl containing approximately 5 ng template DNA, 0.1 mM of each dNTP, 0.2 µM of each primer, and 1 U of *Taq* polymerase (New England BioLabs, Beverly, MA, USA). Multiplex PCRs were carried out with stepwise decreased annealing temperature as follows: 2 min 30 s denaturation at 94 °C, 5 cycles of 30 s at 94 °C, 30 s at 64 °C and 1 min at 72 °C, 5 cycles of 30 s at 94 °C, 30 s at 62 °C and 1 min at 72 °C, 25 cycles of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C with 10 min final extension at 72 °C. Single primer PCRs were performed under identical conditions except for a constant annealing temperature of 56 °C for all 35 cycles. The amplicons were separated with gel electrophoresis in a 2% agarose gel (Invitrogen, Paisley, United Kingdom) and identification was accomplished by comparison of amplicons with those of reference strains (Table 1). For statistical analysis, the frequency of each identified species was compared between stalks collected on corresponding Bt and non-Bt maize plots and between stalks collected in different years, using the Fisher exact test at $P < 0.05$ as implemented in Systat version 9 (Systat Software Inc., Richmond, CA, USA).

Development and application of microsatellites

For assessing the population genetic structure within *Fusarium* species, microsatellite markers were developed from DNA sequences with simple sequence repeats. Such sequences were found either by constructing a TC-repeat enriched clone library of the Swiss *F. culmorum* strain Fc9701 or by screening a *F. graminearum* genome sequence database (Syngenta, Torrey Mesa Research Institute, San Diego, CA, USA) as described in Naef et al. (2006a). Oligonucleotide PCR primers were designed on the flanking regions of repeat sequences with the online service Primer3 (S. Rozen, Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Primer specificity and

Table 2. Newly developed microsatellite loci for *F. graminearum*, *F. culmorum* and *F. crookwellense* in this study

Locus[a]	Primer sequence (5′ → 3′)	Repeat type	Dye label	PCR reaction	Number (and size range in bp) of alleles			Source
					F. gr. (N = 430)[b]	*F. cu.* (N = 57)[b]	*F. cr.* (N = 29)[b]	
MS-Fc1 (1.116/1/1)	F: AAGATACCAGGCCTTGATGG R: AGACTGCATGATTGTTAGCGAG	$(TC)_{17}$	D3	1	7 (141–159)	7 (141–163)	2 (133, 135)	[c]
MS-Fg30 (1.150/3/2)	F: TATGACTGCACGTTTGCTCC R: AGTCTCTAGTGTCGCCGAGG	$(AG)_{14}$	D4	3	14 (184–216)	1 (178)	1 (176)	[d]
MS-Fg98 (1.340/3/2)	F: TCTAGGCCAAACCCAGACAC R: TGTTGCGTTCCTGAAAACAG	$(GCAA)_6$	D4	3	5 (224–240)	1 (220)	1 (220)	[d]
MS-Fg90 (1.183/6/2)	F: GGAACCTTTTGTAGGTTGCG R: TGGGGTGAGGGGAAGTAAC	$(TC)_{15}$	D4	2	39 (127–211)	24 (119–215)	1 (115)	[d]
MS-Fg103 (1.224/4/3)	F: GGTATCCGTACAACCCGATG R: TTCTTTGATTTGGACCGAGG	$(TG)_{19}$	D3	1	24 (223–269)	2 (217, 221)	4 (219–239)	[d, e]
MS-Fg97 (1.224/4/3)	F: ACCACTTTTGGTTACAGGCG R: CCCTTTACTCAAGCTCACGC	$(CCTA)_8$	D2	1	9 (217–269)	1 (209)	1 (209)	[d]
MS-Fg75 (1.286/7/4)	F: TTGGTATGAAAGACTGCCCC R: GAAGCGCAAAAGGTATGCTC	$(GAAA)_{11}$	D3	3	27 (191–295)	15 (207–327)	1 (191)	[d]
MS-Fg60 (1.368/8/4)	F: GAGCCATTCACATGTACCCC R: TCCTCTCGCAAGTGTTGTTG	$(GCCA)_6$	D2	3	8 (147–187)	1 (163)	1 (155)	[d]

[a] Primer name and in brackets Contig No./Linkage group/Chromosome, according to the genome sequence of *Gibberella zeae* strain PH-1 and Gale and Kistler's genetic maps (available online in the *F. graminearum* database from the Broad Institute, Cambridge, MA).

[b] *F. gr.* = *Fusarium graminearum*, *F. cu.* = *Fusarium culmorum*, *F. cr.* = *Fusarium crookwellense*, N = sample size.

[c] Repeat-enriched clone library of *F. culmorum* strain Fc9701.

[d] *F. graminearum* genome database of the Torrey Mesa Research Institute (Syngenta), San Diego, CA, USA.

