ANNEX 10

EXPRESSION OF OPEN READING FRAME 4 (ORF4) IN TUBERS OF AMFLORA SEED POTATOES GROWN IN 2011

BASF
The Chemical Company

BASF Plant Science

REPORT # AMF-11-002

EXPRESSION OF OPEN READING FRAME 4 (ORF4) IN TUBERS OF AMFLORA SEED POTATOES GROWN IN 2011

DATA REQUIREMENT: N/A

AUTHOR:

[*name deleted*]

STUDY COMPLETED ON: FEBRUARY 20, 2012

TEST FACILITY:

SUNGENE GMBH, A BASF PLANT SCIENCE COMPANY
CORRENSSTR. 3
06466 GATERSLEBEN
GERMANY

SUBMITTED BY:

BASF PLANT SCIENCE COMPANY GMBH
CARL-BOSCH-STR. 38
D-67056 LUDWIGSHAFEN
GERMANY

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Company: BASF Plant Science Company GmbH

Company Agent: [*name deleted*] Date: _____

Title: _____

Signature: [*signature deleted*] _____

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PRINCIPAL INVESTIGATOR:

[*name deleted*] _____ _____
Date

SunGene GmbH, a BASF Plant Science Company
Correnssstr. 3
06466 Gatersleben
Germany

SUBMITTER AND SPONSOR REPRESENTATIVE:

[*name deleted*] _____ _____
Date

BASF Plant Science Company GmbH
Carl-Bosch-Str. 38
67056 Ludwigshafen
Germany

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.

PRINCIPAL INVESTIGATOR:

[*name deleted*] _____ _____
Date

SunGene GmbH, a BASF Plant Science Company
Correnssstr. 3
06466 Gatersleben
Germany

SUBMITTER AND SPONSOR REPRESENTATIVE:

[*name deleted*] _____ _____
Date

BASF Plant Science Company GmbH
Carl-Bosch-Str. 38
67056 Ludwigshafen
Germany

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ABBREVIATIONS AND DEFINITIONS

CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
CFR	Code of Federal Regulations (USA)
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act (USA)
gbss	Granule bound starch synthase gene fragment
GBSS	Granule bound starch synthase
LDS	Lithium dodecyl sulfate
NC	Nitrocellulose
*npt*II	Neomycin phosphotransferase II (kanamycin resistance) gene
*orf*4	The open reading frame 4 present within the EH92-527-1 insert
ORF4	The predicted protein encoded by *orf*4
PAA	Polyacrylamide
PCR	Polymerase chain reaction
PIC	Protease inhibitor cocktail

STUDY INFORMATION PAGE

BASF Plant Science Report #:	AMF-11-002
BASF Registration Document #:	Reg. Doc. No. 2012/7000371
Study Title:	EXPRESSION OF OPEN READING FRAME 4 (ORF4) IN TUBERS OF AMFLORA SEED POTATOES GROWN IN 2011
Sponsor:	BASF Plant Science Company GmbH Carl-Bosch-Str. 38 D-67056 Ludwigshafen Germany
Testing Facility	SunGene GmbH, a BASF Plant Science Company Corrensstrasse 3 D-06466 Gatersleben Germany
Principal Investigator:	[*name deleted*]
Study Completion Date:	See date of Principal Investigator's signature on page 3

EXPRESSION OF OPEN READING FRAME 4 (ORF4) IN TUBERS OF AMFLORA SEED POTATOES GROWN IN 2011

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.

Amflora also contains a kanamycin resistance gene (*npt*II), which was used to enable selection for kanamycin resistance during the transformation process. Directly downstream from the *npt*II gene, there is an additional open reading frame named *orf*4. As part of the Amflora post-market environmental monitoring plan (EU Register, 2010), the purpose of this study was to confirm the lack of expression of the ORF4 protein in Amflora seed potato tubers grown at four field locations in Sweden and one location in Germany in 2011.

