Appendix 21

Identification and quantification of allergens in different soybean lines, comparison between Cultivance soybean event 127 and its conventional comparator, Conquista

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Title: Identification and Quantification of Allergens in Different Soybean Lines, Comparison between Cultivance Soybean Event 127 and its Conventional Comparator, Conquista

Project: Cultivance Soybean Event 127

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Executive Summary:

Soybean (*Glycine max* L.) plants have been developed that are tolerant to the imidazolinone class of agricultural herbicides. In the safety assessment of transgenic crop varieties much attention is given to the impact on the allergenic potential of the food that has been modified. Great care is taken to ensure that the newly introduced gene is encoding neither a known allergen nor a protein with potential immuno-cross-reactivity with a known food allergen.
Therefore a proteomic study focusing on allergens present in protein extracts from soybean seed obtained from Conquista and Cultivance Soybean Event 127 greenhouse grown plants in Brazil together with Conquista seed from greenhouse grown plants in the United States was undertaken. Within the normal errors of such study most of the detected allergens do not show significant differences in all three investigated varieties – i.e. the differences between the same varieties (Conquista) grown under different conditions were similar to the differences between Conquista, the nontransgenic closely isogenic line, and the transgenic event Cultivance Event 127.

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Contents

Abbreviations and Definitions

2D gels	Two dimensional gel electrophoresis, isoelectric focusing in the first dimension followed by mass separation in the second dimension
ahasl	imidazolinone-tolerant acetohydroxyacid synthase large subunit
AHAS	Acetohydroxyacid synthase
CHAPS	*3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate*
DTT	dithiothreitol
IEF	Isoelectric focusing
PTFE	Teflon
SDS	sodium dodecyl sulfate
S653N	serine residue at position 653 of *Arabidopsis thaliana* acetohydroxyacid synthase large subunit replaced with asparagine

1. Introduction

Soybean (Glycine max L.) plants have been developed that are tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants were produced by introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) gene (*csr1-2*) from *Arabidopsis thaliana* into the soybean plant genome via biolistics using the PvuII fragment of transformation vector pAC321. This designation, *csr1-2*, has been applied to the *ahasl* gene which contains a mutation resulting in a single amino acid substitution, (S653N), to provide the imidazolinone herbicide tolerance. The seeds examined in this study are from plants that were derived from a single transformation event, referred to as Cultivance Soybean Event 127.

In the safety assessment of transgenic crop varieties much attention is given to the impact on the allergenic potential of the food that has been modified. Great care is taken to ensure that neither a known allergen nor a protein with potential immuno-cross-reactivity with a known food allergen is being encoded by the newly introduced gene. In addition, it is important to assess the impact of the transformation event on the endogenous levels of allergens normally produced by the crop. This can be monitored in several ways including using well characterized sera from patients allergic to the crop in question to see if there is a change in the protein binding profile or by directly monitoring the levels of the specific proteins in the grain. The latter approach was explored in this study with the imidazolinone-resistant soybean, Cultivance Soybean Event 127 and compared to the conventional comparator variety, Conquista.

The Allergenonline database (*http://allergenonline.com*) contains 33 entries for soybean. These include the β–conglycinin family of proteins, the glycinin family, the trypsin inhibitors family and some miscellaneous allergens. The major allergens are also among the major proteins in soybean grain. Several publications[1,2,3] describing proteomic studies (two dimensional [2D] gel electrophroresis separation of proteins with identification of individual protein spots using mass spectral analysis) in soybeans are available in the literature. The authors of ref. 2, Hajduch *et al.*, have also published an interactive web database containing clickable 2D-protein maps of proteins from soybean seeds[4] with multiple annotated spots. The availability of this data renders 2D-polyacrylamide gel electrophoresis a valid tool to examine the content of different allergens in the transgenic and control soybean lines. Furthermore, a proteomic comparison of the proteins in soybean from Cultivance Soybean Event 127 with the conventional comparator variety Conquista would reveal any general unintended effects on protein expression in the transgenic event of interest. Therefore a proteomic study of protein extracts from soybean seed obtained from Conquista and Cultivance Soybean Event 127 greenhouse grown plants in Brazil together with Conquista seed from greenhouse grown plants in the United States was undertaken. This allowed comparison between two varieties that differ only by the newly introduced DNA and between the same variety grown at two different locations to characterize the variability in soybean protein patterns in general. This study focused on both the major allergens, including the glycinins, and the major lectin, soybean agglutinin. Two other major proteins, lipoygenase and sucrose binding protein were monitored as controls.