[e] Previously published by Naef et al. (2006a).

length polymorphism of the amplified fragment were tested with [γ-^{33}P]-labelled PCR primers and polyacrylamide gels, using DNA of different *Fusarium* species (see reference strains listed above), *Microdochium nivale*, *Trichoderma atroviride*, *Pseudomonas fluorescens*, and maize. Polymorphic microsatellite loci were localized in the *F. graminearum* genome by a BLAST search in the *Giberella zeae* strain PH-1 genome sequence (*Fusarium graminearum* database available online at *http://www.broad.mit.edu/annotation/fungi/fusarium/* from the Broad Institute, Cambridge, MA, USA) and subsequent assignment of the resulting sequence number (contig) to a chromosome and a linkage group in Gale and Kistler's genetic maps (available at the same web page). Eight microsatellite loci were selected for population analysis. The alleles at these loci were analyzed with three PCR assays and two fragment analysis runs on a capillary sequencer, using primers labelled with different fluorescence dyes (Beckman-Coulter, Fullerton, CA, USA). PCR 1 contained three multiplexed loci, PCR 2 a single locus, and PCR 3 four multiplexed loci (Table 2). Each reaction was done in a volume of 10 µl containing approximately 5 ng template DNA, 0.1 mM of each dNTP, 1 U of *Taq* polymerase (New England BioLabs), and 0.2 µM of each primer. Amplifications were carried out under the following conditions: 5 min at 94 °C for denaturation, 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 60 s at 72 °C, with a final extension of 10 min at 72 °C. Post-amplification, 2 µl of PCR 1 (3 loci) pooled with 2 µl PCR 2 (1 locus), or 2 µl of PCR 3 solely (4 loci) were mixed with deionized water to reach a final volume of 20 µl. After purification of diluted PCR products with Sephadex G-50 (Amersham Biosciences), 1 µl of the CEQ DNA size standard 400 (Beckman–Coulter) was added. Then, DNA fragments were separated in a CEQ 2000XL DNA analysis system (Beckman–Coulter) and fragment lengths were calculated with the software provided by Beckman-Coulter (version 4.2.0).

Analysis of the genetic structure of F. graminearum, F. culmorum *and* F. crookwellense

Microsatellite analysis was performed with isolates from overwintered maize stalks that were identified as *F. graminearum*, *F. culmorum*, or *F. crookwellense* and with unidentified *Fusarium* spp. isolates that showed *tri5* gene amplification. In addition, five randomly selected isolates of each other identified *Fusarium* species were included into the microsatellite analysis. Multilocus haplotypes were created by assigning microsatellite repeat numbers of each locus to the isolates. *Fusarium graminearum* isolates from stalks of the same maize variety collected in one year were considered as a population. Three levels of pooled populations were created after contingency chi-square tests (Systat) among individual populations revealed no significant differences in allele frequencies ($P > 0.05$): with all isolates from either Bt or non-Bt maize stalks within a collection year, with all isolates from one collection year, and with all *F. graminearum* isolates. Due to the small number of *F. culmorum* and *F. crookwellense* isolates (29 and 19, respectively), only pooled populations with all isolates were investigated for these species.

Maximum possible genotypic diversity was calculated by dividing the genotypic diversity according to Stoddart and Taylor (1988) through sample size N to address the variable sample size of populations. For each multilocus haplotype occurring more than once in a population, the probability P_{sex} of observing it at least as many times under assumption of sexual reproduction was calculated with a binomial expression based on allele frequencies as described by Parks and Werth (1993). After removing repeated multilocus haplotypes in populations, linkage disequilibrium between loci was estimated with the multilocus index of association I_A as indicator for clonal reproduction. The I_A was calculated with MultiLocus ver. 1.2 (P. M. Agapow and A. Burt, Department of Biology, Imperial College at Silwood Park, Ascot, United Kingdom) and compared with the I_A expected under random mating, using 1000 randomizations of individual alleles with linkage group information for loci. Allelic richness and Nei's unbiased gene diversity (Nei, 1987) were calculated without repeated multilocus haplotypes for each locus and averaged over all loci using FSTAT ver. 2.9.3.2 (J. Goudet, Institue of Ecology, University of Lausanne, Switzerland).

Genotypic diversities of *F. graminearum* were compared with a *t*-test (Chen et al., 1994) between Bt and non-Bt populations from single and pooled plots within collection years and between popula-

Table 3. Incidence of different *Fusarium* species and potential trichothecene-producing *Fusarium* (*tri5*) on overwintered stalks of Bt and non-Bt maize varieties[a]