A total of 80 pooled Amflora samples and ten samples of cultivar Bonanza were analysed by Western blotting analysis using ORF4 specific antibodies. Purified ORF4 protein and tuber samples spiked with ORF4 protein served as positive controls for the reaction with the ORF4 antibody. At least 1 ng of protein ORF4 could be detected in all positive controls whereas no ORF4 corresponding polypeptide could be detected in Amflora and Bonanza samples at the limit of detection of 1 ng of the ORF4 protein per 50 µg total protein. These findings confirm the results presented in the Amflora Notification C/SE/96/3501 according to Directive 2001/18/EC and verify the assumption made in the environmental risk assessment.

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.

Directly downstream from the *npt*II gene, there is an additional open reading frame named *orf*4. Bioinformatic analysis predicts that *orf*4 could be transcribed due to its association with *orf*1 (*npt*II). Extensive studies indicated that, although *orf*4 transcript is detectable in the GM potato, there is no corresponding translation into a protein (EFSA, 2006).

The purpose of this study is to determine the absence of expression of ORF4 protein in Amflora seed potato tubers grown at four locations in Sweden and one location in Germany in 2011. The tubers were harvested and sampled as presented in Table 1. Depending on field size, 12 or 22 samples were collected. One sample consists of 10 individual tubers. For each individual sample chips from each individual tuber were taken and combined. A total of 80 pooled Amflora samples were obtained that represent the source material for the protein extraction (80 Amflora samples and ten Bonanza samples used as a control).

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 (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 each with ten potatoes, to give a total of 680 potatoes were analysed. From the German location, twelve pooled samples (120 potatoes in total) were collected. A total of 80 Amflora pooled tuber samples were prepared in this way. In addition, ten pooled samples of tubers, each consisting of ten individual tubers, were taken from the conventional potato variety Bonanza. These ten pooled tuber samples served as control samples for the analysis.

The 90 pooled samples served as the source material for the ORF4 analysis. Using a small tube, a cylindrical sample about 1 mm in diameter and about 1 cm long was taken from each tuber and the peel was cut off. Individual samples were combined into a single sample which was then used for protein extraction.

ORF4 Protein. The *orf4* coding region was cloned into the inducible over-expression vector pCAL-c (Agilent Technologies, Waldbronn, Germany). ORF4 protein was produced in inclusion bodies and purified from 3.5 g *E. coli* cell paste after resuspension in 25 ml of 50 mM Tris-HCl, 2 mM EDTA, pH 9.5, with 25 mg lysozyme. After approximately 30 min at room temperature with gentle rotation, the suspension was sonicated three times for 20 seconds. Following centrifugation at 10,000 x g for 10 minutes, the pellet was resuspended in 25 ml 1% Triton X-100 and sonicated for 20 seconds. After centrifugation for 10 min at 10,000 x g, the pellet was dissolved in 15 ml 8 M Urea, 2 mM EDTA, 5 mM DTT, 50 mM CAPS, pH 10.0. After centrifugation for 10 min at 10,000 x g, the supernatant was dialyzed against two liters of 50 mM Tris-HCl, pH 9.5, two times for 1 hour each. Visualization by Coomassie blue staining of the protein after SDS-polyacrylamide gel electrophoresis indicated a purity of approximately 90% of the 20,000 molecular weight ORF4 protein.

ORF4 Antibodies. Polyclonal antibodies were raised in goat against the bacterial recombinant ORF4 protein. Immunization and purification were performed by Virusys Corporation (Taneytown, MD, USA) using their standard three-month protocol. The ORF4 antibodies in the polyclonal sera were affinity purified using Protein-G affinity resulting in an IgG concentration of 20.3 mg/ml.

Protein Extraction. The pooled samples were frozen in liquid nitrogen and lyophilized. Lyophilized material was ground into a powder, and transferred into a chilled 2 ml tube, then 1.5 ml of pre-cooled TE buffer (50 mM, Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 7.5) was added and mixed by vortexing. The samples were incubated for 10 min on ice and cleared by centrifugation for 10 min at 10,000 g at 4°C. The supernatant was taken as the crude extract and used for protein determination.

Protein Determination. Protein quantification was performed according to Bradford (1976) using the BioRad (Munich, Germany) Protein Assay according to the manufacturers instructions.

Controls. Extracts of non-GM potato tubers (potato variety Bonanza) as well as Amflora spiked with ORF4 protein and purified ORF4 protein.