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[1] E.M. Herman, R.M. Helm, R. Jung, A.J. Kinney *Plant Physiology,* May 2003, Vol. 132, pp. 36 – 43.
[2] M. Hajduch, A. Ganapathy, J.W. Stein, J.J. Thelen *Plant Physiology,* April 2005, Vol. 137, pp. 1397 – 1419.
[3] a) S. Natarajan, C. Xu, H. Bae, T.J., Caperna, W.M. Garrett, *J. Plant Biochemistry & Biotechnology*, July 2006, Vol. 15, 103-108.
b) S. Natarajan, C. Xu, H. Bae, T.J. Caperna, W. M. Garrett, *J. Agric. Food Chem.* 2006, Vol. 54, 3114-3120.
c) S. Natarajan, C. Xu, H. Bae, B.A. Bailey, *J. of Plant Physiology* 2007, Vol. 164, 756-763.
[4] *http://oilseedproteomics.missouri.edu/*

2. Experimental Data

Grain produced in 2005 from the Cultivance Soybean Event 127 eighth filial (F8) generation and its comparator non-transgenic line, Conquista, was obtained from greenhouse-grown plants in Londrina, Brazil. Grain produced in 2005 from greenhouse grown Conquista plants from Research Triangle Park, NC was also used. Plants were grown under a 16 hour light regime at 27°C and 21°C at night.

2.1. Protein Isolation (Phenol Extraction)- Soybean

Protein extraction was performed in "BlueCap"[5] cups with screw-mounted lids. The cups originally contained fine glass beads for grinding. Prior to use these beads were discarded and the empty cups were purified with water and ethanol.

Approx. 20 g of dry soybeans were pre-ground in a coffee grinder. The resulting powder was stored at – 80 °C in 250 mg aliquots until extracted. Extraction was accomplished after the addition of 3 steel balls (4 mm) and 250 µl of extraction buffer (0.9 M Sucrose in 100 mM Tris-HCl, 10 mM EDTA, 1 mM β- mercaptoethanol, pH 8.8) to 250 mg of the pre-ground soy material in a BlueCap-cup and vortexed intensely for 2 min. Commercially available "Phenole"[6] (750 µl) was added and vortexed for 1 min. The cup was transferred into a precooled (-20°C) PTFE-adapter and treated for 30 minutes in a mixer mill[7] (cold room, 4 °C) at the maximum power setting. After centrifugation (14,500 rpm, 16,700 x g, 30 min, 4°C) the complete supernatant including foam was transferred into a new 1.5 ml Eppendorf cup and kept on ice. The pellet (including the steel balls) was re-extracted with 500 µl phenol as before. The resulting supernatants were combined and centrifuged (2 hours, 28,000 rpm, 51,700 x g, 4°C) to separate the phases.

The phenol phase (= upper phase) was transferred drop by drop into 6.25 ml of pre-cooled (-20°C) 0.1 M methanolic ammonium acetate in a 15 ml Falcon tube. The proteins were precipitated overnight in a freezer (-20°C).

