ANNEX 9

EXPRESSION OF OPEN READING FRAME 4 (ORF4) IN TUBERS OF AMLFORA SEED POTATOES GROWN IN 2010

BASF
The Chemical Company

BASF Plant Science

EXPRESSION OF OPEN READING FRAME 4 (ORF4) IN TUBERS OF AMFLORA SEED POTATOES GROWN IN 2010

AUTHOR:

STUDY COMPLETED ON: JANUARY 28, 2011

TEST FACILITY/PERFORMING LABORATORY:
SUNGENE GMBH, A BASF PLANT SCIENCE COMPANY
CORRENSSTR. 3, 06466
GATERSLEBEN, GERMANY

REPORT # M-02-2010

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

SIGNATURE PAGE DELETED

SIGNATURE PAGE DELETED

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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
LDS	Lithium dodecyl sulfate
NC	Nitrocellulose
nptII	Neomycin phosphotransferase II (kanamycin resistance) gene
orf4	The open reading frame 4 present within the EH92-527-1 insert
ORF4	The predicted protein encoded by *orf4*
PCR	Polymerase chain reaction
PIC	Protease inhibitor cocktail

EXPRESSION OF OPEN READING FRAME 4 (ORF4) IN TUBERS OF AMFLORA SEED POTATOES GROWN IN 2010

SUMMARY

The potato variety Amflora (event EH92-527-1) 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 2010.

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 *orf4*. 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 field locations in Sweden and Germany in 2010. Tubers were sampled after harvest from a total of 18 locations in Sweden and one location in Germany. A total of 82 pooled Amflora samples were analysed by western blot analysis using ORF4-specific antibodies raised against bacterial recombinant ORF4 protein. An additional eight pooled samples from the conventional potato variety Bonanza serving as control material were also analysed. No ORF4 polypeptide could be detected in Amflora or Bonanza samples at the limit of detection of 1 ng of the ORF4 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 potato line EH92-527-1 (OECD Unique Identifier BPS-25271-9), also referred to as Amflora potato, 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. The *npt*II gene from *E. coli,* under the control of the nopaline synthase promoter from *Agrobacterium tumefaciens,* allowed selection of the transformant in tissue culture. The potato line with the variety name 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 2010.

Bioinformatic analysis suggested that *orf4* might be transcribed due to its proximity to the *nptII* gene. Extensive studies indicated that, although *orf4* transcript is detectable in Amflora potato, no ORF4 protein is expressed (EFSA, 2006). The purpose of this study was to confirm that no ORF4 protein could be detected in Amflora seed potato tubers grown at field locations in Sweden and Germany in 2010. As outlined in the post-market environmental monitoring plan for Amflora, tubers were sampled and pooled after harvest from a total of 18 locations in Sweden and one location in Germany, and samples were analysed by western blotting analysis using ORF4-specific antibodies.

MATERIALS AND METHODS

Source of Plant Materials. Amflora potatoes were cultivated for seed tuber production at 18 field locations in Sweden (SE01 to SE18) and at one location in Germany (DE01) [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, four pooled samples (consisting of a total of 40 potatoes) were collected, except for location SE07 where six pooled samples were taken, each with either six or seven potatoes, to give a total of 40 potatoes. From the German location, eight pooled samples (80 potatoes total) were collected. A total of 82 Amflora pooled tuber samples were prepared in this way. In

addition, four pooled samples of tubers, each consisting of 10 individual tubers, were taken from the conventional potato variety Bonanza grown in both Kristianstad in Sweden and Baalberge in Germany. These eight pooled tubers samples served as the 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 in a pooled sample and the 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, US) 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. For each location SE01 to SE18 and DE01, one of the four protein extracts from pooled samples of Amflora tubers was spiked with purified bacterial recombinant ORF4 protein as positive controls to test for the reactivity of the ORF4 antibody. Also one of the protein extracts from the pooled samples of Bonanza tubers was also spiked with 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.