Western Blot Analysis. Fifty µg protein of crude protein extracts were diluted with 4x LDS buffer (Invitrogen, Darmstadt, Germany) containing 20 mM DTT and 20 mM PIC (Sigma-Aldrich, Munich, Germany), and loaded on 12% Bis-Tris polyacrylamide gels (BioRad). Molecular weight markers from Fermentas (St. Leon-Rot, Germany) were used to establish approximate molecular weight. Samples were separated at 90 volts and electro-blotted overnight onto nitrocellulose, then incubated with the ORF4 antibody. Blots were blocked with 3% (w/v) non-fat dry milk in 0.1% (v/v) Tween 20, 10 mM Tris-HCl, 150 mM NaCl, pH 7.5 at 30°C. The blocking solution was also used for antibody dilutions. The ORF4 antibody was diluted 1:10,000, and the anti-goat IgG conjugated with alkaline phosphatase (Santa Cruz Biotechnology Inc., Heidelberg, Germany) was used as secondary antibody.

Quantification of gene expression. The detection limit of ORF4 protein has been determined in Amflora tuber extracts by experimental detection of spiked ORF4 in a dilution row experiment. At least 1 ng of ORF4 protein could be detected. Thus extracts of Amflora tuber samples were spiked with 1 ng ORF4 protein to proof the detectability of ORF4 protein in case of its presence.

As long as the ORF4 protein can be detected in spiked samples but not in unspiked extracts of Amflora and non-GM tubers the samples can be considered as free (less than 1 ng) of ORF4 protein.

RESULTS AND DISCUSSION

ORF4 expression in Amflora tubers grown at field locations in Sweden and Germany in 2011 was analyzed by western blot experiments using an ORF4-specific antibody. No expression of the ORF4 protein was detected in a total of 80 pooled Amflora tuber samples from four locations in Sweden and one location in Germany (Table 1 and Figures 1 - 5). Control samples from the conventional potato variety Bonanza, which does not contain the ORF4 sequence, were also found to be negative for ORF4 protein expression (Figure 6). A band of approximately 20,000 molecular weight was visible in those lanes where either microbially produced ORF4 protein was spiked into the tuber protein extracts (Figure 1 - 7) or where the pure protein ORF4 protein was loaded on the gels for western blot analysis (Figure 1 - 7). The detection limit for ORF4 protein when spiked into tuber protein extracts was at about 1 ng per 50 µg of protein extract.
All blots show a cross-reacting band a little smaller than 40 kDa. As this band is also detected in extracts of the conventional potato variety Bonanza it is most likely a tuber protein that cross-reacts by chance with the ORF4 antibody.

CONCLUSIONS

ORF4 protein was not detected in protein extracts from pooled samples of Amflora seed tubers grown at field locations in Sweden and Germany in 2011. When spiked into tuber protein extracts microbially produced ORF protein could be detected at a level of at least 1 ng. It can be concluded that ORF protein is not expressed in Amflora tubers, or the expression is so low that ORF4 protein concentrations remain well below the detection limit of 1 ng ORF4 protein per 50 µg total protein. These findings confirm the results as presented in Amflora Notification C/SE/96/3501 according to Directive 2001/18/EC (EFSA, 2006) and verify the assumption made in the environmental risk assessment.

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EU Register (2010) Post-market monitoring plan for Notification C/SE/96/3501.

Available at: http://ec.europa.eu/food/dyna/gm_register/monitoringplan_eh92-527-1.pdf

Table 1: Tuber material and source used in this study.

	Field Identifier	Field size [ha]	Number of samples (each 10 tubers)
Germany	11STAMDE5UEP001	2	12
South Sweden	11STAMSE5VIN001	5,2	22
South Sweden	11STAMSE5SKA001	2,6	12
North Sweden	11STAMSE5VOJ001	5,4	22
North Sweden	11STAMSE5UNB001	2,3	12
			80

Non-GM control tubers of the cultivar Bonanza with the designation 11FTBONANZ99-CPL-01 were provided by NORIKA GmbH, Groß Lüsewitz, Germany.

