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Endogenous Allergen Assessment of Event SYHT0H2 Soybean: 2-Dimensional Western Blotting with Human Sera

Final Report

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LIST OF ABBREVIATIONS AND ACRONYMS

AQUA®	Absolute quantitation (peptides)
Bradford assay	Total protein determination assay
CAP-FEIA	Pharmacia branded CAP – Fluorenzymeimmunoassay; immunoassay capture test used to measure antigen specific antibody
DTT	dithiothreitol
ELISA	Enzyme-Linked Immunosorbant Assay; antibody-based measure of a target analyte
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
g	gravity
GLPS	Good Laboratory Practices Standards
IEF	Isoelectric focusing
IgE	Immunoglobulin E
IPG	immobilized pH gradient
IUIS	International Union of Immunological Societies
kDa	kiloDalton
L	liter
LPB filter	long pass blue
mA	milliampere
mg	milligram
mL	milliliter
µg	microgram
µL	microliter
ng	nanogram
NBT/BCIP	nitro blue tetrazolium used in conjunction with (5-bromo-4-chloro-3-indolyl-phosphate)
NCBI	National Center for Biotechnology Information
NM	nitrocellulose membrane
PBS-T-M	phosphate buffered saline with Tween 20 and nonfat milk
PMT	photomultiplier tube
Ponceau S	Ponceau S; a non-specific protein stain
QAU	Quality Assurance Unit
RT	Room temperature (25 °C)
s&h	step and hold; a programming method for controlling isolectric focusing
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SOP	standard operating procedure
TIFF	tagged image file format; used to save digital images to file
US EPA	United States Environmental Protection Agency
v/v	in a volume-to-volume ratio
Vhrs	volt hours
w/v	in a weight-to-volume ratio

1.0 EXECUTIVE SUMMARY

Soybean (*Glycine max* [L.] Merrill) has been genetically modified to express the genes *avhppd-03* derived from oat (*Avena sativa* L.) and *pat* from *Streptomyces viridochromogenes*. The gene *avhppd-03* encodes a *p*-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme, designated AvHPPD-03, that catalyzes the formation of homogentisic acid, the aromatic precursor in plastoquinone and vitamin E biosynthesis. In comparison with the native soybean HPPD, the AvHPPD-03 isozyme from oat has lower binding affinity for mesotrione, an herbicide that inhibits HPPD. Expression of *avhppd-03* in the transgenic Event SYHT0H2 soybean plants confers a mesotrione-tolerance phenotype. The gene *pat* encodes the enzyme phosphinothricin acetyltransferase (PAT), which inactivates the herbicide glufosinate, an inhibitor of glutamine synthetase, an enzyme in the nitrogen assimilation pathway. Expression of *pat* confers a glufosinate-tolerance phenotype, which was used as a selectable marker in the development of Event SYHT0H2 soybean.

Soybean seed contains several known allergens, including abundant seed storage proteins, that either cause allergy directly or cross-react with IgE antibody that binds to other known protein allergens. The purpose of this study was to assess the similarity in human serum IgE antibody binding to endogenous allergens contained in seeds of SYHT0H2 soybean and three nontransgenic soybean varieties. The objective was to provide a qualitative assessment of the human serum IgE binding patterns to soybean seed proteins using a 2-dimensional Western blot analysis. A 2-dimensional electrophoretic technique provides a broad spectrum separation of proteins by their molecular weight and by their isoelectric charge. The 2-dimensional separation provides a more discrete isolation of some proteins which have multiple isoforms differing by inherent molecular charge differences as compared with 1-dimensional separation. An IgE binding Western blot using human serum was employed to observe potential differences in the expression pattern of proteins that bind IgE antibody, most of which would represent known soybean allergens.

SYHT0H2 soybean was compared to nontransgenic control soybean and two conventional reference varieties using five sera known to contain IgE antibody specific for soybean proteins. Overall similarity was evident among all four soybean varieties for each of the allergic sera with most comparisons best observed with three sera showing the most varied and intense IgE binding. SYHT0H2 soybean was similar in IgE binding pattern and overall intensity of IgE binding to both the nontransgenic control soybean and the two reference soybean varieties. SYHT0H2 soybean did not show the presence of any shifted or unique protein banding patterns that would indicate a significant difference in endogenous allergen content.

