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Antibiotic resistance marker genes as environmental pollutants in GMO-pristine agricultural soils in Austria

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ABSTRACT

Antibiotic resistance genes may be considered as environmental pollutants if anthropogenic emission and manipulations increase their prevalence above usually occurring background levels. The prevalence of *aph(3′)-IIa/npt*II and *aph(3′)-IIIa/npt*III – frequent marker genes in plant biotechnology conferring resistance to certain aminoglycosides – was determined in Austrian soils from 100 maize and potato fields not yet exposed to but eligible for GMO crop cultivation. Total soil DNA extracts were analysed by *npt*II/*npt*III-specific TaqMan real time PCR. Of all fields 6% were positive for *npt*II (median: 150 copies/g soil; range: 31–856) and 85% for *npt*III (1190 copies/g soil; 13–61600). The copy-number deduced prevalence of *npt*III carriers was 14-fold higher compared to *npt*II. Of the cultivable kanamycin-resistant soil bacteria 1.8% (95% confidence interval: 0–3.3%) were positive for *npt*III, none for *npt*II (0–0.8%). The *npt*II-load of the studied soils was low rendering *npt*II a typical candidate as environmental pollutant upon anthropogenic release into these ecosystems.

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1. Introduction

Agricultural soils are versatile gene exchange platforms by providing the physical matrix for closing the cycle of bi-directional antibiotic resistance gene transfer between human-, animal-, soil- and plant-associated bacteria (Chee-Sanford et al., 2009). Application of manure, unprocessed sewage sludge or treated wastewater to the fields act as drivers for resistance gene transfer and pathogen exchange (Boxall et al., 2004; Gatica and Cytryn, 2013; Marti et al., 2013). These anthropogenic wastes are usually contaminated with antimicrobial agents originating from prophylactic or therapeutic treatments (Boxall, 2004; Chee-Sanford et al., 2009) which support – even if present only at sub-inhibitory concentrations – the fixation of new resistance traits in the affected bacterial populations (Andersson and Hughes, 2014; You and Silbergeld, 2014).

Antibiotics are produced naturally by indigenous soil bacteria in pristine soil habitats (Allen et al., 2010), however, at concentrations typically far below clinically relevant inhibitory concentrations (Gullberg et al., 2011). Antibiotics of anthropogenic origin appearing in concentrations usually not encountered in natural environments are considered by some scientists as environmental pollutants (Martinez, 2009; Tello et al., 2012).

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Similarly, antibiotic resistant bacteria (ARB) under antimicrobial selection pressure may be regarded as environmental pollutants if they carry antibiotic resistance genes (ARG) on mobile genetic elements (MGE) and are introduced into previously non-exposed ecosystems by human activities (Martinez, 2009).

Some authors have extended this concept and propose that antibiotic resistance genes per se may be viewed as environmental pollutants if anthropogenic emission and manipulations raise their prevalence in affected ecosystems above naturally occurring background levels leading to an increased likelihood for adverse effects on human, animal or environmental health (Li et al., 2015; Martinez, 2009; Martínez, 2012; Martinez and Olivares, 2012; Pruden and Arabi, 2012; Pruden et al., 2006; Zhu et al., 2013).

The aminoglycoside phosphotransferase genes *npt*II (*aph*(3′)-*IIa*) and *npt*III (*aph*(3′)-*IIIa*) are the most frequently used antibiotic resistance markers (ARM) in plant gene technology (Miki and McHugh, 2004; Rosellini, 2012). NptII inactivates kanamycin and neomycin - both recently classified as critically important antibiotics for humans and animals (WHO, 2012) – and paromomycin, ribostamycin, lividomycin, butirosin, gentamicin B and isepamicin (Shaw et al., 1993). NptIII additionally phosphorylates amikacin which is a crucial antimicrobial for treating severe human infections and a second line antibiotic in combatting multi-drug resistant tuberculosis (Shaw et al., 1993; WHO, 2014).

Several transgenic crops (e.g. maize MON863, potato EH92-127-1, cotton MON531, MON1445) contain *npt*II genes (EFSA, 2015), which may become accessible for DNA-uptake by competent soil or gut bacteria upon decay of plant cells (de Vries and Wackernagel, 2004). The application of *npt*II has been approved by risk assessment bodies (Bennett et al., 2004; EFSA, 2004, 2007, 2009; FDA, 1998; Gay and Gillespie, 2005; Goldstein et al., 2005) whereas the insertion of *npt*III into the genome of transgenic crops for food and feed use was discouraged (EFSA, 2004).