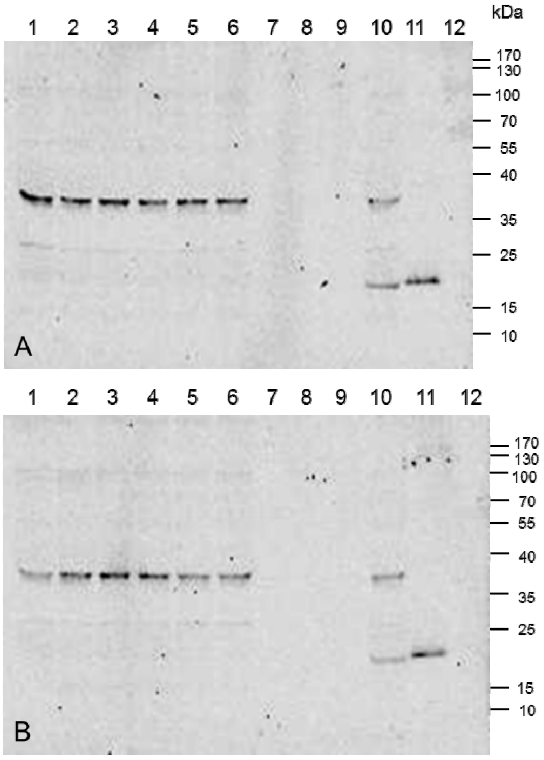

Fig. 1: Western blot analysis to detect protein ORF4 in 12 pooled tuber samples from Germany (blots A and B).
Blot A (lanes 1 – 6) loaded with 6 tuber samples and blot B (lanes 1 – 6) loaded with the remaining 6 tuber samples. One sample of each blot was spiked with 1 ng of protein ORF4 (lanes 10). 1 ng of ORF 4 protein was loaded as a positive control (lanes 11). Lanes 7 to 9: empty. Molecular mass standard (Fermentas) was separated in lane 12 of both blots and is given on the right in kDa.

