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RESEARCH ARTICLE

Detection of feral GT73 transgenic oilseed rape (*Brassica napus*) along railway lines on entry routes to oilseed factories in Switzerland

Mirco Hecht · Bernadette Oehen · Jürg Schulze · Peter Brodmann · Claudia Bagutti

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Abstract To obtain a reference status prior to cultivation of genetically modified oilseed rape (OSR, *Brassica napus* L.) in Switzerland, the occurrence of feral OSR was monitored along transportation routes and at processing sites. The focus was set on the detection of (transgenic) OSR along railway lines from the Swiss borders with Italy and France to the respective oilseed processing factories in Southern and Northern Switzerland (Ticino and region of Basel). A monitoring concept was developed to identify sites of largest risk of escape of genetically modified plants into the environment in Switzerland. Transport spillage of OSR seeds from railway goods cars particularly at risk hot spots such as switch yards and (un)loading points but also incidental and continuous spillage were considered. All OSR plants, including their hybridization partners which were collected at the respective monitoring sites were analyzed for the presence of transgenes by real-time PCR. On sampling lengths each of 4.2 and 5.7 km, respectively, 461 and 1,574 plants were sampled in Ticino and the region of Basel. OSR plants were found most frequently along the routes to the oilseed facilities, and in larger amounts on risk hot spots compared to sites of random sampling. At three locations in both monitored regions, transgenic *B. napus* line GT73 carrying the glyphosate resistance transgenes gox and CP4 epsps were detected (Ticino, 22 plants; in the region of Basel, 159).

Keywords Genetically modified · GM plants · Oilseed rape · *B. napus* · Herbicide resistance · Glyphosate · Seed spillage · Transport · Monitoring

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Introduction

In Switzerland, genetically modified (GM) plants have never been cultivated before and their cultivation is still prohibited until the end of 2017 due to a legal moratorium (The Federal Authorities of the Swiss Confederation 2003, The Federal Office for Agriculture FOAG 2013). Contrasting the EU, the Swiss federal law specifies the accidental 'release' (i.e., spillage and dispersal) of GM plants in the environment as an adverse effect (The Federal Authorities of the Swiss Confederation 2003). Hence, a monitoring concept for analyzing the ecological risks of GM plants, as envisaged by a policy paper by the environmental agencies of Austria, Germany, and Switzerland (Züghart et al. 2011), is needed to document a reference status in Switzerland. Furthermore, it must also consider spillage of GM seeds and accidental presence, dispersal, and persistence of feral GM plants in the environment. In a similar context, Pascher et al. (2011) designed and implemented the BINATS (Biodiversity-Nature-Safety) monitoring program in Austria wherein test areas across the Austrian agricultural landscape were mapped among others for the abundance of hybridization partners of oilseed rape (*Brassica napus* L., OSR).

In the present study, a monitoring concept was developed for Switzerland that aims at identifying hot spots of unintended

spillage and spread of unapproved GM plants, specifically OSR, in the environment.

Due to the unpredictable environmental risks, an application for the cultivation of GM OSR was rejected within the EU in 2005. An unofficial consensus within the EU Member States exists, that cultivation of GM OSR should still be banned. Thus, import of transgenic OSR is approved in the EU only when used for food and feed purposes (European Commission 2012). Nevertheless, its unintended entry into the environment cannot be excluded since, in Switzerland for example, contaminations of approved GM OSR lines are tolerated up to a proportion of 0.5 % without declaration as adventitious presence in seeds and feeding stuff (The Federal Authorities of the Swiss Confederation 2011a, b). At present, contaminations with three GM OSR events (GT73, MS8 × RF3, T45) are possible which all confer resistance to glyphosate and glufosinate, respectively. As shown by investigations in Japan, a cultivation ban does not implicitly prevent the unwanted occurrence and spread of GM OSR in the environment. Herbicide-resistant transgenic *B. napus* plants and seeds were detected and repeatedly confirmed along roadsides and around ports in Japan from 2004 to 2008 (Saji et al. 2005; Aono et al. 2006; Kawata et al. 2009; Nishizawa et al. 2009). Aono et al. (2006) detected stacked events with both glyphosate and glufosinate resistance genes in a proportion of seeds from two plants. Since resistance to multiple herbicides has not been commercially available as of yet, it has most certainly arisen by unintended hybridization of escaped single-trait events. In Canada and the USA, double-herbicide-resistant plants were also observed along transportation routes in GM OSR growing regions (Yoshimura et al. 2006; Knispel et al. 2008; Schafer et al. 2011). In these OSR growing areas, single herbicide resistance traits were detected in 30 to 80 % of feral OSR plants tested. Another significant indication of the escape of transgenic OSR into the environment was the detection of transgenic hybrids between *B. napus* and some of its weedy cross-hybridization partners along a roadside in Vancouver (*B. napus* x *Brassica rapa*, Yoshimura et al. 2006) and close to an OSR processing facility in Japan (*B. napus* x *B. rapa*, *Brassica juncea*, and *Sisymbrium* sp., respectively, Kawata 2010). It is generally acknowledged that these plants originated from GM OSR seed losses during transport. Spillage regularly occurs during transport or transition of germinable seeds along roads, railway lines, or at seed processing factories (Crawley and Brown 2004; von der Lippe and Kowarik 2007; Tamis and deJong 2009; Bailleul et al. 2012). In a 4-year study of the main parameters responsible for the occurrence of the tested feral OSR in France, Pivard et al. (2008) attributed 15 % to spillage during transport activities. Additional factors were seed losses during harvest or sowing of neighboring fields (accounting for 35 % of the overall feral OSR populations) as well as from processes of persistence (35–40 %) and seed spillage from local plants which were lost during harvesting (10 %).