The tube was centrifuged (4000 rpm, 20min, 4°C) and the supernatants discarded. The pellet was resuspended in 1ml of cold (-20°C) 0.1 M methanolic ammonium acetate. The suspension was transferred into a tared Eppendorf cup, vigorously vortexed and placed at -20°C for 15min. The cup was centrifuged (5 min, 28,000 rpm, 51,700 x g, 4°C) and the supernatants were again discarded. Washing with 0.1M methanolic ammonium acetate was repeated for a second time. The resulting pellet was then washed twice with 1 ml of 80% acetone/20% ddH2O (ice cold) and once with 1 ml of 70% ethanol/30% ddH2O (ice cold). All supernatants were discarded. The protein weight was determined after drying the pellet under a stream of nitrogen. 1 ml Lysis buffer [7 M urea, 2 M thiourea, 1% dithiothreitol (DTT), 4% CHAPS, 0.8% ampholine pH 3.5 – 10 (GE Healthcare), Complete protease inhibitor (Roche)] was added to the pellet. The pellet was dissolved by intense stirring for 2 min using a vortexer. To dissolve the proteins completely a second mixing step in an Eppendorf shaker (> 30min, 1,400 rpm, ambient temperature) was performed. The protein solution was stored in 200 µl aliquots at – 80 °C prior to use.

[5] FastPROTEIN BLUE, Cat# 6550-500. MP Biomedicals, 29525 Fountain Pkwy, Solon Ohio 44139, USA
[6] Cat# 0038.1, Carl Roth GmbH, D-76185 Karlsruhe, Germany
[7] Retsch Mixer Mill MM200

2.2. Protein Concentration Determination

The protein content of each sample was determined by means of amido black according to the method of Popov.[8]

2.3. Isoelectric Focussing

2.3.1. Rehydration

The IEF-strips (Immobiline DryStrips pH 4 - 7, 24 cm, GE Healthcare) were hydrated in freshly prepared rehydration buffer [8 M urea, 2, M thiourea, 20 mM DTT, 4% CHAPS, 1% Pharmalyte (pH 3.5 – 10, GE Healthcare)].

Typically 100-150 µg total protein are applied on analytical gels for silver staining. The required amount of protein in solution was diluted with the *rehydration buffer* to a total volume of 450 µl and vortexed well. The solution was spread along the groove of the Immobiline DryStrip Reswelling Tray (GE Healthcare). The protective cover of the IEF strip was removed, and the strip placed carefully into the tray with the gel side down. It is important that the strip has good contact with the sample solution and no air bubbles are trapped between solution and strip. When loading of the strips was complete, the chamber was sealed (to protect from evaporation) and left at ambient temperature over night (approx. 16 h).

2.3.2. Isoelectric focussing

The strips were removed from the chamber, dabbed slightly dry with wet Whatman filter paper No.3 and placed into the "Immobiline strip tray" of the Multiphor chamber (GE Healthcare) with the gel side up. Two electrode strips were soaked with rehydration buffer. Excess buffer was removed with a paper tissue. On the cathode side as well as on the anode side of the strips one of the filter papers was placed on top of the gel. With the electrode in place 90 to 100 ml cover fluid (DryStrip Cover Fluid, GE Healthcare) was added, the electrode chamber was closed and the focussing was performed according to the procedure given in Table 1.

Table 1: Parameters for the isoelectric focussing

voltage (V)	current (max) (mA)	power (max) (W)	pattern	voltage hours (Vh)
500	1	5	Gradient	500
500	1	5	Gradient	2500
3,500	1	5	Gradient	10,000
3,500	1	5	Gradient	45,000

At the end of the focussing the current was interrupted, the chamber opened, the gel strips dabbed slightly on a dry tissue (gel side up!) to remove excess cover fluid.

[8] N. Popov, M. Schmitt, S. Schulzeck, H. Matthies, Acta biol. med. germ. 1975, Vol. 34, 1441-1446.