Stalk collections	Number of stalks	Incidence of fungi[b]									
		F. gram.	*F. aven.*	*F. prol.*	*F. culm.*	*F. crook.*	*F. acum*	*F. subgl.*	*F. vert.*	*F. spp.*	*tri5*
Among years											
2003	322	32%	43%	19%	1%	1%	3%	1%	0%	7%	33%
2004	326	52%	8%	11%	8%	5%	2%	2%	4%	61%	58%
P [c]		**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	0.42	0.73	ND[d]	**< 0.01**	**< 0.01**
Within year 2003											
All non-Bt	161	27%	48%	20%	0%	2%	4%	1%	0%	6%	29%
All Bt	161	37%	39%	18%	2%	0%	1%	1%	0%	7%	38%
P		0.09	0.12	0.78	ND	ND	0.28	1.00	ND	0.65	0.10
Benicia	53	23%	21%	23%	0%	0%	2%	0%	0%	6%	23%
Benicia Bt	55	33%	33%	27%	0%	0%	2%	0%	0%	2%	33%
P		0.29	0.20	0.60	ND	ND	1.00	ND	ND	0.36	0.29
Prelude	50	52%	58%	20%	0%	6%	6%	4%	0%	10%	56%
Prelude Bt	55	47%	42%	22%	5%	0%	2%	0%	0%	7%	49%
P		0.70	0.12	1.00	ND	ND	0.35	ND	ND	0.73	0.56
Nobilis	58	10%	64%	17%	0%	0%	3%	0%	0%	2%	10%
Nobilis Bt	51	29%	41%	4%	0%	0%	0%	2%	0%	14%	31%
P		**0.02**	**0.02**	**0.03**	ND	ND	ND	ND	ND	**0.02**	**0.01**
Within year 2004											
All non-Bt	166	50%	7%	17%	8%	6%	2%	2%	7%	62%	55%
All Bt	160	54%	9%	4%	7%	3%	1%	1%	0%	60%	61%
P		0.44	0.55	**< 0.01**	0.68	0.29	0.37	0.37	ND	0.73	0.31
Benicia	57	49%	2%	19%	18%	4%	0%	0%	21%	53%	58%
Benicia Bt	55	67%	11%	5%	13%	0%	2%	0%	0%	73%	76%
P		0.06	0.06	**0.04**	0.60	ND	ND	ND	ND	**0.03**	**0.05**
Prelude	53	34%	15%	13%	0%	2%	6%	2%	0%	38%	36%
Prelude Bt	55	29%	15%	5%	0%	0%	0%	0%	0%	31%	31%
P		0.68	1.00	0.20	ND	ND	ND	ND	ND	0.54	0.68
DKC 3420	56	66%	5%	18%	7%	13%	2%	5%	0%	95%	71%
DKC 3420 Bt	55	68%	2%	2%	8%	10%	0%	2%	0%	78%	78%
P		1.00	0.62	**0.01**	1.00	0.76	ND	0.62	ND	**0.02**	0.51

[a] Fungi were isolated from overwintered stalks of transgenic Bt maize varieties and corresponding non-Bt maize lines grown in adjacent strip plots in two years.

[b] Percentage of stalks from which a *Fusarium* species or a potential trichothecene-producing *Fusarium* were isolated based on identification with PCR assays. (*gram.* = *graminearum*, *aven.* = *avenaceum*, *prol.* = *proliferatum*, *culm.* = *culmorum*, *crook.* = *crookwellense*, *acum.* = *acuminatum*, *subgl.* = *subglutinans*, *vert.* = *verticillioides*, *tri5* = potential trichothecene-producing *Fusarium* based on the presence of the trichodiene synthase gene).

[c] *P* values represent probabilities of equal incidences on stalks from two different collection years or on stalks from corresponding Bt and non-Bt varieties based on the Fisher exact test. Bold face indicates $P \leq 0.05$.

[d] ND: not determined (the *Fusarium* species was not detected in one or both categories).

tions from different collection years. Genotypic differentiation between pooled populations was further assessed by comparing the frequency distribution of repeated multilocus haplotypes with a chi-square contingency test (Systat). Chi-square tests were used further to compare allele frequencies per locus between Bt and non-Bt pools within years and between year pools. Assuming the stepwise mutation model for microsatellite alleles, genetic differentiation among pooled populations was estimated with the R_{ST} analogue Φ_{ST} (Michalakis and Excoffier, 1996) calculated with ARLEQUIN ver. 2.000 (S. Schneider, D. Roessli, and L. Excoffier, Genetics and Biometry Laboratory, University of Geneva, Switzerland). The null hypothesis of a lack of significant differences between populations was tested with 1000 permutations of individuals among populations.

Microsatellite analysis was performed also for the German wheat head blight *F. graminearum*

population (Miedaner et al., 2001) and the international *F. graminearum* isolates collection. The R_{ST} estimator Φ_{ST} for genetic differentiation between the *F. graminearum* maize stalk population and the wheat head population (Miedaner et al., 2001) was calculated as described above.