In summary, SYHT0H2 soybean is similar to nontransgenic soybeans in its expression of endogenous allergens. There is no evidence that suggests SYHT0H2 soybean has significantly increased expression of endogenous allergens or reacts differently with soybean-reactive serum IgE antibody as compared with nontransgenic soybeans. The data supports the conclusion that SYHT0H2 soybean is similar to a nontransgenic, near-isogenic control soybean variety and unlikely to have greater allergenic potential than nontransgenic soybean varieties.

2.0 INTRODUCTION

Soybean (*Glycine max* [L.] Merrill) has been genetically modified to express the genes *avhppd-03* derived from oat (*Avena sativa* L.) and *pat* from *Streptomyces viridochromogenes*. The gene *avhppd-03* encodes a *p*-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme, designated AvHPPD-03, that catalyzes the formation of homogentisic acid, the aromatic precursor in plastoquinone and vitamin E biosynthesis. In comparison with the native soybean HPPD, the AvHPPD-03 isozyme from oat has lower binding affinity for mesotrione, an herbicide that inhibits HPPD. Expression of *avhppd-03* in the transgenic Event SYHT0H2 soybean plants confers a mesotrione-tolerance phenotype. The gene *pat* encodes the enzyme phosphinothricin acetyltransferase (PAT), which inactivates the herbicide glufosinate, an inhibitor of glutamine synthetase, an enzyme in the nitrogen assimilation pathway. Expression of *pat* confers a glufosinate-tolerance phenotype, which was used as a selectable marker in the development of Event SYHT0H2 soybean.

Soybean seed contains abundant seed storage proteins as well as several lower abundance proteins that cause allergy directly or cross-react with IgE that recognizes other known protein allergens. A health concern that is addressed for genetically modified (GM) food crops is increased allergy potential due to potential changes in endogenous allergen content. Presumptively, if endogenous allergen content were to increase for a particular variety of soybean then exposure would be increased and a soybean allergic individual could be at greater risk for an allergic reaction. To date, there is no evidence that natural variation in endogenous soybean seed allergen expression increases allergy risk for soybean sensitive individuals.

Specific allergen IgE antibody binding is a typical *in vitro* assay used in clinical settings to assess the patient allergen response. Serum IgE binding assays are usually considered limited in their capacity to quantify target allergens in a food or other complex sample because each serum donor will have IgE antibodies with different titer, specificity, and affinity for allergen binding. IgE binding to proteins separated by electrophoresis and detected by Western blotting methods is generally considered a qualitative method for observing specific protein allergen binding from plant extracts, because true quantitation is difficult without protein standards. To date, there have been no determinations of individual soybean allergen exposure thresholds (i.e., minimum exposure concentrations or total exposure amounts) whereby clinicians are able to predict allergy risk based on the amount of individually consumed soybean allergens. Therefore, the risk for allergy in response to an exposure to any one of the soybean allergens cannot be predicted by discrete measures of endogenous allergens in soybean seed, at present.

The purpose of the study was to assess the overall similarity between SYHT0H2 soybean and nontransgenic soybean comparators by human serum IgE binding to proteins extracted from seed and separated by 2-dimensional SDS-PAGE. Conventional nontransgenic soybean varieties provide comparators that represent commercially available soybeans. A nontransgenic, near-isogenic control variety provides a direct comparator to SYHT0H2 soybean. Five soybean-reactive human sera and a negative control serum were used to evaluate IgE binding patterns to soybean proteins by Western blotting.

3.0 MATERIALS AND METHODS

3.1 Test, Control, Reference Soybean Varieties

Transgenic test and nontransgenic control soybean plants were field-grown concurrently in Hawaii in the year 2009 and were subjected to the same standard local agronomic procedures, growing conditions, harvesting, and grain storage conditions. The SYHT0H2 soybean seed test material is indicated for its pedigree in Appendix A. Conventional reference varieties were grown in Hawaii in the year 2009 (Table 1). Note that the reference variety, "BPR99402 /99022-A01-16277-01", is abbreviated as BPR99402 throughout. Isolation procedures were taken to avoid the intermixing of grain types. Reference soybean varieties were selected to represent soybean varieties currently available on the commercial market. Grain lots of the test and control varieties were characterized by real-time polymerase chain reaction (PCR) testing to confirm identity and purity prior to use in this study.