2.4. SDS-Polyacrylamide Gelelectrophoresis (PAGE) – 2nd Dimension

2.4.1. Equilibration of the Gel Strips

The gel strips are placed into the grooves of the equilibration tray with the gel side up and 6 ml of the DTT equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl, pH 8.8, 15 mg/ml bromophenol blue, 10 mg/ml DTT) are added to each strip and agitated for 15 minutes on a rotary shaker. The buffer is discarded and the strips agitated for 15 minutes with the iodoacetamide equilibration buffer (equivalent to the DTT equilibration buffer except contains 260 mM iodoacetamide instead of DTT). The buffer is again removed by decanting. Excess equilibration buffer is removed by dabbing the gel strips with wetted tissue (Whatman filter paper).

2.4.2. Electrophoresis

The second gel dimension is performed in the Ettan DALT*twelve* electrophoresis chamber from GE Healthcare. Self-cast gels (24 x 20 cm, 1 mm thick) with 12.5 % polyacrylamide and a (2,5ml/gel) stacking zone (6 % polyacrylamide) were used. The electrode buffer system consists of 25 mM Tris, 0.2 M glycine and 0.1% SDS

The Ettan chamber is equipped with the buffer and the required amounts of gels within glass plates (maximum 8 gels per run). The groove between the glass plates from each gel is filled with low melting agarose (1.2 %, 40 °C). The IEF gel strips were carefully inserted into the liquid agarose layer; all air bubbles were removed. The upper buffer compartment was filled, the chamber closed and the electrophoretic run performed according to the parameters given in Table 2:

Table 2: Parameter for the SDS-PAGE in the Ettan-Dalt *twelve*

step	pump	Power per gel (W)	Temp. (°C)	time (min)	comments
1	Auto	4	20	75	const. Power
2	Auto	14	20	120	const. Power
3	Auto	20	20	approx. 240	const. Power

After the dye front has reached the bottom of the gels the current is switched off. The gels are released from the glass plates and stored in the fixing solution (40 % methanol, 10 % acetic acid, 50 % H_2O) under constant shaking for a period of at minimum 2 hours, usually overnight.

2.5. Silver Staining and Image Analysis

The silver staining was performed in a home made staining device based on the published procedure of Blum *et al.*, 1987.[9]

Image analysis was conducted with the *ImageMaster platinum* software package from GE Healthcare (version 5.0.0.0).

[9] Blum, H., Beier, H., Gross, H.J., Electrophoresis 1987, Vol. 8, 93-99.