In the present work, a risk targeted monitoring of GM OSR along railway lines from the Swiss borders with Italy and with France to the respective oilseed processing factories was developed and implemented.

Materials and methods

Monitoring concept

To identify "hot spots" for the monitoring of unapproved GM plants in the environment, a selection process was developed. In a first step, cases of unintended dispersal of GM plants into the environment were gathered based on literature review (Fig. 1). The literature was screened for information on the sources of the GM plants. The mentioned sources were compiled and assessed by expert opinion for their relevance for Switzerland (Fig. 1, step 1). The possible answers were "*yes*", "*no*", and "*no knowledge available*". No source could be excluded after this step.

From this preliminary list, the relevant sources were identified by using a selection matrix, where the source was weighted against several criteria (Fig. 1, step 2). Similar to the previous step, the possible answers were "*yes*", "*no*", or "*no knowledge available*". Criteria that received a "yes" in the first round were afterwards weighted according to their impact on the source: "*strong impact*" or "*weak impact*". "No knowledge available" was assessed as a "*strong effect*". This procedure resulted in a list of hot spots for unintended escape of GM plants in Switzerland such as import, discharge, and transport (inland and transit), processing of food and feed and adventitious presence of GM material in seeds. In a similar selection procedure, the GM plants relevant for a monitoring were identified (Fig. 1, step 2). Therefore, GM plants with a market approval in Switzerland and the EU or those used in large-scale field trials in the EU and Switzerland were compiled. No focus was given on the single events.

Subsequently, the relevant plants were identified on the basis of questions related to its potential to persist in Switzerland (Fig. 1, step 3). The possible answers were "*yes*", "*no*", and "*no knowledge available*". This selection process resulted in the list of the following most relevant plants: OSR, sunflower (*Helianthus annuus)*, alfalfa (*Medicago sativa)*, potato (*Solanum tuberosum*), flax (*Linum usitatissimum*), and sugar beet (*Beta vulgaris*). OSR was chosen as the most relevant species for monitoring for its potential to germinate outside arable fields, to establish feral populations (Pessel et al. 2001; Pivard et al. 2008; Beckie and Warwick 2010; Schafer et al. 2011) and for its ability to cross-hybridize with wild as well as cultivated relatives (Chèvre et al. 2004; Hails and Morley 2005; Ford et al. 2006; FitzJohn et al. 2007; Elling et al. 2010; Huangfu et al. 2011).

Fig. 1 Selection procedure to guide the monitoring activities aiming at the detection of the spread of GM plants in Switzerland. The procedure started with a compilation of possible sources for GM plants based on a literature review. In step 1, each source from this list was assessed according to its relevance for Switzerland. In step 2, external impacts (strong or weak impact) on the respective source were taken into account. Based on this information, the relevant sites for a monitoring could be selected. In step 3, the GM plant, that is relevant for a monitoring, was chosen according to environmental criteria. Based on the selection process, a monitoring for *B. napus* was conducted in Switzerland with a focus on transport routes from unloading point at the border to Swiss oilmills

Therefore, according to the selection process, import, discharge, transport (inland and transit), and processing of OSR were identified as most relevant for monitoring. We decided to focus on railway transportation lines rather than on highways because imported and domestic OSR is transported mainly by freight cars to different processing facilities.

Monitoring sites

The two largest oilseed processing facilities in Switzerland (Oleificio Sabo, Manno; Ölmühle Muttenz, Muttenz) and their main railway supply lines from the Swiss border were chosen as sampling areas. While parts of these railways are used exclusively for goods traffic (transit cargo and delivery to the oil mills), others are shared with passenger transportation. In Southern Switzerland (Ticino), this comprises a railway line of a total length of 36.7 km from Chiasso, close to the Italian border, via Taverne, Vedeggio to Manno, where an oil mill exists (Fig. 2). The second railway line is situated in Northern Switzerland (region of Basel). The test line covered altogether 14.8 km, from the French border in Basel (border crossing St. Louis) to Muttenz as well as from the Rhine port Birsfelden to Muttenz, where another OSR plant is located. Additionally, two loading/unloading sites in the Rhine port of Basel (Kleinhüningen) were sampled (Fig. 2).

The sampling concept considered the detection of a continuous leakage of OSR seeds as well as the survey of hot spots with the highest risk of seed spillage along the testing routes. To check for continuous leakage, the entire length of the identified railway lines was divided into subsections of 500 m. Subsequently, several subsections were randomly selected for sampling (Table 1). The subsections were first screened for accessibility. In cases where a subsection was too dangerous to sample because of railway tubes or bridges, the sampling was shifted in one of the adjacent subsections. In such a case, the subsection exhibiting a switch yard or a narrow bend was the preferred segment. If no switch yard or narrow bend was present, the less dangerous site was chosen. We considered switch yards, areas with increased switch points and narrow bends along the railway routes as regions where the spillage of seeds occurs with a higher probability, i.e., as hot spots. In addition, the entry lines, surroundings, and the premises of the oilseed processing facilities as well as the loading/unloading points of the Rhine port of Basel were included in the sampling and considered as hot spots.