3.2 Experimental Design

The experimental design consisted of comparing the IgE binding of five soybean allergic sera to soybean seed extracts prepared from four soybean varieties. Total protein extracts prepared from each of the four soybean varieties were loaded onto polyacrylamide gels to separate the proteins electrophoretically; each gel contained one soybean extract. In a separate transfer procedure, the separated proteins in each gel were transferred to a nitrocellulose Western blot membrane for probing with IgE-containing human sera samples to detect reactivity of serum IgE antibody. A control serum shown not to contain soybean reactive IgE antibody was used as a Western blotting control.

3.3 Serum Source and Characterization

Soybean-reactive human sera were selected for use in the IgE Western blotting by determining specific IgE reactivity to soybean protein extracts. A negative control serum that did not specifically bind soybean proteins was used to identify any non-specific IgE binding that may be present as an artifact in the Western blot assay. Sera were sourced from a clinical laboratory, ViraCor-IBT Laboratories, LLC[1] and patient-specific information was removed. IgE binding screening data (CAP) for sera (both soybean positive and the negative control) were produced by ViraCor-IBT Laboratories (Table 2).

Sera positive for IgE antibody reactivity to soybean proteins were identified by standard clinical allergy CAP testing. Sera that produced a CAP test result against soybean with a value >0.35 kU/L and had a CAP class (Flag class) of ≥3 were considered positive for soybean IgE binding. Sera were screened by Western blot (data not shown) to determine which soybean positive sera (from an initial pool of 20) showed an appropriate level of IgE binding to soybean protein allergens. A nontransgenic soybean variety, "Williams 82", was used to determine antibody and serum reagent dilutions and protein loading conditions in a pre-study evaluation. Five sera that had high titers (Table 2) as well as high levels of specific reactivity to soybean proteins by Western blotting were considered the acceptable for

[1] ViraCor-IBT Laboratories, 11274 Renner Blvd, Lenexa, KS 66219

comparing IgE binding to test, control, and reference soybean varieties (McClain and Thelen 2012).

A negative control serum that showed no IgE binding to soybean by CAP (CAP result < 0.35 kU/L) and showed no IgE binding in pre-study Western blot assays was considered appropriate as a negative control for this study.

3.4 Soybean Protein Extraction for Electrophoresis

Total protein was extracted from seed of each soybean variety. This procedure was performed using previously described methods (Hajduch *et al*. 2005, Lee *et al*. 2010). Briefly, five soybean seeds from each variety were weighed and pulverized for 30 seconds using a Waring blender. The samples were then weighed. Equal parts of 1) extraction buffer and 2) Tris-buffered phenol pH 8.0 were added in a 1:30 ratio (mL buffer mix : mg sample). Samples were vortexed and homogenized and samples were placed on a rocking platform at 4 °C for 30 min and then centrifuged. The separated upper phenol phase was transferred to a new tube and five volumes of ice-cold precipitation solution (0.1 M ammonium acetate in 100% methanol) were added. The samples were vortexed and incubated overnight at -20 °C to precipitate the protein. Protein was pelleted by centrifugation and then washed twice with 0.1 M ammonium acetate in methanol, twice with ice-cold 80% (v/v) acetone in water, and once in 70% (v/v) ethanol in water. Protein pellets were stored in 70% (v/v) ethanol at -20 °C. Protein was resuspended in 50 mM Tris-HCl, pH 8.0 and 8 M urea media followed by quantification using the Bradford assay (Bradford 1976).

Protein quantitation: Protein pellets from each sample were resuspended in 250 µL of IEF buffer (GE Healthcare Life Sciences, Piscataway, NJ U.S.A.) for 1 h at room temperature (RT) with gentle vortexing. Solubilized protein was centrifuged for 15 min at 16,000 × *g* and supernatant decanted to a fresh tube and the protein from each sample was quantified using the EZQ protocol (Invitrogen Life Sciences, Grand Island, NY U.S.A.). Briefly, 2 µL from each sample extract was removed to a fresh tube and 18 µL IEF buffer added. Following vortexing, 10 µL was removed to a fresh tube and 10 µL of IEF buffer was added and the tubes vortexed. Fluorescent signal was read from each sample and each dilution of a standard curve using a FLA5000 laser imager (Fuji Film, Japan) using 375 volts on PMT, 435 nanometer laser, and LPB filter, 100 µm resolution. The 1:10 and 1:20 dilutions of sample protein concentrations (µg/g) were interpolated from the standard curve using serial dilutions of a known amount of ovalbumin protein standard.

3.5 Western Blotting Procedure

3.5.1 2-dimensional protein separation by isoelectric focusing and SDS-PAGE:

Samples of each soybean protein extract were subjected to 2-dimensional SDS-PAGE according to detailed laboratory protocols.