RESULTS AND DISCUSSION

ORF4 expression in Amflora tubers grown at field locations in Sweden and Germany in 2010 was analyzed by western blot experiments using an ORF4-specific antibody. No expression of the ORF4 protein was detected in a total of 82 pooled Amflora tuber samples from locations in Sweden (SE01 to SE18) and the location DE01 in Germany (Table 1 and Figures 1 - 11). 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 (Table 1 and Figure 12). 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 - 12, lane 10) or where the pure protein ORF4 protein was loaded on the gels for western blot analysis (Figure 1 - 12, lane 11). The detection limit for ORF4 protein when spiked into tuber protein extracts was at about 1 ng per 50 µg of protein extract. One background band of about 40,000 molecular weight is evident in all tuber protein extracts. 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 2010. 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. 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.

REFERENCES

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

EFSA (2006) Opinion of the Scientific Panel on Genetically Modified Organisms on an application (Reference EFSA-GMO-UK-2005-14) for the placing on the market of genetically modified potato EH92-527-1 with altered starch composition, for production of starch and food/feed uses under Regulation (EC) No 1829/2003 from BASF Plant Science. EFSA Journal 324 :1-20. Available under:http://www.efsa.europa.eu/de/efsajournal/pub/324.htm

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. Summary of Western Blot Analysis of ORF4 Protein Expression in Pooled Tuber Samples from Locations in Sweden and Germany in 2010

Potato Variety	Location	Field Identifier	Number of Pooled Samples Analysed	Number of Pooled Samples Positive for ORF4
Amflora	SE01	10AMFLORA-JB1	4	0
Amflora	SE02	10AMFLORA-JB2	4	0
Amflora	SE03	10AMFLORA-LBS1	4	0
Amflora	SE04	10AMFLORA-LBS2	4	0
Amflora	SE05	10AMFLORA-LBS3	4	0
Amflora	SE06	10AMFLORA-EH1	4	0
Amflora	SE07	10AMFLORA-EH2	6	0
Amflora	SE08	10AMFLORA-ML2	4	0
Amflora	SE09	10AMFLORA-ML3	4	0
Amflora	SE10	10AMFLORA-ML4	4	0
Amflora	SE11	10AMFLORA-ML5	4	0
Amflora	SE12	10AMFLORA-ML6	4	0
Amflora	SE13	10AMFLORA-ML7	4	0
Amflora	SE14	10AMFLORA-ML8	4	0
Amflora	SE15	10AMFLORA-RN1	4	0
Amflora	SE16	10AMFLORA-RN2	4	0
Amflora	SE17	10AMFLORA-RN3	4	0
Amflora	SE18	10AMFLORA-RN4	4	0
Amflora	DE01	10AMFLORA-N1&N2	8	0
Bonanza	Kristianstad (SE)		4	0
Bonanza	Baalberge (DE)		4	0

Figure 1. Western Blot Analysis of ORF4 Protein Expression in Pooled Amflora Tuber Samples from Locations SE01 and SE02 in 2010

Lanes 1-4: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE01. Lanes 5-8: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE02. Lane 9: empty. Lane 10: 50 µg of protein extracted from one pooled Amflora tuber sample from location SE02 spiked with 1 ng ORF4 protein. Lane 11: 1 ng ORF4 protein. Lane 12: Molecular weight markers ($x10^{-3}$) as indicated.