*Npt*II and *npt*III fulfil several criteria consistent with a classification as environmental pollutants as suggested by Martinez and Olivares (2012): *Npt*II was originally identified as a resistance determinant on transposon Tn*5* from an *Escherichia coli* isolate (Beck et al., 1982), *npt*III originates from a conjugative plasmid of *Enterococcus faecalis* (Trieu-Cuot and Courvalin, 1983). Both ARM genes derive, thus, from human clinical sources and are associated with mobile genetic elements. In the transgenic plant genome they are present as artificial constructs of anthropogenic origin. Crop cultivation leads to ARM gene exposure of large crop growing areas most likely increasing the abundance of similar resistance alleles in the extracellular DNA fraction of the affected soils (de Vries et al., 2003). DNA uptake and recombination in competent soil bacteria may result in the formation of new resistance determinants adversely interfering with antimicrobial chemotherapy of infectious diseases if acquired by human or animal pathogens (Woegerbauer et al., 2015). However, quantitative information on the naturally occurring prevalence of npt*II* and npt*III* in affected agricultural soils is lacking.

Here we test the hypothesis that ARGs per se can act as environmental pollutants for two ARM genes (npt*II*, npt*III*; present in transgenic plants) in a defined environment (GMO-pristine agricultural soils) providing quantitative data for fields selected to represent Austrian maize and potato growing regions. The obtained results establish the naturally occurring abundances of these resistance genes in the respective soils. The data can be applied for a comprehensive assessment of the impact caused by an exposure of these environments with these ARM genes and are useful for their assignment as environmental pollutants.

2. Materials and methods

2.1. Selection of test fields

Fifty maize and fifty potato fields were representatively selected from Austrian maize and potato growing regions (Figs. 1 and 2). For an in depth analysis of physicochemical properties and microbiological characterization 5 reference fields for each cultivated crop (maize: M1 – M5; potato: K1 – K5) were chosen (details: Supplementary Text S1).

2.2. Soil sampling and processing

From each test field a composite soil sample comprising of ten single soil extractions from the rhizosphere of ten individual crop plants (extraction layer: 0–25 cm) was obtained shortly before harvest (6.8. – 9.9.2011) following a predefined, universally deployable sampling scheme (Fig. S1). A total of approximately 750 g of the composite soil sample were carefully mixed and stored at 8 °C–10 °C during transportation. Approx. 50 g of each crude batch were sieved (grid size: 2 mm) and used for DNA extraction and cultivation of soil bacteria. Samples were processed within 1–2 days at the collection center (AGES, Spargelfeldstrasse 191, 1220 Vienna). To avoid cross-contamination throughout the whole soil sampling and processing procedures a strict safety protocol was adhered including use of disposable gloves and hypochlorite decontamination of the devices. The physicochemical soil properties were analyzed according to national guidelines (ÖNORM1061, 1080, 1083, 1085, 1087, 1095) in a certified laboratory (Tables S1 and S2). Additionally the following data were collected for each composite soil sample: preceding crop, organic/inorganic fertilizer, soil type and humidity, and weather conditions during sampling.

2.3. Total DNA extraction from soils

DNA from 4 × 250 mg (wet weight) of a homogenized and sieved composite soil sample was extracted using the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA USA) according to the manufacturer's instructions. The eluted four DNA extracts per composite sample were pooled and stored at −20 °C until further analysis. Quantity and quality of the purified DNA were determined using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and agarose gel electrophoresis. Samples were excluded and re-extracted if the $OD_{280/260}$ ratio was below 1.8 and/or extensive low molecular weight smearing occurred on the gel.

DNA extraction and purification efficiency was evaluated in a test soil from a maize field spiked with a defined number of *Erwinia amylovora* CFBP 6449 (10^8 cells per gram soil). A logarithmic dilution series of the Powersoil DNA extracts was quantified in four replicates using an *E. amylovora* specific TaqMan assay (Gottsberger, 2010). The mean DNA recovery from soil using this procedure was approx. 60%. No inhibition was detectable at a 10^{-1} dilution of the Powersoil DNA eluate.

2.4. Detection of nptII and nptIII in total soil DNA

Tests for *npt*II and *npt*III gene targets were performed simultaneously in a single well on the LightCycler 480 real-time PCR platform (Roche; Vienna, Austria) using 96-well microtitre plates. PCR TaqMan double assays were prepared according to the manufacturer's recommendations (Ingenetix; Vienna, Austria). In brief the amplification mix contained 5 µl of 2× LC480 Probes Master (Roche), 0.5 µl of the *npt*III mix (0.6 µM primer each, 0.2 µM 5′YEE labelled probe) and 0.5 µl *npt*II mix (0.6 µM primer each, 0.2 µM

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