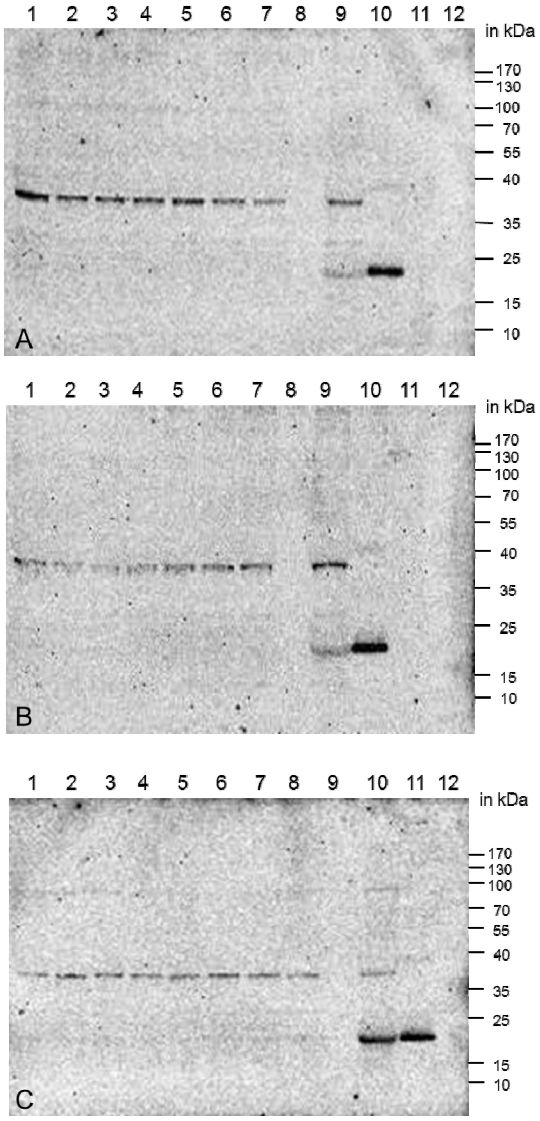

Fig. 2: Western blot analysis to detect protein ORF4 in 22 pooled tuber samples from South Sweden – field denomination: 11STAMSE5VIN001 (bots A to C). Blot A (lanes 1 – 7) loaded with 7 tuber samples and blot B (lanes 1 – 7) loaded with other 7 tuber samples and Blot C (lanes 1 - 8) loaded with the remaining 8 tuber samples. One sample of each blot was spiked with 1 ng of protein ORF4 (lanes 9 of blots A and B; Lane 10 of blot C). 1 ng of ORF 4 protein was loaded as a positive control (lanes 10 in blot A and B; lane 11 in blot C). Lane numbers not covered in the legend were empty. Molecular mass standard (Fermentas) was separated in lanes 12 of all three blots and is given on the right in kDa.

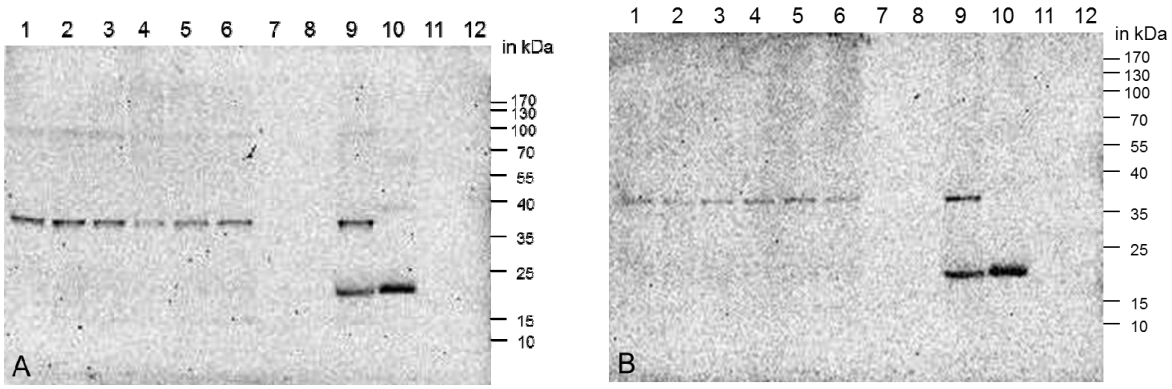

Fig. 3: Western blot analysis to detect protein ORF4 in 12 pooled tuber samples from South Sweden – field denomination: 11STAMSE5SKA001 (bots A and B).
Blot A (lanes 1 – 6) loaded with 6 tuber samples and blot B (lanes 1 – 6) loaded with the remaining 6 tuber samples. One sample of each blot was spiked with 1 ng of protein ORF4 (lanes 9). 1 ng of ORF 4 protein was loaded as a positive control (lanes 10). Lanes 7, 8 and 11: empty. Molecular mass standard (Fermentas) was separated in lane 12 of both blots and is given on the right in kDa.