In Ticino, the railway tracks where closed during our sampling, and the sampling was conducted as planned. In Basel, the rail traffic was always in progress. While following our sampling plan we had to decide at each subsection, whether the sampling was possible or too dangerous. During the first sampling, we had to make adjustments according to the safety guidelines of Swiss

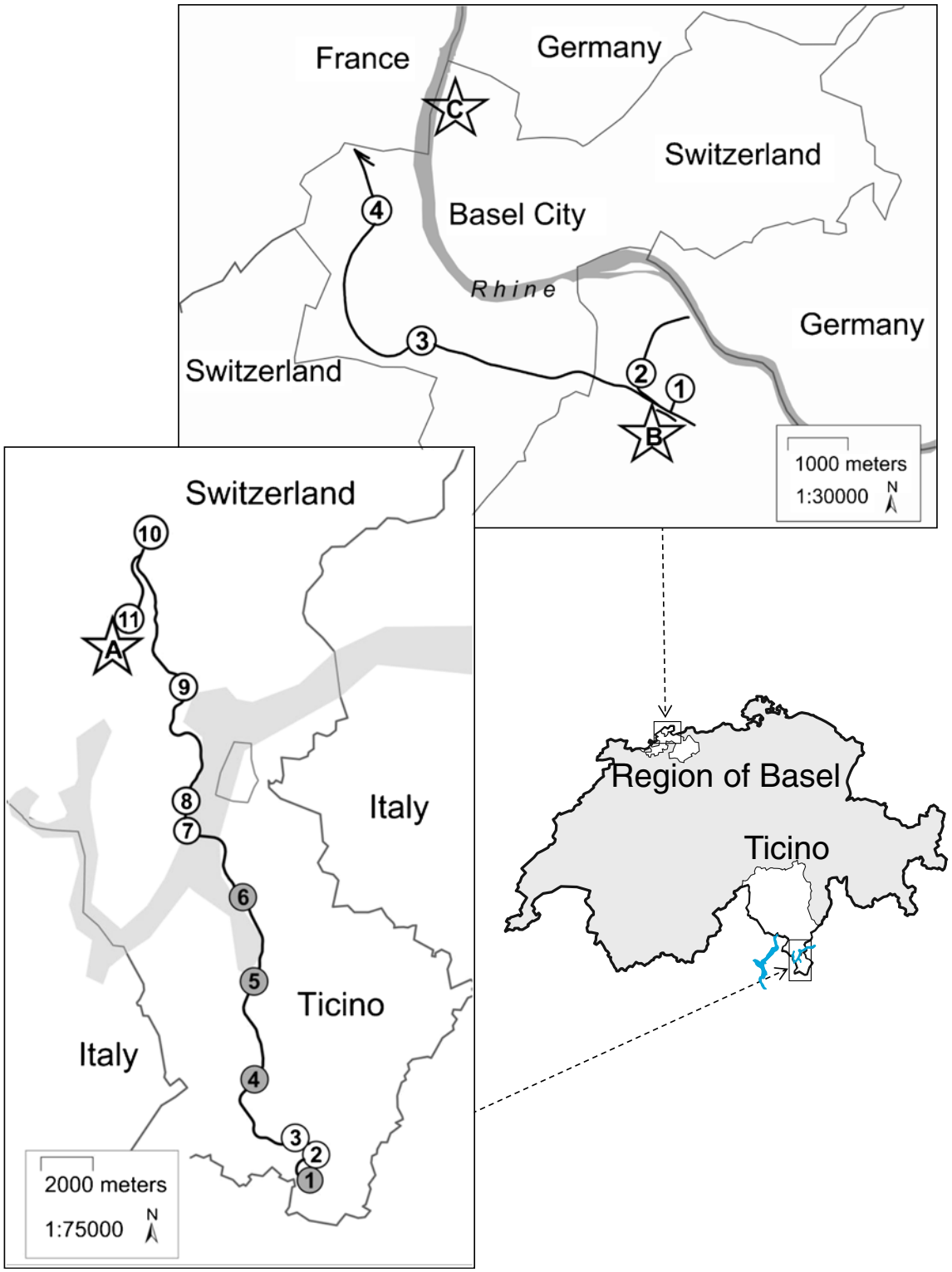

Fig. 2 Map of the monitoring areas in region of Basel and Ticino situated in Northern and Southern Switzerland, respectively. Sampling was performed along the route indicated by the *black line*. Sampling sites are marked with *numbers in circles*: Ticino: *1* Chiasso, *2* segment Chiasso–Balerna, *3* Balerna station, *4* Mendrisio station, *5* Capolago, *6* Marrogia, *7* Melide station, *8* segment Lugano-Paradiso–Melide, *9* Lugano station, *10* Taverne, 11 Vedeggio; region of Basel: *1* Muttenz yard station, *2* segment Muttenz–Rhine port of Birsfelden, *3* Basel station (SBB), *4* segment border crossing St. Louis–St. Johann station–tunnel entrance Kannenfeldplatz. *Grey shaded numbers* specify sites where no OSR was found at any monitoring occasion. *Capital letters* indicate oilseed mills in Manno (**A**), in Muttenz (**B**), and (un)loading points at the Rhine port of Basel (**C**)

Table 1 Sampling sites in Southern and Northern Switzerland and the number of (GM) OSR plants and cross-hybridization partners collected on these sites