Immunoassay quantification of Bt protein and mycotoxin DON in maize stalks

After fungal isolations, the first above-crown internode of air-dried maize stalks were ground in a ZM1 centrifuge mill (Retsch, Haan, Germany) with a ring sieve with slot openings of 0.1 mm. Powder of stalks of the same variety were pooled and mixed thoroughly. The content of the *Fusarium* mycotoxin deoxynivalenol (DON) was quantified with the commercial ELISA kit Ridascreen Fast DON (R-Biopharm, Darmstadt, Germany) in three sub-samples of 30 mg from the pooled powder. For all Bt maize varieties, the content of Cry1Ab Bt protein was quantified with the commercial ELISA kit Cry1Ab QuantiPlate kit (EnviroLogix Inc., Portland, ME, USA) in three subsamples of 30 mg from the pooled powder prepared as described above. Means were compared with Fisher's least significant difference (LSD) test (Systat).

Results

Analysis of Fusarium *species abundance on overwintered maize stalks*

Fusarium isolates from overwintered maize stalks were identified with two multiplex PCRs and three individual PCRs with those primer pairs that did not amplify under multiplex conditions (Table 1). In about 90% of all cases, PCR with the *Fusarium* genus-specific primer pair confirmed our visual pre-selection of isolates based on mycelium morphology. *Fusarium* spp. strains were isolated from 431 of the 648 collected overwintered maize stalks (67%). Many stalks bore more than one *Fusarium* isolate, resulting in a total number of 1028 isolates. PCR analysis with species-specific primers showed that 62% of the stalks were colonized by more than one species. For both collection years together, the three most abundant species were *F. graminearum* (isolated from 42% of the stalks), *F. avenaceum* (26%), and *F. proliferatum* (15%). The other identified species, *F. acuminatum, F. crookwellense*, *F. culmorum*, *F. subglutinans*, and *F. verticillioides*, were each isolated from less than 5% of the stalks. Strains that belonged to genus *Fusarium* but did not amplify with one of the species-specific primers were isolated from 34% of the stalks. These isolates showed diverse colony morphology, suggesting that they belong to several different *Fusarium* species. None of the saprophytic *Fusarium* isolates was identified as *F. poae*. From the 393 potential trichothecene-producing isolates (amplification with Tox5 primers), 83% belonged to *F. graminearum*, 7% to *F. culmorum*, 3% to *F. crookwellense*, and 8% to unidentified *Fusarium* spp. While all *F. graminearum* isolates amplified Tox5, amplification failed for 10% and 44% of *F. culmorum* and *F. crookwellense* isolates, respectively. All of the nine isolates identified as *F. verticillioides* were potential fumonisin producers (amplification with Fum5 primers).

Comparison of *Fusarium* communities that were recovered in the years 2003 and 2004 showed several significant differences in fungal incidences on maize stalks (Fisher exact tests, Table 3). The frequency of unidentified *Fusarium* spp. was significantly higher in 2004 than in 2003 (on 58% and 33% of stalks, respectively). *Fusarium graminearum* was the dominant identified species in 2004 (on 52% of the stalks), but only the second most abundant species in 2003 (on 32% of the stalks). This corresponded with the trend for potential trichothecene-producing *Fusarium* (Tox5-positive isolates on 33% and 58% of the stalks, respectively). In 2003, the most abundant species was *F. avenaceum* on 43% of the stalks but present significantly less on only 8% of the stalks in 2004. *Fusarium proliferatum* was significantly more common in 2003 than in 2004 (on 19% and 11% of the stalks, respectively). Significantly lower incidences in 2003 than in 2004 were also observed for the less frequent species *F. culmorum* and *F. crookwellense*.

Comparisons of the incidences of *Fusarium* species between Bt and corresponding non-Bt maize stalks revealed no consistent differences over multiple hybrid pairs and both years (Table 3). However, for *F. proliferatum*, a lower abundance on Bt than on non-Bt maize stalks was observed for all hybrid pairs in 2004 (significant for Benicia and DKC 3420) and for the Nobilis hybrid pair in 2003 (Table 3). Significantly higher incidence on Bt than

Table 4. Genotypic and genetic diversity parameters[a] for *F. graminearum*, *F. culmorum* and *F. crookwellense* populations examined at 8 microsatellite loci

