ANNEX 9

CONFIRMATION OF IDENTITY OF AMFLORA SEED POTATOES GROWN IN 2011 VIA PCR ANALYSIS

BASF
The Chemical Company

BASF Plant Science

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CONFIRMATION OF IDENTITY OF AMFLORA SEED POTATOES GROWN IN 2011 VIA PCR ANALYSIS

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d) (1) (A), (B), or (C).

Company: BASF Plant Science Company GmbH

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The study described in this volume was not conducted in compliance with the OECD Principles of Good Laboratory Practice or the GLP Principles of German Chemikaliengesetz (Chemicals Act) and does not meet the United States Environmental Protection Agency Good Laboratory Practice Standards [40 CFR Part 150 (FIFRA)]. The data generated by BASF Plant Science Company GmbH in support of product safety comply with generally accepted scientific procedures. Record keeping is consistent with procedures used throughout the research community. This report accurately presents the raw data developed during the studies.

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CERTIFICATION OF AUTHENTICITY

We, the undersigned, hereby declare that this study was performed under our supervison according to the procedures described herein, and that this report provides a true and accurate record of the results obtained.

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ABBREVIATIONS AND DEFINITIONS

BHQ	QPCR quencher Black Hole Quencher
CFR	Code of Federal Regulations (USA)
CRL	Community Reference Laboratory (now European Reference Laboratory, or EURL)
Ct	Fractional PCR cycle number when fluorescence is greater than the predetermined threshold
dCt	Difference between the Ct value of the EH92-527-1 event-specific target and the potato endogenous reference gene
DE	Germany
FAM	QPCR fluorophore 6-carboxyfluorescein
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act (USA)
gbss	Granule bound starch synthase gene from potato
gDNA	Genomic DNA
qPCR	Quantitative PCR
IRMM	Institute for Reference Materials and Measurements, Geel, Belgium
PCR	Polymerase chain reaction
ROX	QPCR passive reference dye sulforhodamine
SE	Sweden
TET	QPCR fluorophore tetrachlorofluorescin

STUDY INFORMATION PAGE

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CONFIRMATION OF IDENTITY OF AMFLORA SEED POTATOES GROWN IN 2011 VIA PCR ANALYSIS

SUMMARY

The amylopectin potato EH92-527-1, variety Amflora, has been genetically modified for increased amylopectin content in the tuber starch via transformation with a gene fragment encoding granule bound starch synthase (*gbss*) from potato in antisense orientation. This modification leads to the silencing of the amylose synthesizing enzyme in the potato tuber. In March 2010, Amflora was approved for commercial cultivation in the European Union and was grown for seed potato production at locations in Sweden and Germany in 2011.

As part of the Amflora post-market environmental monitoring plan the purpose of this study was to demonstrate the presence of the EH92-527-1 insert and thereby confirm the identity and genetic stability of Amflora seed potato tubers grown at field locations in Sweden and Germany in 2011. Tubers were sampled after harvest from a total of four locations in Sweden and one location in Germany, DNA was extracted from a total of 80 pooled tuber samples and used as matrix in an EH92-527-1 event-specific qPCR assay. Additional four pooled samples from the conventional potato variety Bonanza serving as control material were also analysed. All pooled samples of Amflora potato tubers proved to be positive for the presence of the EH92-527-1 insert. These results confirm the identity of Amflora seed potatoes grown at locations in Germany and Sweden in 2011, and consequently genetic stability of the EH92-527-1 insert and verify the assumption made in the environmental risk assessment contained in Amflora Notification C/SE/96/3501.

INTRODUCTION

The amylopectin potato EH92-527-1, variety Amflora, has been genetically modified for increased amylopectin content in the tuber starch. The mother starch potato variety Prevalent was transformed with a construct containing a gene fragment encoding granule bound starch synthase (*gbss*) from potato in reverse (antisense) orientation under the control of the potato *gbss* promoter. A kanamycin resistance gene from *Escherichia coli* under the control of the nopaline synthase promoter from

Agrobacterium tumefaciens allowed selection of the transformant in tissue culture. The amylopectin potato EH92-527-1, variety Amflora, was approved for commercial cultivation in the European Union in March 2010 and was cultivated for seed potato production in Sweden and Germany in 2011.