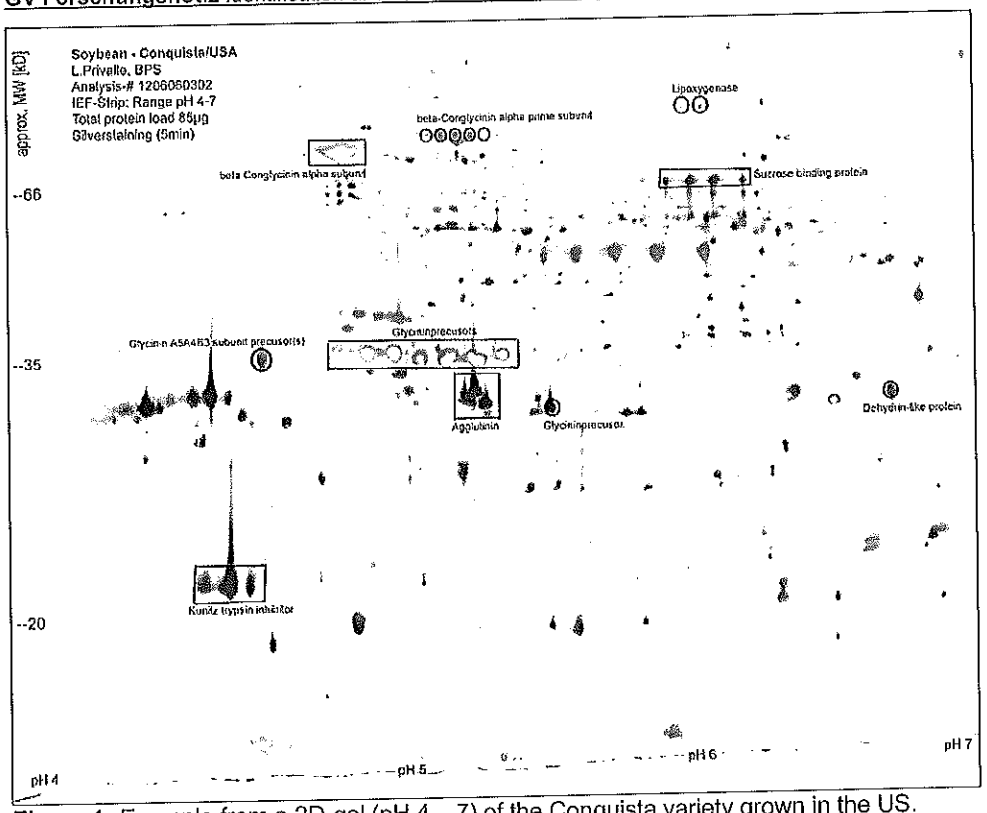

Figure 1: Example from a 2D-gel (pH 4 – 7) of the Conquista variety grown in the US.

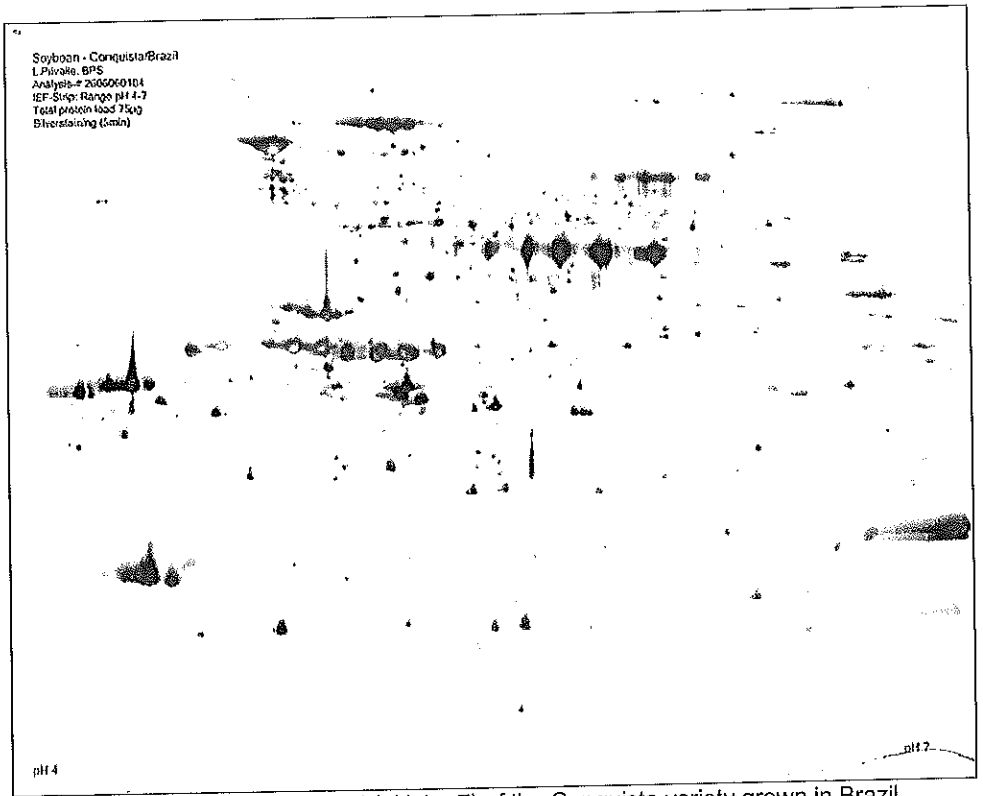

Figure 2: Example from a 2D-gel (pH 4 – 7) of the Conquista variety grown in Brazil.