Sampling sites (number/letter on Fig. 1)	Type of sampling site	Species	Sampled plants (no.)		GM plants (no.)
			2011	2012	2011/2012
Southern Switzerland (Ticino)					
Chiasso (1)	H	–	0[a]	0	–
Chiasso–Balerna (2)	R	*B. napus*	1[a]	0	–
		D. tenuifolia	2[a]	0	–
Balerna station (3)	R	*B. napus*	0	2	–
Mendrisio station (4)	H	–	0[a]	0	–
Capolago (5)	R	–	0[a]	0	–
Maroggia (6)	R	–	0[a]	0	–
Melide station (7)	H	*B. napus*	0[a]	8	–
Lugano-Paradiso–Melide (8)	R	*B. napus*	0[a]	6	–
Lugano station (9)	H	*B. napus*	13	14	13/9
Taverne (10)	H	*B. napus*	15	22	–
Oilmill Sabo, Manno (off-site, A, Vedeggio, 11)	H	*B. napus*	35	231	–
Oilmill Sabo, Manno (on-site, A)	H	*B. napus*	50	61	–
		R. raphanistrum	0	1[b]	–
Total Ticino		*B. napus*	114	344	22
		Other species	2	1	–
Northern Switzerland (region of Basel)					
Rhine port of Basel (Kleinhüningen, 5)	H	*B. napus*	Ns	198	ns/46
		D. tenuifolia	ns	28	–
Border crossing St. Louis–St. Johann station–tunnel entrance Kannenfeldplatz/(4)	H	*B. napus*	1[a]	141	0/113
		D. tenuifolia	0[a]	3	–
		S. officinale	2[a]	1	–
Basel station (Basel SBB, 3)	H	*B. napus*	0	8	–
Muttenz–Rhine port of Birsfelden (2)	H	*B. napus*	27	245	–
		S. arvensis	1	0	–
Muttenz yard station (1)	H	*B. napus*	7	145	–
		D. tenuifolia	2	0	–
Oilmill Muttenz (off-site, A)	H	*B. napus*	107	165	–
		D. tenuifolia	1	0	–
Oilmill Muttenz (on-site, A)	H	*B. napus*	78	414	–
Total region of Basel		*B. napus*	220	1,316	159
		Other species	6	32	–

H hotspot, *R* random, *ns* not sampled

[a] Only sampled at one time point

[b] Not analyzed

Federal Railways SBB. Therefore, it was not possible to follow the randomly selected subsections as planned, but all the subsequent samplings where executed according to adjustment done in this first sampling.

Sampling

All samplings were carried out twice each in 2011 and 2012 with the exception of the Rhine port of Basel (Kleinhüningen) which was sampled only in 2012. In Southern Switzerland (Ticino), the sampling took place on the following days: 2011: June 10th, June 11th, and September 29th; 2012: April 19th, April 20th, May 8th, September 17th, and September 18th. In Northern Switzerland (region of Basel), sampling was conducted on the following days: 2011: June 20th and October 10th; 2012: May 24th, May 31st, October 10th, and October 11th. The test sites were screened for the presence of *B. napus* and the following potential crossing partners *B. rapa*,

Brassica nigra, *Brassica oleracea*, *Diplotaxis tenuifolia*, *Sinapis arvensis*, *Sinapis alba*, *Raphanus raphanistrum*, *Erucastrum gallicum*, *Sisymbrium officinale*, *Sisymbrium orientale*, *Sisymbrium irio*, *and Hirschfeldia incana*. Small seedlings ('two leaf stage'), flowering plants, mature plants with developed seeds, and plants that were already fully senescent were collected. After sampling, plant material was stored at 4 °C until processed.

The sampling sites and the location of each plant sampled were mapped using GPS (data not shown).

DNA extraction

Before storing the plants at −80 °C, seeds were collected and leaf discs (Ø=10 mm) were stamped with a hole puncher. If possible, plant material from ten plants was pooled with keeping leaves and seeds in separate pools. Each pool consisted of either a maximum of 20 seeds (two seeds/plant) or ten leaf disks. If pools tested positive for one or more genetic modification markers, DNA was extracted from each individual plant belonging to these pools. The frozen plant material was ground to powder in liquid nitrogen using mortar and pestle. A maximum of 100 mg ground leaf material or 20 mg ground seeds per pool was used to extract DNA with the DNeasy Plant Mini Kit (Qiagen, Basel, Switzerland) in combination with the extraction robot QIAcube (Qiagen) according to the manufacturer's standard procedure. DNA concentration was measured by spectrophotometry (Nanodrop ND-1000, Fisher Scientific AG, Wohlen, Switzerland) and set to a concentration of 2 ng μl^{-1}.