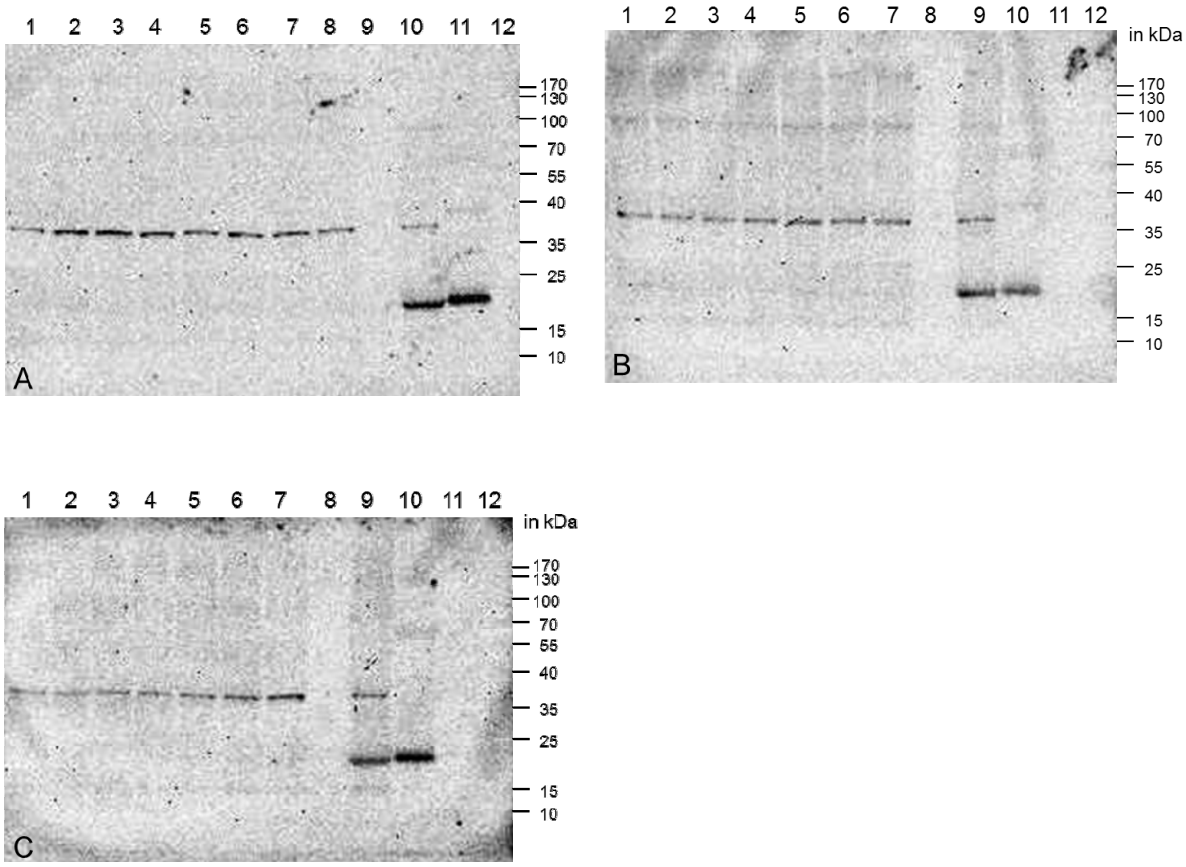

Fig. 4: Western blot analysis to detect protein ORF4 in 22 pooled tuber samples from North Sweden - field denomination: 11STAMSE5VOJ001 (blots A to C).
Blot A (lanes 1 – 8) loaded with 8 tuber samples and blot B (lanes 1 – 7) loaded with other 7 tuber samples and Blot C (lanes 1-7) loaded with the remaining 7 tuber samples. One sample of each blot was spiked with 1 ng of protein ORF4 (lane 10 in blot A; lanes 9 in blots B and C). 1 ng of ORF 4 protein was loaded as a positive control (lane 11 in blot A; lanes 10 in blots B and C). Lane numbers not covered in the legend were empty. Molecular mass standard (Fermentas) was separated in lanes 12 of all three blots and is given on the right in kDa.