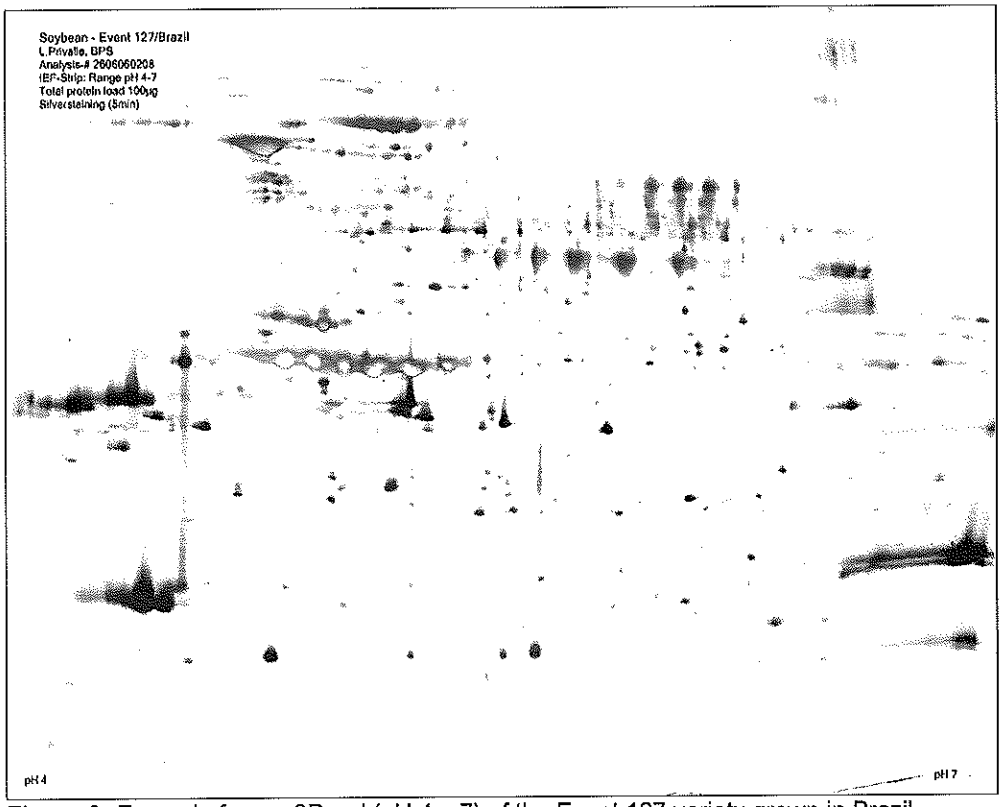

Figure 3: Example from a 2D-gel (pH 4 – 7) of the Event 127 variety grown in Brazil.

3. Results and Discussion

From each of the three soybean varieties three replicate analytical gels were prepared. The gels had protein patterns that looked very similar to the published gels. Thus several spots could be identified as allergens simply by means of pattern comparison of our gels with the internet database as well as with the literature (Figure 1).[1-3] The gel images of all three varieties, Conquista US, Conquista Brazil and Event 127 show also a high similarity (>90 %) to each other (see Figures 1 – 3).

The ratio of relative intensities of the spots for allergens (Kunitz trypsin inhibitor, dehydrin-like protein, β-conglycinin alpha subunit, glycinin A5A4D3 subunit precursor, glycinin precursor [as a chain], glycinin precursor [spot –shaped] β-conglycinin alpha prime subunit, soybean lectin [agglutinin]) and two nonallergens (sucrose binding protein and lipoygenase) were determined between the Conquista lines from different locations as well as between Conquista, the nontransgenic comparator, and the transgenic Cultivance soybean Event 127 (Table 1). The results indicate that within the inherent variability of the method of analysis there were no significant differences between the allergen contents in Conquista and Cultivance soybean Event 127 grown in the same site on the one hand and the two Conquista varieties on the other hand, grown either in a green house in the US or in a green house in Brazil.