Real-time PCR analysis of plant DNA

DNA was amplified and analyzed on a Rotor-Gene Q real-time PCR cycler (Qiagen) with the following cycling conditions: initial heating for 15 min at 95 °C followed by 50 cycles of amplification of 15 s at 95 °C and 60 s at 60 °C. All real-time PCR reactions were carried out in a 25 μl reaction volume containing 1× QuantiTect multiplex PCR Master Mix (Qiagen), 10 ng template DNA, and the primers and probes specified below (all purchased from Microsynth, Balgach, Switzerland). The plant-specific sequence of the actin gene and the false negative control (FNC) fragment were amplified in a duplex real-time PCR: act-F (5′-CAA GCA GCA TGA AGA TCA AGG T-3′, at a final concentration of 0.13 μM), act-R (5′-ACA ATC TGT TGG AAA GTG CT GAG-3′, 0.13 μM), act-P (5′-ROX-CCT CCA ATC CAG ACA CTG TAC TTY CTC TC-BHQ2-3′, 0.035 μM, Laube et al. 2010), fnc-F (5′-CGT CAC ATC GGT AGA CGA ACT AA-3′, 0.13 μM), fnc-R (5′-TTC AAG TCC TGA GCG GTT GTA A-3′, 0.13 μM), fnc-P (5′-JOE-ACC TAA CGC AGC AAC TTA TCG ACC GTT CAC TT-BHQ1-3′, 0.035 μM). To test the samples for the phosphinotricin acetyltransferase genes bar and pat from *Streptomyces hygroscopicus* and *Streptomyces viridochromogenes*, respectively, conferring a resistance against the herbicide glufosinate, as well as the glyphosate oxidoreductase gene gox from *Ochrobactrum anthropi* and the enolpyruvylshikimate-3-phosphate synthase gene CP4 epsps from *A. tumefaciens* CP4 mediating glyphosate resistance, the following primer and probes were used in a tetraplex real-time PCR reaction: bar-F (5′-CTG CAC CAT CGT CAA CCA CTA C-3′, 0.4 μM); bar-R (5′-GAT AGC GCT CCC GCA GAC-3′, 0.4 μM), bar-P (5′-FAM-CGT ACC GAG CCG CAG GAA CCG CAG GAG T-BHQ1-3′, 0.2 μM) for the amplification of a 110 bp fragment of the bar gene (GenBank® Acc.Nr. X05822.1: 222–331); pat-F (5′-CGC GGT TTG TGA TAT CGT TAA C-3′, 0.5 μM), pat-R (5′-TCT TGC AAC CTC TCT AGA TCA TCA A-3′, 0.5 μM), pat-P (5′-CY5-AGG ACA GAG CCA CAA ACA CCA CAA GAG TG-BHQ2-3′, 0.2 μM, Zeitler et al. 2002); gox-F (5′-CCG TGG AGG TTG GGA ACT T-3′, 0.5 μM), gox-R (5′-CCC TTG GTA AAG GCG TGA GA-3′, 0.5 μM), gox-P (5′-ROX-CTG ATG CAT TGC GTG ATT TCG ATC CTA AC-BHQ2-3′, 0.2 μM) for the amplification of a 107 bp fragment of the gox gene (Acc.Nr. BD008400, based on a conventional PCR reaction, Matsuoka et al. 2002); epsps-F (5′-CCA ATG GGT CGT GTG TTG AA-3′, 0.6 μM), epsps-R (5′-TTG GCG TTG GAG TCT TTG GT-3′, 0.6 μM), epsps-P (5′-JOE-AGA CGG TGA TCG TCT TCC AGT TAC CTT GC-BHQ1-3′, 0.2 μM, Zeitler et al. 2002). For the detection of the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (nos) terminator from *A. tumefaciens*, using the following primers and probes: 35S-F (5′-GCC TCT GCC GAC AGT GGT-3′, 0.64 μM), 35S-R (5′-AAG ACG TGG TTG GAA CGT CTT C-3′, 0.64 μM), 35S-FAM (5′-FAM-CAA AGA TGG ACC CCC ACC CAC G-BHQ1-3′, 0.16 μM, The Federal Authorities of the Swiss Confederation 2001); NOS-F (5′-ATG ACG TTA TTT ATG AGA TGG GTT TTT A-3′, 0.64 μM), NOS-R (5′-TTG CGC GCT ATA TTT TGT TTT C-3′, 0.64 μM), NOS-YY (5′-YY-AGA GTC CCG CAA TTA TAC ATT TAA TAC GCG A-BHQ1-3′, 0.16 μM, The Federal Authorities of the Swiss Confederation 2001). Detection of the GT73 event-specific sequence: GT73F1 (5′-TCA TAC TCA TTG CTG ATC CAT GTA GA-3′, 0.3 μM), GT73R1 (5′-AAG CTT ATA CGA AGG CAA GAA AAG G-3′, 0.9 μM), GT73TMP1 (5′-FAM-TTC CCG GAC ATG AAG ATC ATC CTC CTT C-DABCYL-3′, 0.2 μM, Mazzara et al. 2007). All primers and probes which were developed in-house were designed using Primer Express® Software (Applied Biosystems) and tested in silico for specificity and cross-reactivity using blast from the National Center for Biotechnology Information (Altschul et al. 1990).

Reference DNA

As positive control for real-time PCR, reference plasmids were used containing the target sequences for the detection

of either actin, bar, pat, gox, and CP4 epsps or 35S and nos-T. The reference sequences (not shown) were synthesized and cloned into the vector pCB2.1 by Eurofins MWG Operon (Ebersberg, Germany). The reference plasmids were used at a final amount of 10^3 copies per 25 μl PCR reaction. As an internal false negative control (FNC) a random synthetic DNA sequence of 111 bp length (ACG TCA CAT CGG TAG ACG AAC TAA TCG CTT AGG ATC CAA TAC CTA ACG CAG CAA CTT ATC GAC CGT TCA CTT CTA CTA AAG CTT CGT TTA CAA CCG CTC AGG ACT TGA ACA) was designed and cloned into the pGEM-T vector system (Promega, Wallisellen, Switzerland). The sequence does not exhibit significant homology with any known database sequence. As a positive control for the detection of GT73 OSR genomic GT73 DNA was used.

Results

Sampling

Southern Switzerland (Ticino) Of the 36.7 km railway line from Chiasso to Manno, 11.5 % (4.2 km) were sampled three times as scheduled and on one occasion a reduced sampling took place. Four hundred sixty-one individuals of OSR plants including their wild relatives were collected within the 2 years: 72 individuals at railway hot spots, 11 individuals at random sites along the railway lines, and 378 individuals in the vicinity of the oilseed facility (112 on-site and 266 off-site; Table 1). With the exception of three plants (1× *R. raphanistrum*, 2× *D. tenuifolia*) all sampled plants belonged to the species *B. napus* (458 plants, 99 %). At eight out of 12 sites (67 %) in Ticino, OSR could be found in at least one of the two surveyed years. While at four sites during the two monitoring years neither OSR nor any relatives were found, there were four sites where OSR plants were growing in both years. Compared to the random sampling along the railway lines, the vast majority of the sampled plants (450 out of 461, 98 %) was found at risk hot spots like unloading areas of the oilseed factories and switching yards of the examined hot spots (Table 1).