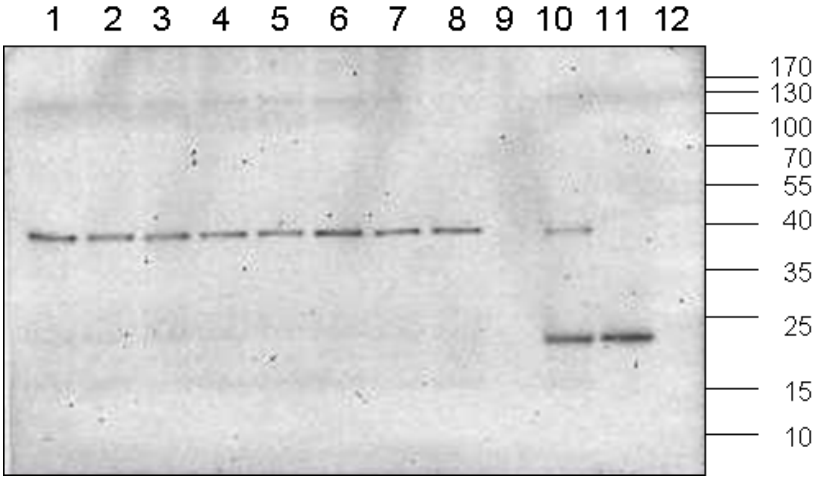

Figure 2. Western Blot Analysis of ORF4 Protein Expression in Pooled Amflora Tuber Samples from Locations SE03 and SE04 in 2010

Lanes 1-4: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE03. Lanes 5-8: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE04. Lane 9: empty. Lane 10: 50 µg of protein extracted from one pooled Amflora tuber sample from location SE03 spiked with 1 ng ORF4 protein. Lane 11: 1 ng ORF4 protein. Lane 12: Molecular weight markers ($x10^{-3}$) as indicated.

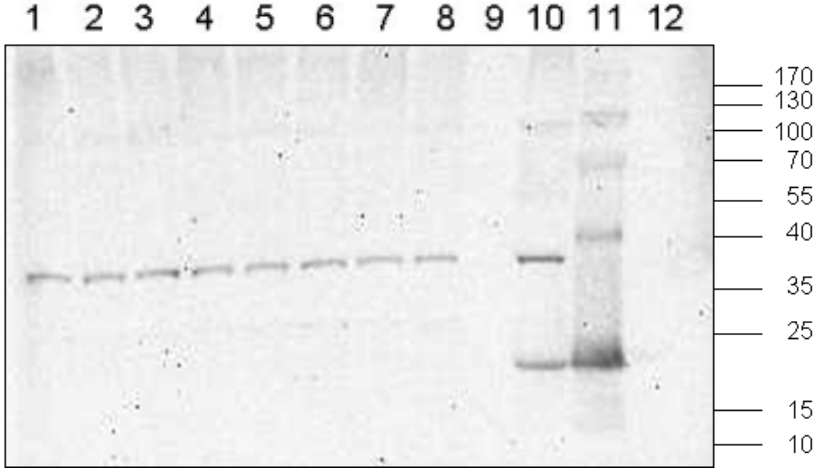

Figure 3. Western Blot Analysis of ORF4 Protein Expression in Pooled Amflora Tuber Samples from Locations SE05 and SE06 in 2010

Lanes 1-4: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE05. Lanes 5-8: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE06. Lane 9: empty. Lane 10: 50 µg of protein extracted from one pooled Amflora tuber sample from location SE05 spiked with 1 ng ORF4 protein. Lane 11: 1 ng ORF4 protein. Lane 12: Molecular weight markers ($x10^{-3}$) as indicated.

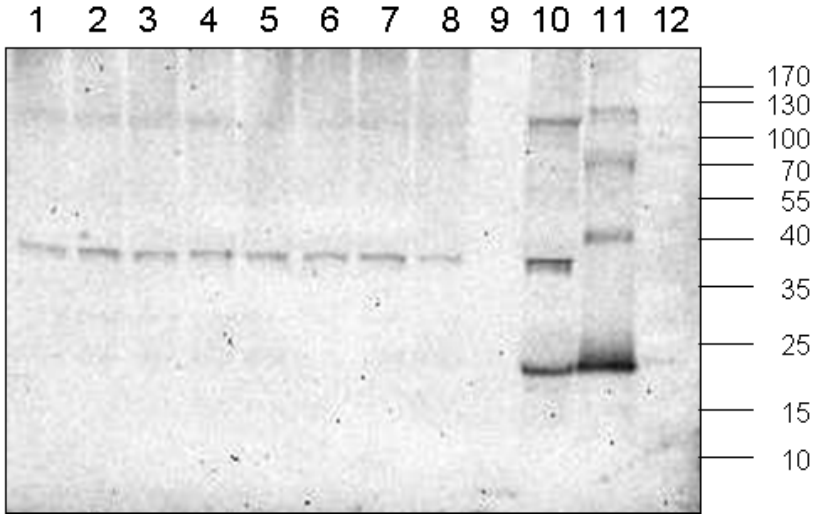

Figure 4. Western Blot Analysis of ORF4 Protein Expression in Pooled Amflora Tuber Samples from Location SE07 in 2010