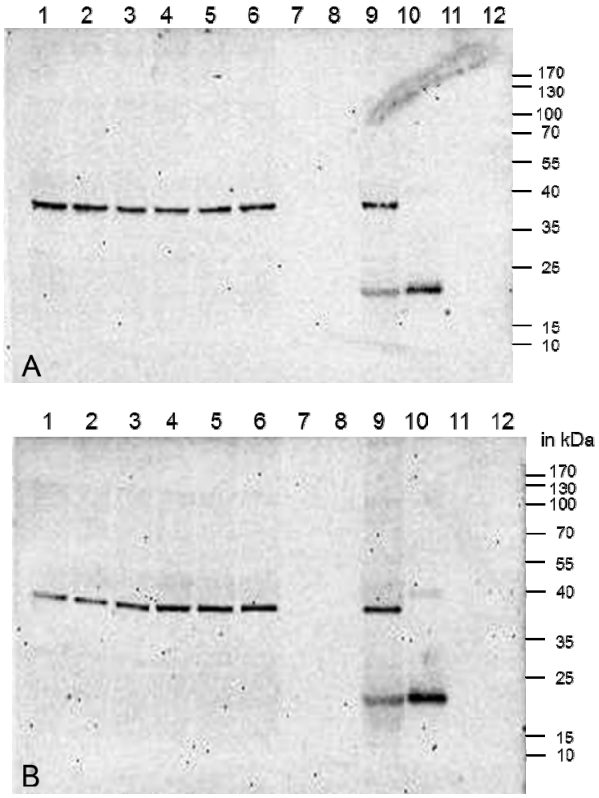

Fig. 5: Western blot analysis to detect protein ORF4 in 12 pooled tuber samples from North Sweden – field denomination: 11STAMSE5UNB001 (blots A and B).
Blot A (lanes 1 – 6) loaded with 6 tuber samples and blot B (lanes 1 – 6) loaded with the remaining 6 tuber samples. One sample of each blot was spiked with 1 ng of protein ORF4 (lanes 9). 1 ng of ORF 4 protein was loaded as a positive control (lanes 10). Lanes 7, 8 and 11: empty. Molecular mass standard (Fermentas) was separated in lane 12 of both blots and is given on the right in kDa.

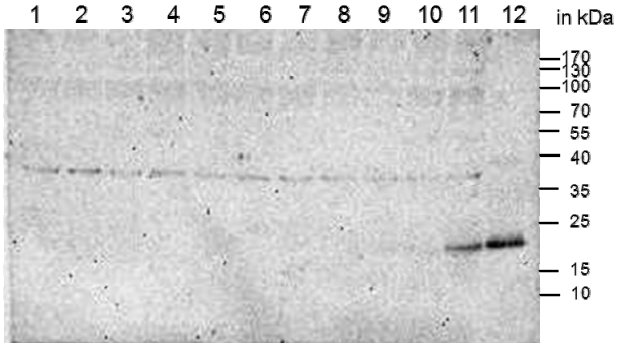

Fig. 6: Western blot analysis to detect protein ORF4 in 10 pooled control tuber samples of cv. Bonanza (lanes 1 to 10). One sample was spiked with 1 ng of protein ORF4 (lane 11). 1 ng of ORF 4 protein was loaded as a positive control (lane 12). Molecular mass standard (Fermentas) is given on the right in kDa, it run in a second gel in the same electrophoresis apparatus at the same time.

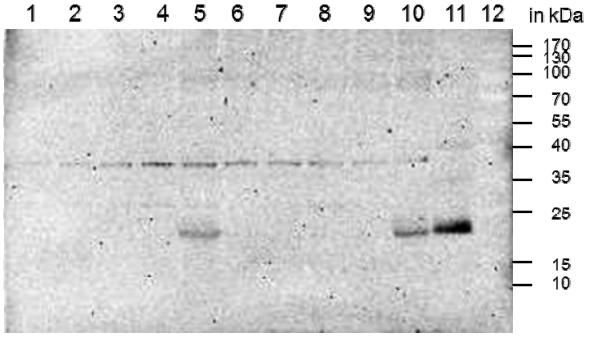

Fig. 7: Control blot for ORF4 protein detection in selected Amflora and Bonanza tuber pool samples to compare the mobility of extracted proteins. Loading as follows:

Lane	Cultivar	Location	Field denomination	Comment
1	Amflora	North Sweden	11STAMSE5UNB001	Pooled sample as in Fig. 5 A, lane 1
2	Amflora	South Sweden	11STAMSE5SKA001	Pooled sample as in Fig. 3 A, lane 1
3	Amflora	South Sweden	11STAMSE5VIN001	Pooled sample as in Fig. 2 A, lane 1
4	Amflora	North Sweden	11STAMSE5VOJ001	Pooled sample as in Fig. 4 A, lane 1
5	Amflora	North Sweden	11STAMSE5VOJ001	Sample as in lane 4 spiked with 1 ng ORF4 protein
6	Bonanza	-	-	Pooled sample as in Fig. 6, lane 7
7	Bonanza	-	-	Pooled sample as in Fig. 6, lane 8
8	Bonanza	-	-	Pooled sample as in Fig. 6, lane 9
9	Bonanza	-	-	Pooled sample as in Fig. 6, lane 10
10	Bonanza	-	-	Sample as in lane 9 spiked with 1 ng ORF4 protein
11	-	-	-	1 ng of purified ORF protein
12	-	-	-	Molecular mass standard - Fermentas