Northern Switzerland (region of Basel) The sampling in the northern part of Switzerland was not executed as scheduled due to the fact that some sites were not accessible and one sampling site was considered as too dangerous to be entered (despite the mandatory safety measures). Therefore, the sampling had to be adapted to the local conditions. Instead of the planned randomly selected sites of 500 m, 5.7 km of railway tracks were sampled representing 38.5 % of the tracks in the sampling area.

At the sampling sites in Northern Switzerland, we collected a total of 1,574 plants (Table 1). Thereof, 226 plants were found at the Rhine port of Basel, 583 plants were collected at railway hot spots and 765 plants originated from the premises and the vicinity of the oilseed factory (492 and 273, respectively). On a former parking area next to the oilseed factory, where transport trucks used to wait before the delivery of their load, we spotted OSR on each sampling occasion. The parking was not in use for more than 3 years and there was no treatment to reduce the vegetation. The individual plants occurred in different growth stages and some could be detected in following sampling. We assume this is a persistent, self-reproducing OSR population. Of the 1,574 sampled plants, 1,538 were identified as *B. napus* (98 %). The remaining samples were closely related species like *D. tenuifolia* (34×), *S. arvensis* (1×), *S. officinale* (3×).

The overall sampling line in the region of Basel was shorter compared to the line in Ticino. OSR could be found at all sampling sites in both monitored years, with substantially more specimens in 2012. The observed OSR plant numbers increased more than 13-fold at railway hot spots (40 plants in 2011 vs 543 in 2012) and approximately threefold (186 vs 579) at the site of the oilseed company.

Most OSR growing along the railway lines was detected in the ballast next to or in between the rails (Fig. 3). At the oilseed facilities, OSR could be found close to rail and truck unloading areas, where it was growing in the ballast, in concrete cracks or even in dense vegetation (e.g., lawn; Fig. 3).

Detection of herbicide-resistant OSR

DNA extracts from sample pools were analyzed for the presence of p35S and tNOS gene regulators as well as gox, CP4 epsps, bar, and pat transgenes (all conferring herbicide resistance) by real-time PCR. In both years and regions, transgenic OSR could be found (Fig. 2, Table 1). Three sample pools containing 13 plants collected in Southern Switzerland (station of Lugano, Ticino) in autumn 2011 proved positive for the resistance transgenes gox and CP4 epsps (average cT of positive pools 22, data not shown). Both transgenes confer resistance against the herbicide glyphosate (the active ingredient in among others Roundup®). In spring and autumn 2012, nine gox and CP4 epsps positive OSR plants (five and four plants, respectively, data not shown) were detected once more at the same location. Furthermore, the two glyphosate resistance transgenes were as well detected in pools originating from Northern Switzerland (port of Basel and station St. Johann, region of Basel). For all sample pools affected, DNA was extracted from individual plants and reanalyzed by real-time PCR for the gox and CP4 epsps genes as well as for the event-specific DNA sequence of the *B. napus* transgenic line GT73. As a result, 22 (Ticino) and 159 (region of Basel) *B. napus* individuals were identified to carry these three markers (cT values 20, 21, and 23, respectively, vs. cT of plant-specific actin 17, data not shown).

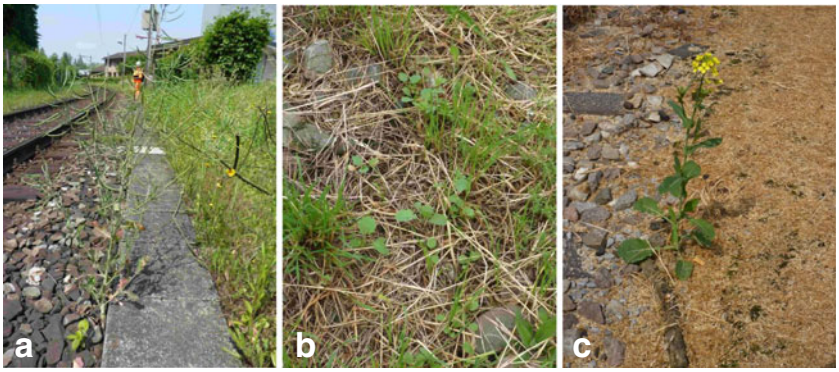

Fig. 3 OSR growing observed **a** in the gravel next to railway lines and **b** with competition of dense vegetation in the green verge adjacent to the railway gravel. **c** Transgenic GT73 OSR next to the railway line gravel surviving a recent application of glyphosate treatment

In Ticino, all GM OSR plants were collected on the same site, the railway station of Lugano. These plants represent the vast majority of all collected plants at this site (22 out of 27; 81 %, Table 1). The plants, which differed in their developmental stage (from seedlings to mature plants), were growing on a dead-end rail track at the northern end of the station platform. In the region of Basel, GM OSR was found in the port of Basel as well as between the French border and the station of St. Johann. At these sites the percentage of transgenic vs non-GM OSR was 23 % (46 out of 198; port of Basel) and 80 % (113 out of 141; station St. Johann), respectively (Table 1). The GM OSR plants in the port of Basel were located at two ship loading/unloading points between railway tracks (Fig. 3). The GM OSR plants close to the French border were found on a siding belonging to the station of St. Johann.