Lanes 1-6: 50 µg of protein extracted from each of 6 pooled Amflora tuber samples from location SE07. Lanes 7-9: empty. Lane 10: 50 µg of protein extracted from one pooled Amflora tuber sample from location SE07 spiked with 1 ng ORF4 protein. Lane 11: 1 ng ORF4 protein. Lane 12: Molecular weight markers ($x10^{-3}$) as indicated.

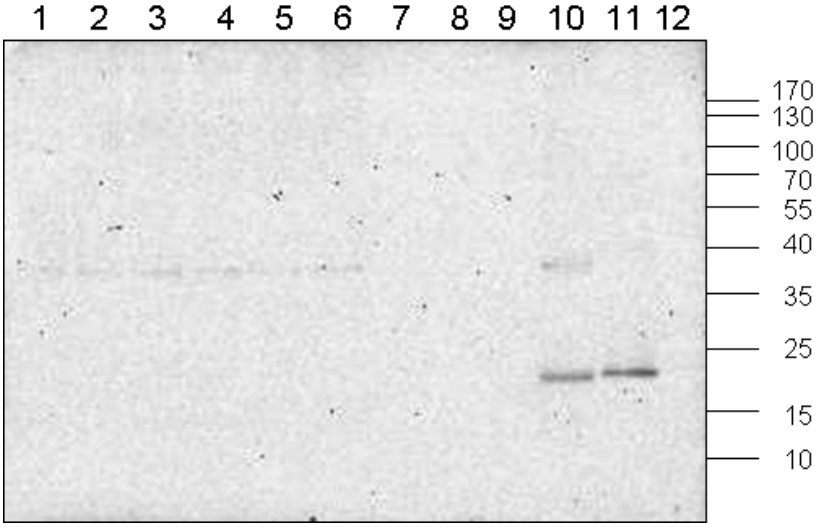

Figure 5. Western Blot Analysis of ORF4 Protein Expression in Pooled Amflora Tuber Samples from Locations SE08 and SE09 in 2010

Lanes 1-4: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE08. Lanes 5-8: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE09. Lane 9: empty. Lane 10: 50 µg of protein extracted from one pooled Amflora tuber sample from location SE08 spiked with 1 ng ORF4 protein. Lane 11: 1 ng ORF4 protein. Lane 12: Molecular weight markers ($x10^{-3}$) as indicated.

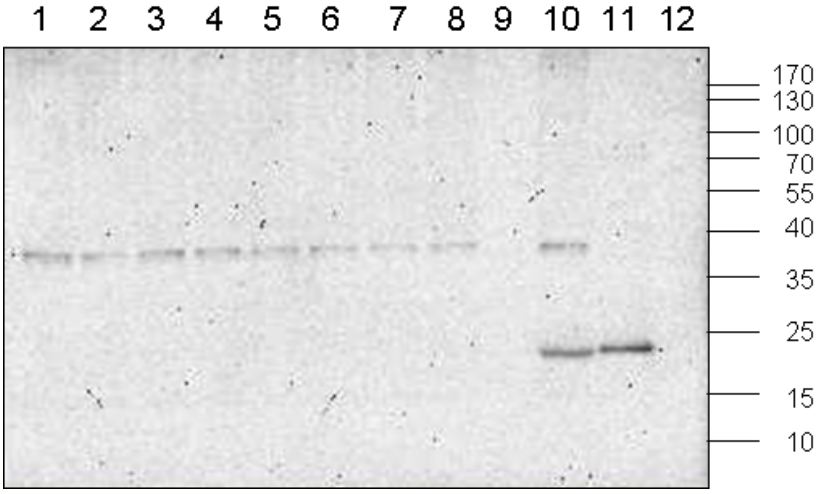

Figure 6. Western Blot Analysis of ORF4 Protein Expression in Pooled Amflora Tuber Samples from Locations SE10 and SE11 in 2010