Population	*N*	*M*	repMLH	G_0/N	I_A[b]	R_t[b]	H[b]
F. graminearum from overwintered maize stalks							
2003 & 2004[c]	325	246	46**[d]	0.55	0.28* [e]	4.15	0.70
2003[c]	110	100	9**	0.83	0.21*	4.00	0.68
2004[c]	215	163	32**	0.56	0.36*	4.19	0.71
Non-Bt 2003[c]	45	44	1**	0.96	0.26*	3.93	0.67
Bt 2003[c]	65	58	6**	0.80	0.17*	3.95	0.68
Non-Bt 2004[c]	105	91	12**	0.77	0.37*	4.04	0.70
Bt 2004[c]	110	84	17**	0.59	0.44*	4.19	0.70
Benicia 2003	13	13	0	1.00	0.14	3.55	0.61
Benicia Bt 2003	20	20	0	1.00	0.11	3.62	0.65
Prelude 2003	26	25	1**	0.93	0.21*	3.99	0.70
Prelude Bt 2003	29	24	5**	0.74	0.15	3.96	0.71
Nobilis 2003	6	6	0	1.00	0.06	3.32	0.72
Nobilis Bt 2003	16	16	0	1.00	0.36*	3.67	0.68
Benicia 2004	29	29	0	1.00	0.81*	3.83	0.67
Benicia Bt 2004	42	36	6**	0.78	0.55*	4.00	0.68
Prelude 2004	36	28	6**	0.64	0.30*	3.99	0.69
Prelude Bt 2004	21	18	3**	0.78	0.02	3.93	0.70
DKC 3420 2004	40	37	3**	0.87	0.25*	4.07	0.73
DKC 3420 Bt 2004	47	39	7**	0.72	0.77*	4.10	0.69
F. graminearum from wheat heads							
Sersheim 1992	70	37	22**	0.39	0.25*	4.03	0.69
F. culmorum from overwintered maize stalks							
2003 & 2004	29	24	5*	0.74	0.10	2.67	0.34
F. crookwellense from overwintered maize stalks							
2003 & 2004	19	4	2	0.13	−0.31	1.38	0.19

[a] *N*: sample size; *M*: number of unique multilocus haplotypes; repMLHs: number of repeated multilocus haplotypes; G_0/N: maximum possible genotypic diversity (Stoddart and Taylor, 1988); I_A: index of association; R_t: allelic richness; *H*: Nei's (1987) gene diversity.
[b] I_A, R_t, and *H* were calculated for populations without repeated multilocus haplotypes.
[c] Populations from individual plots were pooled after exclusion of significant differences in allele frequencies with contingency chi-square tests at $P \leq 0.05$.
[d] For each repeated multilocus haplotype, the propability P_{sex} of its multiple encounter in a sexually reproducing population was calculated from microsatellite allele frequencies in the population (Parks and Werth, 1993). * and ** indicate $P_{sex} \leq 0.05$ and 0.01 for all repMLHs.
[e] The probability *P* of observing the multilocus association I_A under the assumption of random mating was estimated with 1000 randomly recombined datasets. *, indicates $P \leq 0.05$.

on non-Bt stalks were observed for *F. graminearum*, Tox5-positive, and unidentified *Fusarium* spp. isolates on stalks of Nobilis in 2003 and Benicia in 2004. For *F. avenaceum*, opposing trends were observed on different Bt/non Bt hybrid pairs (Nobilis 2003 and Benicia 2004). *Fusarium verticillioides* was isolated only from non-Bt stalks of Benicia in 2004, resulting in different incidences between non-Bt and Bt stalks and between years.

Microsatellite markers

Genetic markers with a variable number of simple sequence repeats were developed to study the population genetic structure of *Fusarium* species on overwintered maize stalks. Eight loci were selected from 28 tested loci with more than seven simple sequence repeats. PCR with primers designed on the flanking regions of these loci amplified a single DNA fragment of variable length with genomic DNA of *Fusarium* strains of the section *Discolor* (*F. graminearum*, *F. culmorum*, and *F. crookwellense*, Table 2). The primers of these loci did not amplify a fragment with genomic DNA from other tested organisms, including the *Fusarium* species *F. acuminatum* (section *Gibbosum*), *F. avenaceum* (section *Roseum*), *F. poae* (section *Sporotichiella*), *F. oxysporum* (section

Table 5. Differences in genotypic diversities and in distribution of repeated multilocus haplotypes (repMLH) between *F. graminearum* subpopulations[a]

Compared populations	Genotypic diversity[b]			repMLH distribution[c]		
	t	df	*P*	χ^2	df	*P*
2003 vs. 2004	3.49	318	< **0.01**	60.71	45	0.06
Bt 2003 vs. non-Bt 2003	1.53	108	0.13	1.86	8	0.99
Bt 2004 vs. non-Bt 2004	1.88	213	0.24	24.72	31	0.78
Benicia 2003 vs. Benicia Bt 2003	∞ [d]	31	1.00	ND[e]		
Prelude 2003 vs. Prelude Bt 2003	1.39	53	0.17	ND		
Nobilis 2003 vs. Nobilis Bt 2003	∞ [d]	20	1.00	ND		
Benicia 2004 vs. Benicia Bt 2004	2.64	69	**0.01**	ND		
Prelude 2004 vs. Prelude Bt 2004	0.85	55	0.40	ND		
DKC 3420 2004 vs. DKC 3420 Bt 2004	1.17	85	0.25	ND		

[a] Subpopulations were isolated from overwintered Bt and non-Bt maize stalks in two collection years.
[b] Differences in genotypic diversities according to a *t*-test (Chen et al., 1994) on the maximum possible genotypic diversity G_0/N (Stoddart and Taylor, 1988). Multilocus haplotypes were based on alleles at eight polymorphic microsatellite loci.
[c] Differences in distribution of repeated multilocus haplotypes according to a chi-square test on frequencies of repMLHs.
[d] The *t*-values became infinite because all haplotypes were unique to one population.
[e] ND: not determined (too few repeated multilocus haplotypes).