Discussion

In both border regions in Switzerland, OSR plants were frequently found along the railway import transportation routes to the oilseed manufacturer as well as in the area of the Rhine port of Basel. Highest numbers of *B. napus* plants occurred in the proximity of oilseed plants (on- and off-site). These locations represent likewise the only sampling sites, where seeds are regularly exposed for handling. In Ticino, second highest numbers of feral OSR were found at the sampling spot closest to the oilseed company (Taverne). Similar correlations between the abundance of OSR plants or seeds along transportation routes and the distance to oilseed processing facilities, grain silos, or ports have been established in earlier studies. Yoshimura et al. (2006) showed an exponential decline of feral OSR density and infestation area with increasing distance from grain terminals at the port of Vancouver, Canada. In Japan, feral OSR was found mainly along transportation routes leading away from harbors, where OSR was imported, towards the OSR mills (Kawata et al. 2009). Furthermore, a Dutch study identified unloading at ports, overland transport to processing plants, and disposal of seed-cleaning waste as the major sources of OSR seed spillage (Tamis and deJong 2009). Taverne is located north of the OSR plant; all other sites in the monitoring area of Southern Switzerland are situated in the south. While substantial amounts of conventional OSR are delivered to the oilseed factory in Manno from the north of Switzerland, only a marginal amount is imported from Italy by train (personal communication A. Cè, Oleificio Sabo, Switzerland). Since the entire OSR harvest in Switzerland is processed at mainly four oil mills (situated in Manno, Muttenz, Horn (Eastern Switzerland) and Etoy (Western Switzerland)), the railway route north of Manno is used exclusively for delivery of inland produced OSR to the Manno oil mill. This suggests that the plants found south of Manno originated from transport spillage of OSR seeds imported into or transited through Switzerland. Those north of Manno are thought to derive from spillage of inland produced OSR seeds that are processed at the oil mill. This interpretation corresponds with previous observations of increased incidences of feral OSR reflecting the direction of OSR seed transports (Crawley and Brown 2004; Kawata et al. 2009; von der Lippe and Kowarik 2007). Crawley and Brown (2004) observed significantly more OSR plants growing in the verge adjacent to the M25 motorway around London in the direction towards an oilseed processing facility rather than in the opposite verge (direction away from the facility).

At the monitored sites in Northern Switzerland, next to the premises of the oilseed processing factory in Muttenz, OSR plants were found on a former parking area that was unused for a couple of years according to the operating company. These OSR plants established a perennial population. Our findings of cumulative occurrence of OSR along the observed route therefore correlated with hot spots of (former) handling activities. Other prominent sources of feral OSR at these locations, such as fields with OSR (Pivard et al. 2008), could be excluded: with an exception of one sampling site (Balerna, Ticino, where only two OSR plants were found) which is in the vicinity of a test field of OSR no fields of OSR were documented in the two monitored regions.

In addition to the observation of feral non-GM OSR populations along the described transportation routes, also transgenic OSR was detected. In each of the monitored regions, we detected at least one location of glyphosate-resistant GT73 OSR, thus proving the suitability of the monitoring concept

for the present study. The stations of Lugano and St. Johann as well as the Rhine port of Basel were identified likewise by a study (Schoenenberger and D'Andrea 2012) commissioned independently by a different body. In the other study, which was conducted during the same period of time, Schoenenberger and D'Andrea (2012) sampled 79 railway stations and yards throughout Switzerland and Liechtenstein. The locations were chosen in order to cover the Swiss railway system for goods traffic with isolated samplings as densely as possible within their project. This contrasts with the approach presented in this study in which sampling was performed according to a risk-based monitoring and therefore exclusively along transportation routes to oilseed processing facilities and at OSR processing sites. This difference is reflected in the number of sampled plants (1,574 this study vs 2,453 Schoenenberger and D'Andrea 2012) and particularly the number of sampling sites (19 vs 79) which were both considerably lower for this study. Thus, both approaches accomplished the detection of the three locations of GM OSR (and of GMO in general) in Switzerland for the first time, however, monitoring at risk hot spots as presented here has emerged as the more efficient approach for detecting GM OSR.

The only other documented case of presence of GT73 OSR in the environment in Europe took place in Belgium 2008, where a single GT73 plant was found as far as 100-km away from a GT73 OSR test field (Berben 2009). To our knowledge, all other surveys of GM OSR along transportation routes conducted in Europe took place in Germany and remained negative (personal communication, A. Belter, State Ministry for the Environment Saxony Anhalt and N. Hess, Institute for Hygiene and Environment Hamburg, Germany). It remains therefore difficult to conclude, whether the escape of GM OSR into the environment represents a very rare event in Europe or actually occurs rather more frequently. Low sampling numbers or low sampling frequencies could be a possible reason for not finding more GM plants in the past surveys.

None of the three locations of GT73 OSR presented in this study corresponded to the sites of highest abundance of feral OSR. Therefore, rather than from delivery tours to the oilseed mill by train, these GM OSR plants likely originate from spilled seeds of GT73 cargoes transiting Switzerland or of conventional OSR contaminated with GT73 OSR seeds imported into Switzerland. Since GM OSR is not allowed for import as food and feed use and for cultivation (The Federal Authorities of the Swiss Confederation 2003, 2011a), the importers and processors of oilseed have installed common guidelines to avoid the import of GM material (trade organization Swiss granum). These guidelines are based on segregation, traceability, and testing.