Lanes 1-4: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE10. Lanes 5-8: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE11. Lane 9: empty. Lane 10: 50 µg of protein extracted from one pooled Amflora tuber sample from location SE11 spiked with 1 ng ORF4 protein. Lane 11: 1 ng ORF4 protein. Lane 12: Molecular weight markers ($x10^{-3}$) as indicated.

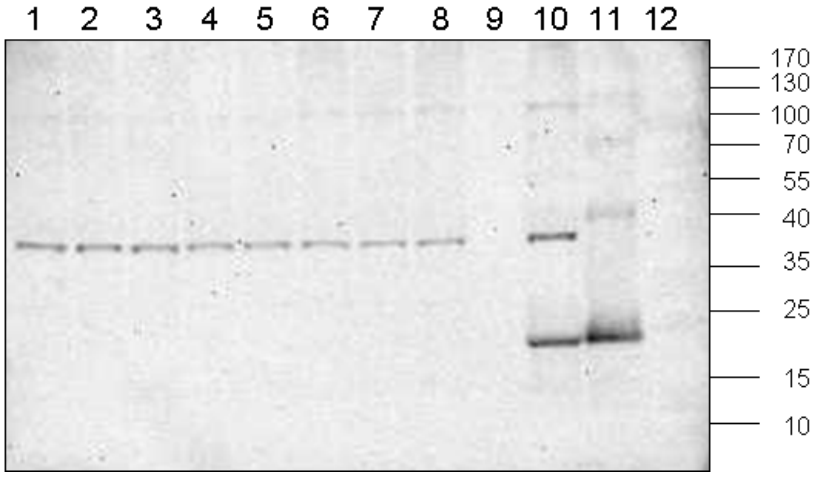

Figure 7. Western Blot Analysis of ORF4 Protein Expression in Pooled Amflora Tuber Samples from Locations SE12 and SE13 in 2010

Lanes 1-4: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE12. Lanes 5-8: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE13. Lane 9: empty. Lane 10: 50 µg of protein extracted from one pooled Amflora tuber sample from location SE13 spiked with 1 ng ORF4 protein. Lane 11: 1 ng ORF4 protein. Lane 12: Molecular weight markers ($x10^{-3}$) as indicated.

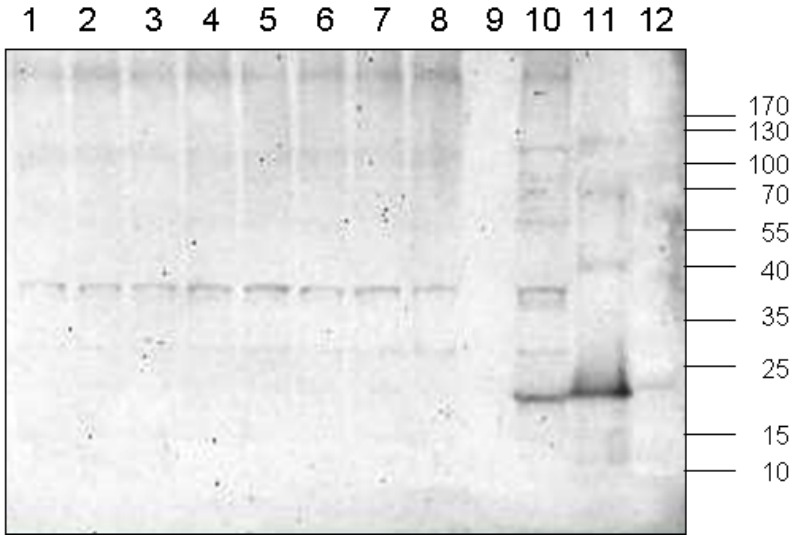

Figure 8. Western Blot Analysis of ORF4 Protein Expression in Pooled Amflora Tuber Samples from Locations SE14 and SE15 in 2010

Lanes 1-4: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE14. Lanes 5-8: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE15. Lane 9: empty. Lane 10: 50 µg of protein extracted from one pooled Amflora tuber sample from location SE15 spiked with 1 ng ORF4 protein. Lane 11: 1 ng ORF4 protein. Lane 12: Molecular weight markers ($x10^{-3}$) as indicated.