First Dimension: IEF was performed using an IPGPhor instrument (Amersham instrument, GE Healthcare, Piscataway, NJ, U.S.A.). To perform the focusing of proteins in seed extracts, a total of 50 µg protein was used to rehydrate immobilized pH gradient (IPG) strips as follows: 350 µg protein was removed from concentrated stock and diluted to 840 µL with

IEF buffer. Each IPG strip was removed from -20 ^{o}C storage, allowed to equilibrate to RT for 10 min, and rehydrated with 120 µL of protein solution for 1 h at RT. Mineral oil (2 mL) was used to cover the rehydrated strips and focusing was conducted. Briefly, the IEF protocol included a 10 h active rehydration at 50V, immediately followed by a 250V "step&hold" (s&h) with a rapid ramping for 250 volt-hours (Vhrs), 1000V s&h for 1000Vhrs, gradient ramp to 5000V over 1 hr, then 5000V s&h for 12,000 Vhrs. A 500V hold was used prior to putting strips on 2nd dimension.

Second Dimension (SDS-PAGE): – IPG strips were removed and excess mineral oil removed by blotting onto paper. Strips were then placed in IEF equilibration trays and incubated for 10 min on a rocking platform at RT in buffer (50 mM Tris, pH 6.8; 6 M urea; 30% (v/v) glycerol; 5% (w/v) SDS; 0.01% (w/v) bromophenol blue); this solution was supplemented with 2% (w/v) DTT and then in a sequential manner, 2.5% (w/v) iodoacetamide to reduce and alkylate the proteins, respectively. Equilibration buffer was removed and strips placed in 1X SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Strips were placed on top of a SDS-PAGE gel (12% acrylamide, isocratic) and sealed with agarose overlay solution (60 mM Tris-HCl, pH 6.8; 60 mM SDS; 0.5% (w/v) agarose; 0.01% (w/v) bromophenol blue). Separation of protein by molecular weight was performed by applying electrical current for 30 min at 5 mA constant current, followed by 3.5 h at 20 mA constant current. Each gel was stopped immediately after the dye front ran off the bottom of the gel.

After electrophoresis, gels were equilibrated for 15 min at RT in transfer buffer (1X = 25 mM Tris; 192 mM glycine, 0.005% (w/v) SDS, 20% (v/v) methanol). Equilibrated gels were layered onto wet nitrocellulose and transfer was conducted at 100 mA for 16 h at RT. Following transfer, the nitrocellulose membrane was removed and immediately placed in Ponceau S stain (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid), and digitally imaged.

3.5.2 Serum IgE binding by Western blotting:

The nitrocellulose membranes (NM) produced for each soybean variety, as described above, were each probed with one of the six serum samples. Before probing with serum, each NM was blocked with phosphate buffered saline, 0.3% (v/v) Tween-20, 1% (w/v) nonfat milk (PBS-T-M). Each NM was then probed with human sera (1:100 dilution) in PBS-T-M. Each NM was washed with PBS-T-M and then washed with PBS-T. In preparation for secondary antibody probing, NMs were then blocked in PBS-T-M. Each NM was probed with mouse anti-human IgE-alkaline phosphatase conjugated secondary antibody (Southern Biotech, Birmingham, Alabama 1:1000 dilution). The NMs were washed with PBS-T-M followed by washing with PBS-T. The NM were equilibrated in pH 9.5 buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) and developed with NBT/BCIP colorimetric reagents. Detection of IgE bound to soybean proteins was performed by developing each NM with an alkaline phosphatase substrate solution. The phosphatase detection procedure was terminated at a fixed time for all membranes.

3.5.3 Recording results

Digital images (TIFF format) were captured of each nitrocellulose membrane stained by Ponceau S prior to the Western blot procedure to assess protein content by visual analysis and to account for protein loading differences or any other general differences between

soybean varieties. Digital images were also captured of each corresponding Western blot to assess IgE binding to soybean proteins by visual analysis of spot intensity and location.

4.0 RESULTS

4.1 SDS-PAGE and Western Blot Analysis of Soybean Protein Extracts

A comparison of total protein staining and IgE antibody binding by 2-dimensional SDS-PAGE and Western blotting was made among the seed extracts from SYHT0H2 soybean, the nontransgenic, near-isogenic control ("Jack" variety) and two nontransgenic soybean reference varieties (BPR99402 and NE0800097). This study provides a qualitative comparison among the four soybean varieties to assess whether the content of endogenous allergens differs between SYHT0H2 transgenic soybean and nontransgenic soybean varieties.