Most of the allergens show up in the gels as more than one spot, as many of them are heteromeric, differentially glycosylated or partly deamidated. The staining intensities of the corresponding spots were summed up to calculate the total protein amount of each allergen (Table 1). Protein staining intensity ratios approaching one indicate little change between Cultivance soybean Event 127 and control Conquista, or between Conquista grown in two

different locations. The variation in staining intensity of protein spots between the two different sources of Conquista resulted in ratios ranging from 0.2 – 2.7 with the lowest (the five-fold difference) being associated with the lipoxygenase spot and the almost three-fold difference with the glycinin precursor. The average difference from the value of 1.00 for all protein staining ratios for the Conquista to Conquista comparison was 0.48 The variation in relative intensity of bands between Cultivance soybean Event 127 and Conquista was smaller (0.5 – 2), both representing a two-fold change, one increasing and the other decreasing. Also, the average difference from the value of 1.00 for all protein staining ratios for the Cultivance soybean Event 127 to Conquista comparison was 0.29. These results suggest that more variation is associated with growing the same variety in different locations than is associated with the presence of the transgene, when the transgenic and conventional lines are grown at the same location.

One major source of variation in the quantification of changes in allergen levels involves the differential staining of spots due to wide variation in prevalence. Many allergens are the major protein components of their source crop. For instance the soybean allergen Glycinin itself as well as its precursors and subunits were among the major spots and thus to a large extent are overstained in the silver gels. This could give rise to misquantifications.

The observed differences in case of *Sucrose Binding protein* and *Glycinin A5A4B3 subunit precursor* were attributed to the fact that in each case one of the multiple spots was missing in some of our gels (e.g. some of the *Glycinin A5A4B3 subunit precursor* spots are missing in some of the Conquista gels, but not in all).

Nevertheless within the normal errors of such a pilot study most of the detected allergens do not show significant differences in all three investigated varieties – i.e. the differences between the same varieties (Conquista) grown under different conditions were similar to the differences between Conquista, the nontransgenic closely isogenic line, and the transgenic event Cultivance Event 127. We consistently detected a high similarity between the gels of all three varieties examined (see figures 1 – 3). In the central regions nearly all gels look alike. Only small differences were recognized in the less reliable border areas of the gels ("border effects").

Table 1: Ratio of different proteins, determined by means of image analysis (integration of the corresponding spots in silver stained 2D-gels)

protein name	Event-127/ Conquista gels 103/101	Event-127/ Conquista gels 104/102	Event-127/ Conquista Average from two technical replicas	Conquista USA/ Conquista Brazil (single experiment)	
• Agglutinin	0.7	1.1	**0.9±0.2**	1.5	
• Sucrose Binding protein	0.8	3.2	**2.0±1.2**	0.8	
• Lipoxygenase	0.3	0.7	**0.5±0.2**	0.2	
• Kunitz Trypsin inhibitor	1.4	1.6	**1.5±0.1**	0.9	partly overstained
• Dehydrin-like protein	1.2	0.9	**1.1±0.2**	1.2	
ß-Conglycinin alpha subunit	0.8	1.2	**1.0±0.2**	0.6	overstained
Glycinin A5A4D3 sub. prec.	0.6	2.4	**1.5±0.9**	0.5	
Glycinin prec. (as a chain)	1.2	1.2	**1.2±0.0**	1.1	completely overstained
Glycinin prec. (Spot-shaped)	1.0	1.0	**1.0±0.0**	2.7	
ß-Conglycinin alpha prime sub.	0.7	1.3	**1.0±0.3**	0.7	
• Glycinin in total (incl. Precursors + subunits)	0.9	1.3	**1.1±0.2**	0.9	all Glycinin-related spots were summed up