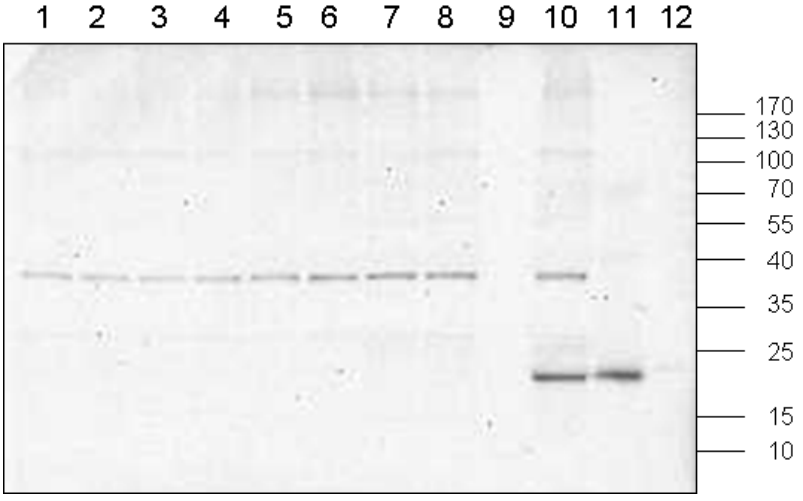

Figure 9. Western Blot Analysis of ORF4 Protein Expression in Pooled Amflora Tuber Samples from Locations SE16 and SE17 in 2010

Lanes 1-4: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE16. Lanes 5-8: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE17. Lane 9: empty. Lane 10: 50 µg of protein extracted from one pooled Amflora tuber sample from location SE16 spiked with 1 ng ORF4 protein. Lane 11: 1 ng ORF4 protein. Lane 12: Molecular weight markers ($x10^{-3}$) as indicated.

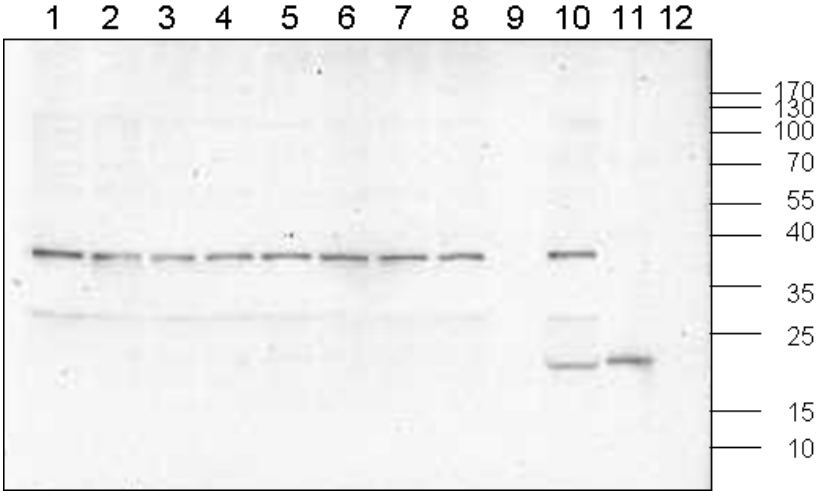

Figure 10. Western Blot Analysis of ORF4 Protein Expression in Pooled Amflora Tuber Samples from Location SE18 in 2010

Lanes 1-4: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location SE18. Lanes 5-9: empty. Lane 10: 50 µg of protein extracted from one pooled Amflora tuber sample from location SE18 spiked with 1 ng ORF4 protein. Lane 11: 1 ng ORF4 protein. Lane 12: Molecular weight markers ($x10^{-3}$) as indicated.