Each Ponceau S stained "blot set" (Figure 1) corresponds to each set of those same Western blot membranes indicated for each serum in Figure 2 (*e.g.*, Ponceau blot set 1 = serum 1). There were minor variations in individual protein spots across the blots for the four soybean varieties, but overall loading and transfer to the blotting membranes appeared consistent across each of set of blots as shown by Ponceau S staining. An evaluation of IgE antibody binding from soybean-reactive sera to soybean proteins from SYHT0H2 soybean, the nontransgenic, near-isogenic control and the BPR99402 and NE0800097 references showed broad reactivity to a variety of proteins in soybean seed extracts (Figure 2). An acceptable level of IgE binding was observed for all five sera to soybean proteins at various molecular weights and isoelectric charge positions with each serum showing general consistency in IgE binding across all four soybean varieties. There were a few cases where differences were observed in the intensity of IgE binding to a given protein spot or where there was a spot present in one variety while not visible in one or more of the other varieties for that same serum. Some protein spots were noted as exhibiting more or less IgE binding for a given serum, but this appears due to slight variations in the amount of protein that was physically present on the blotting membrane as noted in the Ponceau S stained blots.

The minor variability in IgE binding observed was most obvious for those sera that showed the most numerous and most intense IgE binding; e.g., sera 1, 3 and 5 (Figure 2). The most notable difference in apparent IgE binding was for the nontransgenic control and NE0800097 soybean varieties with regard to the two glycinin precursor proteins located between 20 kDa and 24 kDa on the far alkaline side (right side) of the blot for serum 3; neither variety displayed the number of IgE-bound proteins or intensity of binding as the SYHT0H2 or BPR99402 varieties. This difference in IgE binding is not apparent for serum 1 which exhibits equivalent binding to both glycinin precursor proteins across the four soybean varieties. Inspection of the Ponceau S stained blot membranes for the nontransgenic, near-isogenic control show that the glycinin precursor proteins (glycinin G1 and G2 (Mooney and Thelen 2004)) appear to not be as enriched on the nontransgenic control blotting membrane as compared to other three soybean varieties (Figure 1). There are some highly alkaline protein spots in BPR99402 in the blot for serum 3 that share at least one common spot in the nontransgenic control and the NE0800097 soybean varieties; none of these spots are apparent in SYHT0H2.

Other observations show that protein spots (molecular weight range of 29 – 45 kDa) are present for SYHT0H2 soybean and not apparent in the nontransgenic control for serum 1 and 3. A similar result occurs for serum 5 for protein spots located between 66 kDa - 97 kDa. However, differences between SYHT0H2 soybean and the nontransgenic, near-isogenic control are not reflected in the two references; the SYHT0H2 soybean always has a matching protein spot in one or both of the reference soybean varieties for these same IgE binding protein spots indicating similarity between SYHT0H2 soybean and at least one of the nontransgenic varieties. Other minor variations in the visible intensity of individual protein spots from one blot to another were not considered significant in terms of IgE reactivity to those proteins. There were no unique IgE binding protein spots present in SYHT0H2 soybean seed or in seed of the other three varieties that would indicate a significant difference in protein allergen content or IgE binding capacity.

Soybean seed analyses by 2-dimensional gel electrophoresis separation and serum IgE blotting identified multiple IgE binding proteins which allowed a side-by-side comparison of four soybean varieties. As expected, there was variation in apparent protein content observed in individual protein spots from the seed extracts among the four soybean varieties. In general, Mooney and Thelen (2004) showed that many soybean proteins differ in apparent abundance between varieties when using 2-dimensional gel electrophoresis. The authors also noted that seed storage proteins, many of which are known allergens, effectively create a large dynamic range in protein expression within seed because storage proteins are so prominent relative to the other proteins in a total seed protein extract. The broad dynamic range of the soybean seed proteome means detecting and quantifying lower-abundance allergens is very challenging. In addition, 2-dimensional gel electrophoresis is a difficult method with which to achieve reproducibility in detection of individual protein spots. Thus, detecting many of the soybean seed proteins simultaneously in a total soybean protein extract using 2-dimensional electrophoresis has to be acknowledged as a qualitative assessment of protein expression.