Elegans), *F. proliferatum*, *F. subglutinans*, and *F. verticillioides* (all section *Liseola*), the fungi *T. atroviride* and *M. nivale*, the bacterium *P. fluorescens*, and the plant *Zea mays*. According to Gale and Kistler's genetic maps (available online in the *F. graminearum* database from the Broad Institute, Cambridge, MA, USA), these eight microsatellite loci were distributed over all four chromosomes and over six linkage groups in the *F. graminearum* genome (Table 2).

Population genetic structure of F. graminearum, F. culmorum *and* F. crookwellense

All isolates of *F. graminearum*, *F. culmorum*, and *F. crookwellense* from overwintered maize stalks showed PCR amplification of the eight selected microsatellite markers. Amplification failed for isolates of other *Fusarium* spp., including Tox5 positive isolates, confirming the marker specificity observed with the reference strains mentioned above. The 325 *F. graminearum* isolates, originating from 273 maize stalks, were composed of 200 unique multilocus haplotypes (MLHs) and 46 repeated MLHs (Table 4). Multiple *F. graminearum* isolates from single stalks were genetically different on 33 maize stalks and genetically identical on nine maize stalks. Identical MLHs were found also on different maize stalks: 17 MLHs were shared among stalks within single strip plot collections, three MLHs were shared between strip plot collections within the same year, and 17 MHLs were shared between the two collection years. These repeatedly isolated MLHs of *F. graminearum* were unlikely to result from random mating ($P_{sex} < 0.01$; Parks and Werth, 1993) in 7 of 12 single plot collections and in all pooled collections (Table 4), indicating that they were asexually produced clones. Predominant asexual reproduction of the *F. graminearum* population on maize residues was further supported by significant multilocus gametic disequilibrium in 7 of 12 clone-corrected single plot populations and in all clone-corrected pooled populations ($P \leq 0.05$ for I_A with 1000 randomizations, Table 4).

The maximum possible genotypic diversity observed varied between 0.56 and 1 among *F. graminearum* isolates from maize stalk collections within strip plots (Table 4). Comparisons of genotypic diversities revealed no significant differences between corresponding Bt and non-Bt populations except for the hybrid pair Benicia in 2004 with a significantly lower genotypic diversity on Bt maize stalks (Table 5). A comparison between collection years revealed a significantly higher genotypic diversity in the pooled *F. graminearum* population from 2003 than from 2004 ($P < 0.01$, *t*-test, Table 5). The frequency distribution of repeated MLHs differed between collection years ($P = 0.06$, contingency chi-square test, Table 5) but not between pooled Bt and non-Bt populations within years. Allelic richness and

Table 6. Concentration of the Cry1Ab Bt protein and the *Fusarium* mycotoxin deoxynivalenol (DON) in air dried field-overwintered maize stalks [a]

Varieties	Cry1Ab Bt protein content (μg mg^{-1})[b]		DON content (μg mg^{-1})[b]	
	2003	2004	2003	2004
Benicia	not measured	not measured	0.00 ± 0.002 A	0.04 ± 0.033 A
Benicia Bt	0.11 ± 0.026 b	0.01 ± 0.001 a	0.08 ± 0.061 A	0.09 ± 0.067 A
Prelude	not measured	not measured	0.27 ± 0.076 AB	0.62 ± 0.160 BC
Prelude Bt	0.00 ± 0.001 a	0.00 ± 0.001 a	0.26 ± 0.067 AB	0.92 ± 0.291 C
Nobilis	not measured		0.00 ± 0.000 A	
Nobilis Bt	0.12 ± 0.020 b		0.04 ± 0.036 A	
DKC 3420		not measured		0.27 ± 0.092 AB
DKC 3420 Bt		0.10 ± 0.005 b		0.56 ± 0.143 BC

[a] Maize stalks of transgenic Bt and corresponding non-Bt maize varieties from the previous cropping season were collected from the soil surface in spring 2003 and 2004.
[b] Cry1Ab and DON concentrations were determined by quantitative ELISA. Values are means (±standard error of mean) of three sub-samples from a homogenized powder of the first above crown internode of 50 randomly collected maize stalks. Values followed by the same letter are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Nei's genetic diversity (Nei, 1987) were similar in *F. graminearum* single plot populations and in pooled populations (Table 4) and ranged from $R_t = 3.55$ to 4.19 and from $H = 0.61$ to 0.73, respectively. Comparisons of allele frequencies at microsatellite loci and R_{ST} estimates for genetic differentiation revealed no evidence for a substructure in the *F. graminearum* population between years or between Bt and non-Bt stalks within years. The highest R_{ST} value over all loci was 0.02 between Bt and non-Bt populations in 2003, which was not significant according to 1000 permutations of individuals among populations ($P = 0.32$).