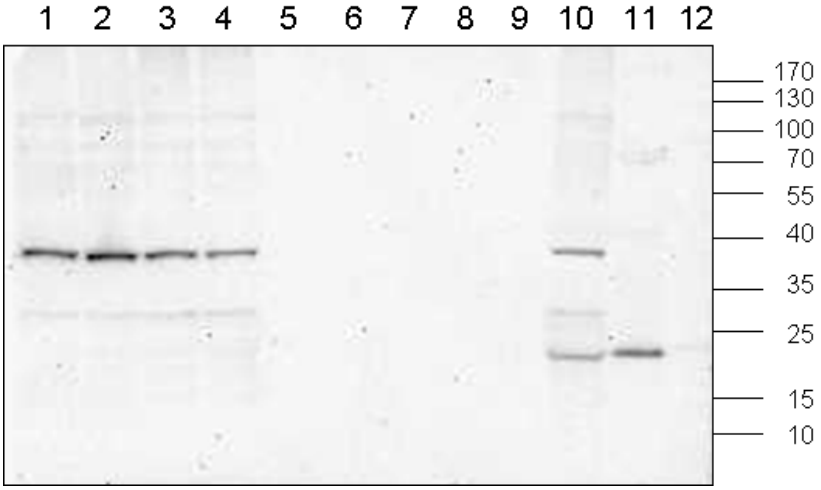

Figure 11. Western Blot Analysis of ORF4 Protein Expression in Pooled Amflora Tuber Samples from Location DE1 in 2010

Lanes 1-8: 50 µg of protein extracted from each of 4 pooled Amflora tuber samples from location DE1. Lane 9: empty. Lane 10: 50 µg of protein extracted from one pooled Amflora tuber sample from location DE1 spiked with 1 ng ORF4 protein. Lane 11: 1 ng ORF4 protein. Lane 12: Molecular weight markers ($x10^{-3}$) as indicated.

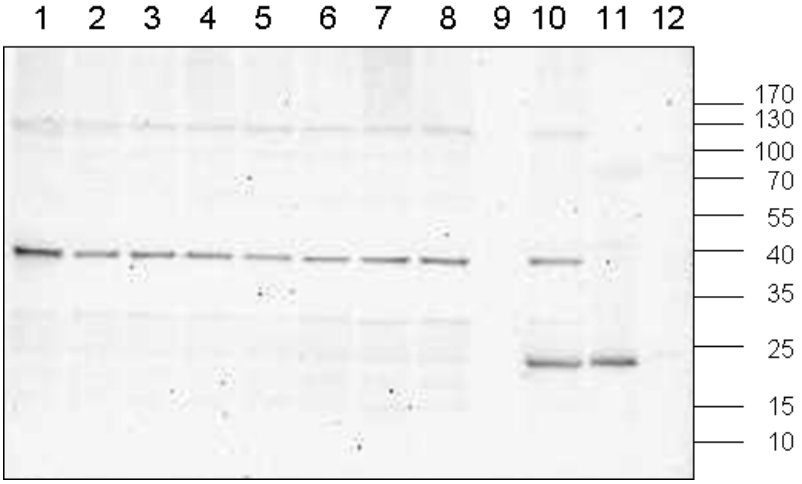

Figure 12. Western Blot Analysis of ORF4 Protein Expression in Pooled Bonanza Tuber Samples from Locations Kristianstad and Baalberge in 2010.

Lanes 1-4: 50 µg of protein extracted from each of 4 pooled Bonanza tuber samples from location Kristianstad. Lanes 5-8: 50 µg of protein extracted from each of 4 pooled Bonanza tuber samples from location Baalberge. Lane 9: empty. Lane 10: 50 µg of protein extracted from one pooled Bonanza tuber sample from location Baalberge spiked with 1 ng ORF4 protein. Lane 11: 1 ng ORF4 protein. Lane 12: Molecular weight markers ($x10^{-3}$) as indicated.