However, at the stations of St. Johann and Lugano, GT73 OSR plants were detected on or close to dead-end haulage tracks, where no through traffic happens but freight cars are put on a siding. Although none of these locations are used to store OSR, cleaning of empty freight cars might be performed during temporary stops after unloading (personal communication U. Grützmacher, Securitrans AG, Bern, Switzerland). At the Rhine port of Basel, GT73 OSR was found exclusively at loading/unloading points. A domestic source could therefore be excluded. In addition, no other GM crops for food and feed use have been imported into Switzerland since 2007 (The Federal Office for Agriculture FOAG 2012). The GM OSR population might therefore originate from an earlier event indicating an established seed bank. At all three sites, the occurrence of GT73 OSR might also represent a contamination of imported crops other than OSR. For example, Canadian OSR producers were advised to implement crop rotations with cereals, especially with wheat (Canola council 2011, Schoonover 2012). Therefore, imported cereals may contain seeds from volunteer populations of GM OSR. In such a case, residues in the transporters or non-GM seeds, food, and feed imports containing GM OSR seeds below the 0.5 % declaration threshold for adventitious traces of GM plants (The Federal Authorities of the Swiss Confederation 2011a, b) could be responsible for the entry of GT73 OSR into the environment. Contrary to the feral transgenic OSR populations found in Japan (Saji et al. 2005; Aono et al. 2006; Kawata et al. 2009; Nishizawa et al. 2009), where GM OSR is imported as food and feeding stuff, the origin of the three Swiss feral GT73 OSR populations is thus less traceable, since there is no documented import of GM OSR into Switzerland.

In Switzerland, railway tracks are regularly treated with a glyphosate-containing herbicide by the operating company Swiss Federal Railways SBB. This happens on safety grounds (to avoid destabilization of gravel by plant growth) and is based on a unique permit (The Federal Authorities of the Swiss Confederation 2005). These management conditions, which are also practiced by other countries (e.g., Austria, Germany), may well promote the establishment of glyphosate-resistant GM OSR. Gravel beds create an ideal fallow habitat for GM herbicide-resistant OSR to germinate. Once emerged, GM OSR plants are positively selected by glyphosate applications (Londo et al. 2010). These circumstances were likely to promote the growth of GT73 OSR plants along Swiss railway tracks. Similarly, Yoshimura et al. (2006) observed a greater frequency of occurrence of glyphosate-resistant OSR in the area of Vancouver where glyphosate is used around grain elevators, as compared to the rural monitoring region of Saskatchewan in which glufosinate is commonly applied. However, herbicide selection pressure did not play a crucial role along transportation routes in Japan and the USA where the persistence of feral herbicide-resistant OSR was observed previously (Aono et al. 2006; Kawata et al. 2009; Nishizawa et al. 2009; Schafer et al. 2011).

Even without the application of herbicide, herbicide-resistant OSR prevails for up to 5 years post cultivation within cultivated fields, adjacent ruderal habitats or road sides (Warwick et al. 2008; Beckie and Warwick 2010; Knispel

and McLachlan 2010; Munier et al. 2012). Long-time persistence of GM OSR increases the risk of out-crossing of GM traits to wild relatives. Warwick et al. (2008) detected spontaneous hybridization between glyphosate-resistant *B. napus* and wild *B. rapa* in 2001. Although the number of hybrids decreased over time, F_1 and backcross hybrids and one introgressed individual were observed for at least 3 years after the absence of selection pressure. If herbicide was applied, selection for resistance could have led to an increased persistence of GM OSR and further gene flow (Stewart et al. 1997; Londo et al. 2010).

For the presented findings of transgenic OSR in Switzerland, persistence, hybridization, and poor information on transport, particularly of transit cargo, make it practically impossible to trace the time (year), the extent (number of seeds), or the origin (transit cargo vs import cargo, GMO cargo vs GMO contaminated non-GM OSR cargo vs contaminant of other source) of the spillage of GM OSR. By applying the estimations by Tamis and deJong (2009) of a 0.1 % loss of OSR seeds during transport, seed losses from imported, non-GM OSR to Switzerland in 2011 add up to around 7.5 tons (The Swiss Federal Customs Administration FCA 2012). Supposing a contamination of non-GM OSR with GM OSR as small as 0.001 %, 75 g, or more than 20,000 GM OSR seeds would have been lost during 2011 in Switzerland, and potentially 200 transgenic ones could have germinated thereof (assuming 1 % germination rate). The occurrence of feral transgenic GT73 OSR at three locations in Switzerland where the particular herbicide (glyphosate) was applied, for which a tolerance is conveyed, is therefore not entirely surprising.

In accordance to the Swiss Release Ordinance (The Federal Authorities of the Swiss Confederation 2008), local authorities and operators are urged to eliminate feral transgenic GT73 OSR populations found at the three locations. In these habitats, management has to occur primarily by repeated up-rooting of OSR plants (Garnier et al. 2006). These actions are supported by the directive to eradicate any plants growing on the railway tracks in Switzerland for safety reasons (e.g., gravel bed stability).

Based on the example of OSR, our findings confirm the importance and eligibility of monitoring (transgenic) plant populations along transportation lines. In addition, the concept used to identify "hot spots" lead to an efficient and targeted monitoring. To get more in-depth and continuous understanding of the situation in Switzerland, a consecutive monitoring study is planned to include roads frequently used by heavy truck traffic in addition to railway transportation routes.

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