The *F. graminearum* population on maize stalks was compared with a *F. graminearum* population collected from a naturally contaminated wheat field 100 km distant about 10 years earlier (Miedaner et al., 2001). Microsatellite analysis of the 70 wheat isolates revealed 37 unique and 22 repeated multilocus haplotypes. Seven of 35 pairs of isolates originating from different spikelets within single heads showed different MLHs. Isolates with identical MLHs were found not only within heads but also among different heads within and between sampling points. Significantly overrepresented repeated MLHs and significant multilocus genetic association indicated a high contribution of asexual dispersal also in this *F. graminearum* population (Table 4). Six MLHs isolated from overwintered maize stalks were found also in the wheat-pathogenic population. Size corrected genotypic diversity was significantly lower on wheat heads than on maize stalks ($P < 0.01$, *t*-test for pooled populations) but allelic richness and Nei's genetic diversity in the clone-corrected populations were similar ($R_t = 4.03$ and 4.15 and $H = 0.69$ and 0.70). Allele frequency distributions differed between the two populations for four of the eight microsatellite loci ($P \leq 0.05$, contingency chi-square tests) but the genetic differentiation based on R_{ST} estimator Φ_{ST} was only 0.04. However, this differentiation was close to statistical significance ($P = 0.07$ according to 1000 permutations).

The pooled *F. culmorum* and *F. crookwellense* populations from overwintered maize stalks consisted of unique and repeated MLHs (Table 4). For *F. culmorum*, the null hypothesis of random mating could be rejected for the repeated multilocus haplotypes ($P_{sex} \leq 0.05$) but not for the association of loci ($P = 0.88$, according to 1000 randomizations, Table 4). Neither test supported clonal reproduction in the *F. crookwellense* population. Allelic richness and genotypic diversity were lower in the *F. culmorum* and the *F. crookwellense* populations than in the *F. graminearum* population from maize stalks, corresponding to the lower number of polymorphic loci in these species (Table 2).

Content of Bt protein and mycotoxin DON in overwintered maize stalks

The analysis of Cry1Ab protein content in overwintered maize stalks revealed Bt protein levels

between 0 and 120 ng per mg dry sample (Table 6). Significant differences in the content of Bt protein were found between maize stalks of different Bt hybrids within collection years and between stalks of Benicia Bt from two different years. The overwintered maize stalks were further analyzed for the DON content (Table 6). The DON concentrations varied between 0 and 920 ng per mg dry sample. Significant differences in the DON concentration were found between maize hybrids but not between corresponding Bt and non-Bt maize lines.

Discussion

Fungi of the genus *Fusarium* were isolated from 67% of overwintered maize stalks collected from the soil surface in a field trial with different Bt/non-Bt maize hybrid pairs. A new combination of multiplex and single primer PCR assays allowed efficient identification of agronomically important *Fusarium* specimens, namely, two groups of potentially mycotoxigenic *Fusarium* spp. and nine maize- or wheat-pathogenic *Fusarium* species (Table 1). The three most abundant species were *F. graminearum*, *F. avenaceum*, and *F. proliferatum*. Their prevalence on the collected stalks (42%, 26%, and 15%, respectively) corresponded with other maize residue surveys (Windels et al., 1988; Cotten and Munkvold, 1998), indicating their high saprophytic competitiveness on maize residues. Other species, including *F. acuminatum, F. crookwellense*, *F. culmorum*, *F. subglutinans*, and *F. verticillioides*, were isolated from less than 5% of the stalks. In the two collection years, different incidences were observed for several species (Table 3), most likely reflecting different species preferences for changing weather conditions. No consistent differences were found in the comparison of species incidences between corresponding Bt and non-Bt maize stalks (Table 3). However, a significantly reduced *F. proliferatum* incidence on Bt maize stalks was found in three Bt/non-Bt hybrid pair comparisons (Nobilis in 2003, Benicia in 2004, and DKC 3420 in 2004, Table 3). A reduced proportion of *F. proliferatum* has been reported in the maize stalk rot disease on Bt maize plants (Gatch and Munkvold, 2002), suggesting that a reduced *F. proliferatum* infestation on maize plants may cause a reduced *F. proliferatum* inoculum on maize residues. But, this beneficial side effect of maize Bt transformation appears to depend on the Bt maize hybrid as well as on the collection year (no differential *F. proliferatum* incidence on Bencia Bt/non-Bt in 2003, Table 3). Furthermore, the sometimes significant reduction of *F. proliferatum* incidence was often accompanied by an increased *F. graminearum* incidence (Table 3), indicating that the absence of one species may open a niche for another species. The shift towards more *F. graminearum* on Bt maize stalks of Nobilis in 2003 and Benicia in 2004 corresponded with a shift towards more mycotoxigenic isolates (Table 3) because all *F. graminearum* isolates possessed the trichodiene synthase gene *tri5* (Niessen and Vogel, 1998). However, this increase was not correlated with increased DON concentrations in pooled stalk samples (Table 6), which indicates that mycotoxin contamination in residues cannot be predicted solely from the presence of toxigenic species, as suggested for wheat heads (Edwards et al., 2001).