The purpose of this study was to demonstrate the presence of the EH92-527-1 insert and thereby confirm the identity of Amflora seed potato tubers grown at field locations in Sweden and Germany in 2011. As outlined in the post-market environmental monitoring plan for Amflora (EU Register, 2010), tubers were sampled and pooled after harvest from a total of four locations in Sweden and one location in Germany. Aliquots of pooled tuber samples were lyophilized, ground into powder and stored at room temperature until analysis. DNA from the samples was extracted and analysed by a real-time TaqMan® qPCR assay specific for event EH92-527-1.

MATERIALS AND METHODS

Source of Plant Materials. Amflora potatoes were cultivated for seed tuber production at four field locations in Sweden and at one location in Germany in 2011 (Table 1). The sampling followed the outline provided in the post-market environmental monitoring plan for EH92-527-1 potato (EU Register, 2010), which calls for a total of 80 pooled samples consisting of 10 individual tubers each collected from the seed potato production fields. At locations in Sweden, 68 pooled samples (consisting of a total of 680 potato tubers) were collected. From the German location, 12 pooled samples (120 potato tubers total) were collected. A total of 80 Amflora tuber pools were prepared in this way. In addition, four pooled tuber samples, each consisting of 10 individual tubers, were taken from the conventional potato variety Bonanza provided by NORIKA GmbH, Groß Lüsewitz, Germany. These four pooled samples served as the control samples for the analysis.

The 84 pooled samples served as the source material for the qPCR analysis. A small tissue slice was taken from each of the tubers in a pool and combined into a single sample resulting in 84 pooled samples consisting of 10 tissues slices each for DNA extraction.

For the 5-stage dilution series used as a positive control for the qPCR, gDNA was isolated from EH92-527-1 certified reference material ERM-BF421b purchased from the IRMM (Geel, Belgium). As negative control gDNA was isolated from certified reference material ERM-BF421a consisting of non-GM potato tissue (Prevalent).

DNA Extraction. For each sample, a small piece of the flesh of each of the ten individual tubers in the pooled sample was cut off and combined in a 2 ml tube. Samples were lyophilized and homogenized. Approximately 50 mg of freeze-dried tuber meal was used for extraction with CTAB buffer (Carlson et al., 1991). After incubation and centrifugation, the clear supernatant was mixed with magnetic beads from QIAGEN (MagAttract 96 DNA Plant Core Kit, order number: 67165, QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. After washing, genomic DNA bound on magnetic beads was eluted with 1:10 AE buffer (QIAGEN). Two independent gDNAs samples were isolated and analysed from each tissue pool. In all cases, the results obtained from the duplicate samples were equivalent.

DNA Quantification. The concentration of gDNA isolated by this method was on average around 80ng/µl, as determined by Nanodrop measurement (Thermo Fisher Scientific Inc., Waltham, MA USA). The DNA concentration of each individual sample was not measured. As duplex qPCR reactions were performed, the presence in each individual sample of a typical DNA amount was confirmed by the Ct value of qPCR reaction of the endogenous reference gene.

Quantitative Real-time PCR Setup. TaqMan® assays (Applied Biosystems, Carlsbad, CA USA) were performed in a 96 well plate format on an ABI PRISM® 7900 sequence detection system (Applied Biosystems) using a BASF Plant Science internally validated event-specific real-time qPCR TaqMan® assay. Assay conditions and primers used are described in the Appendix. For the EH92-527-1 part of the duplex assay, primer and probe sequences were as described in JRC (2009). All analyses were performed as duplex PCR assays using an endogenous potato gene as reference. As these assays were performed as duplex reactions, while the JRC (2009) method is based on a simplex reaction, an alternate potato endogenous reference gene was used to generate this data.

The analysis produced a Ct value for both the event-specific target and the potato endogenous reference gene for each pool analysed. A dCt value, representing the difference between the Ct value for the EH92-527-1 event assay and the Ct value for the endogenous reference gene, was calculated for each sample. Results for the presence of the EH92-527-1 event in each sample were calculated as either positive or negative based on the dCt value for each sample.

Controls. Each 96 well PCR plate included a 5-stage dilution series of gDNA isolated from EH92-527-1 potato certified reference material ERM-BF421b (IRMM, Geel) as positive control, two wells with isolated gDNA from certified reference material ERM-BF421a (IRMM, Geel) as a negative control for the EH92-527-1 amplicon and one well without any DNA template as a negative control for the reference endogene amplicon. Four pools of the conventional Bonanza potato tubers were analyzed as additional negative controls.