The population structure of the *F. graminearum* isolates was assessed with newly developed polymorphic microsatellite markers (Table 2). About 80% of maize stalks bearing more than one *F. graminearum* isolate were colonized by different multilocus haplotypes (MLHs), suggesting saprophytic competition among haplotypes within stalks. Over all maize stalks, 200 MLHs were unique but 46 MLHs were shared among stalks and 17 MLHs were found in both collection years. All repeated MLHs were unlikely to occur more than once under assumption of random mating, pointing to an asexual component of reproduction in the *F. graminearum* population on maize residues. Association of microsatellite loci (Table 4) further supported the significance of asexual reproduction. Comparisons of genotypic diversities and frequencies of repeated MLHs revealed evidence for selection operating on haplotypes between collection years but not between Bt and non-Bt maize stalks (Table 5). We concluded that use of maize Bt hybrids has not yet resulted in noticeable selection of *F. graminearum* haplotypes.

Despite the observed haplotypic difference between collection years, there were no significant allelic differences, indicating that other evolutionary forces such as reproductive system and/or gene flow affected the genetic structure of the *F. graminearum* population. The assumption of

random mating was rejected but the performed tests (Table 4) cannot exclude a combination of asexual and sexual mode of reproduction. A sexual component of *F. graminearum* reproduction on maize residues (Sutton, 1982) could explain a low impact of haplotype selection on the allelic pool because recombination rearranges alleles between haplotypes. Sexually produced wind-dispersed ascospores could further support gene flow between strip plots which prevents substructuring within the saprophytic *F. graminearum* population.

Our observation of six shared haplotypes and genetic similarity between a maize residue population and a wheat head blight population from 100 km distant fields supports the assumptions of either that both populations originated from the same population recently or that genetic exchange exists between saprophytic and pathogenic *F. graminearum* populations over large distances. Clones can be dispersed over a large distance by ascospores (de Luna et al., 2002) from self-fertilization of homothallic strains. Interregional genetic exchange has also been found in US populations of *F. graminearum* (Zeller et al., 2004), but our observations are the first genetic evidence for the role of maize residues as a genetic diversity reservoir for wheat-pathogenic *F. graminearum*.

Microsatellite data were also generated for *F. culmorum* and *F. crookwellense* isolates from overwintered maize stalks. Sample sizes were too small to perform a meaningful comparison between years or between Bt and non-Bt isolates, but the data indicated lower genetic diversities in these species than in *F. graminearum*. This observation corresponds with the purely asexual reproduction assumed for these two species. However, the clonal nature of our *F. culmorum* and *F. crookwellense* populations was not statistically supported, indicating that the reduced number of polymorphic microsatellites (four and two, respectively) was insufficient for assessing the mating system. It remains to be determined with larger sample sizes and additional genetic markers whether the low microsatellite polymorphism represents a low genetic diversity in these species.

The fate of the transgene product in the environment is of general interest. We detected traces of the Cry1Ab Bt protein in overwintered maize stalks of Benicia Bt (=X0920 RT), Nobilis Bt (=Novelis), and DKC 3420 Bt (=TXP 138), all containing the transformation event MON810. The concentrations were only about 1% of the amount measured in autumn (A. Naef, unpublished data). This agrees with another report of persistence of a small part of Cry1Ab protein in slowly decomposing maize parts (Baumgarte and Tebbe, 2005). The absence of Bt protein in Prelude Bt (=Valmont) stalks corresponds with its transformation event Bt176 which causes no cry gene expression in non-green plant parts such as the lower stalk (Biotech Crop Database available online at *http://www.agbios.com* from AGBIOS, Merrickville, Canada).

In conclusion, we presented a new multiplex PCR assay for efficient identification of agronomically important *Fusarium* species and eight polymorphic microsatellite markers for *F. graminearum* that can be multiplexed in PCR and fragment analysis. Together with the published microsatellite markers for *F. graminearum* (Giraud et al., 2002; Suga et al., 2004), these tools will facilitate future population studies. The investigation of *Fusarium* species composition on overwintered maize stalks revealed no consistent differences between Bt and corresponding non-Bt maize residues. The effects of Bt transformation were minor compared to the effect of stalk collection year, suggesting that environmental conditions have a stronger impact on the saprophytic competition among *Fusarium* species than maize Bt transformation. The same conclusion can be drawn for haplotype competition within *F. graminearum* because our population genetic analysis revealed evidence for genotypic differences between stalk collection years but not between Bt and non-Bt maize stalks. The genetic similarity of our saprophytic and a wheat-pathogenic *F. graminearum* population further emphasized the importance of maize residues as a diversity reservoir for pathogenic *F. graminearum*.

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