RESULTS AND DISCUSSION

Isolated gDNA from pooled samples of Amflora potato tubers was analysed using an EH92-527-1 event-specific duplex qPCR detection method. The qPCR raw data were calculated and a dCt value was obtained for each sample. Samples positive for the presence of the EH92-527-1 insert showed dCt values in the range 0 to -1, whereas the dCt values for the negative control samples were greater than +10. The dCt values thereby allowed positive confirmation on the identity of the Amflora potato samples.

The summary of the qPCR analysis results for the pooled samples of Amflora potato tubers and the Bonanza control tubers are shown Table 1. All samples from all field locations proved to be positive for the EH92-527-1 event, while none of the Bonanza tuber samples were found to contain the EH92-527-1 event.

CONCLUSIONS

All pooled samples of Amflora potato tubers proved to be positive for the presence of the EH92-527-1 insert, when analysed using an event-specific qPCR detection method for EH92-527-1 potato. Thus, these results confirm the identity and thereby the genetic stability of Amflora seed potatoes grown at locations in Germany and Sweden in 2011 and verify the assumption made in the environmental risk assessment contained in Amflora Notification C/SE/96/3501.

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Table 1. Presence of the EH92-527-1 Insert in Pooled Tuber Samples Harvested in Sweden and Germany in 2011

Potato Variety	Location	Field Denomination	Number of Pooled Samples Analysed	dCt Values of Pooled Samples*	Number of Pooled Samples Positive for EH-92-527-1
Amflora	Germany	11STAMDE5UEP001	12	-0.8 -0.9 -0.8 -0.6 -0.7 -0.7 -0.6 -0.5 -0.7 -0.6 -0.4 0.8[1]	12
Amflora	South-SE	11STAMSE5VIN001	22	-0.8 -0.9 -0.8 -0.9 -0.7 -0.9 -0.8 -0.8 -0.3 -0.5 -0.4 -0.8 -0.8 -0.7 -0.8 -0.3 -0.4 -0.1 -0.7 -0.4 -0.6 -0.4	22
Amflora	South-SE	11STAMSE5SKA001	12	-0.9 -0.7 -0.5 -0.7 -0.9 -0.7 -0.6 -0.8 -0.9 -0.8 -0.6 -0.7	12
Amflora	North-SE	11STAMSE5VOJ001	22	-0.4 -0.6 -0.8 -0.4 -1.0 -0.8 -0.8 -0.4 -0.6 -0.4 -0.7 -0.7 -0.8 -0.3 -0.5 -0.6 -0.6 -0.4 -0.5 -0.8 -0.9 -0.8	22
Amflora	North-SE	11STAMSE5UNB001	12	-0.9 -0.8 -0.8 -0.4 -0.4 -0.5 -0.6 -1.0 -0.7 -0.8 -0.7 -0.8	12
Bonanza		11FTBONANZ99-CPL-01[2]	4	10.8 10.8 11.1 11. 10.7 10.9 11.8 11.(	0

* Values represent means of two measurements;

[1] Rerun resulted in dCt value of -0.8;

[2] Internal seed lot identifier (control variety, ordered from NORIKA GmbH, Groß Lüsewitz, Germany)

APPENDIX

Real-time PCR Assay Conditions

Reaction Conditions

Initial denaturation	95°C for 300 seconds	1 cycle
Amplification	95°C for 15 seconds/60°C for 60 seconds	40 cycles
Format	The qPCR reaction was performed in 10µl final assay volume containing 8µl Master Mix and 2µl gDNA.	

Components of Event-Specific EH92-527-1 Part of the Duplex Assay

Primers

event 527-bf1 5'-GTG TCA AAA CAC AAT TTA CAG CA-3'

St527-R1 5'-TCC CTT AAT TCT CCG CTC ATG A-3'

Probe

St527-FAM 1 5'-FAM-AGA TTG TCG TTT CCC GCC TTC AGT T-BHQ-3'

Table A. Preparation of the Master Mix for the Duplex Detection Assay

Component	Final Concentration
Jumpstart Taq Readymix*	1x
$MgCl_2$	7 mM
ROX	600 nM
EH92-527-1 Primer event 527-bf1	900 nM
EH92-527-1 Primer St527-R1	900 nM
EH92-527-1 TaqMan Probe	100 nM
Endogene Primer event 527-bf1	900 nM
Endogene Primer St527-R1	900 nM
Endogene TaqMan Probe	100 nM

* Sigma, P2893-400RXN