4.2 Western Blot Controls

A Ponceau S and Western blot of a non-allergic serum control is shown in Figure 3; the Western blot is free of visible IgE binding indicating good specificity of the Western blot procedure used in this study for the detection of human IgE-binding to soybean proteins. A determination of potential cross reactivity between IgE antibody and immunoglobulin G (IgG) antibody in human serum was determined for the alkaline phosphatase-conjugated anti-human IgE antibody. This analysis was performed prior to the antibody being used for the Western blotting to ensure specific detection of IgE antibody (Figure 4). These results demonstrated that the anti-human IgE detection antibody was highly selective for human IgE and did not cross-react with human IgG antibody.

5.0 CONCLUSIONS

In this study, qualitative Ponceau S protein staining showed consistency in the number of observed proteins across all four soybean varieties indicating a general level of similarity in soybean protein expression and the ability to consistently apply the same amount of total soybean protein to 2-dimensional PAGE. Individual serum IgE does not necessarily bind the same proteins compared with other sera and the specificity of a serum's IgE for one or more of

the soybean allergens is expected to vary naturally. A qualitative assessment of human serum IgE antibody binding to soybean proteins also showed consistency in IgE binding across all four soybean varieties for each of the five soybean reactive sera. A reduced level of Ponceau S staining was noted for some known proteins, particularly the glycinin precursor proteins. The impact on IgE binding to these glycinin precursor proteins was most apparent for serum 3 where there was decreased staining intensity for these proteins. In this case, the nontransgenic control and NE0800097 soybean varieties either did not express these proteins to the extent observed in the other two soybean varieties or, the technical variability of the 2-dimensional gel electrophoresis and the electrophoretic transfer procedures resulted in a blot membrane that was less enriched for the glycinin precursor proteins. Nevertheless, SYHT0H2 soybean showed similar IgE binding compared to nontransgenic soybean. Serum 1, in contrast to serum 3, showed an overall higher apparent affinity for these same proteins and equivalent IgE binding to the glycinin precursor proteins.

A "weight-of-evidence" approach relying on evaluating all of the sera, in total, provided the best estimation of IgE binding amongst the soybean varieties. SYHT0H2 did not show the presence of any unique protein spots that were not also present in the nontransgenic control or one of the reference varieties. In many cases, proteins displaying differences in IgE binding appeared to be expressed at very low levels and are likely near the limit of detection for IgE binding detection. The overall impact was slightly variable IgE binding, thus translating into some protein spots being visible for one soybean variety and invisible in another. Overall, the serum IgE binding to SYHT0H2 soybean proteins was considered consistent with the overall level of IgE binding to the nontransgenic soybean varieties.

In summary, SYHT0H2 soybean showed a high degree of similarity in the assessment of IgE binding to endogenous soybean proteins when compared to nontransgenic soybean varieties. Both SYHT0H2 soybean and the nontransgenic control shared overall similarity with the nontransgenic reference soybean varieties and support the conclusion in McClain and Thelen (2012) that SYHT0H2 contains similar levels of endogenous allergens as nontransgenic soybean comparators. Taken together, the data in this study strongly supports the conclusion that SYHT0H2 is not likely to have greater allergenic potential than conventional, nontransgenic soybean varieties.

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TABLE 1 List of test, control and reference substances for the 2-dimensional Western blot analysis

Material ID No.	Material Type	Identification
10SG900903	Test	SYHT0H2
09SG050867	Nontransgenic control	JACK
09SG061490	Nontransgenic	BPR99402 /99022-A01-16277-01
09WC001421	Nontransgenic	NE0800097

TABLE 2 Serum IgE binding screening scores produced by ViraCor-IBT Laboratories for sera used in this study

Serum Sample	IgE Binding Score to Soybean (CAP-FEIA), kU/L	Class[1]
Control Serum	<0.10	0
Soybean Positive Serum 1	85.70	5
Soybean Positive Serum 2	32.10	4
Soybean Positive Serum 3	98.00	5
Soybean Positive Serum 4	25.30	4
Soybean Positive Serum 5	53.40	5

[1]Concentration (kU of Allergen/liter serum) of specific IgE antibody categorized into a whole-number rating; class 0 - 6 over the range of 0.1 to > 100 kU/L.

FIGURE 1 Series of Ponceau S stained blotting membrane sets

Each membrane set contains a blot for each of the soybean varieties and each set is matched to the corresponding serum in Figure 2.

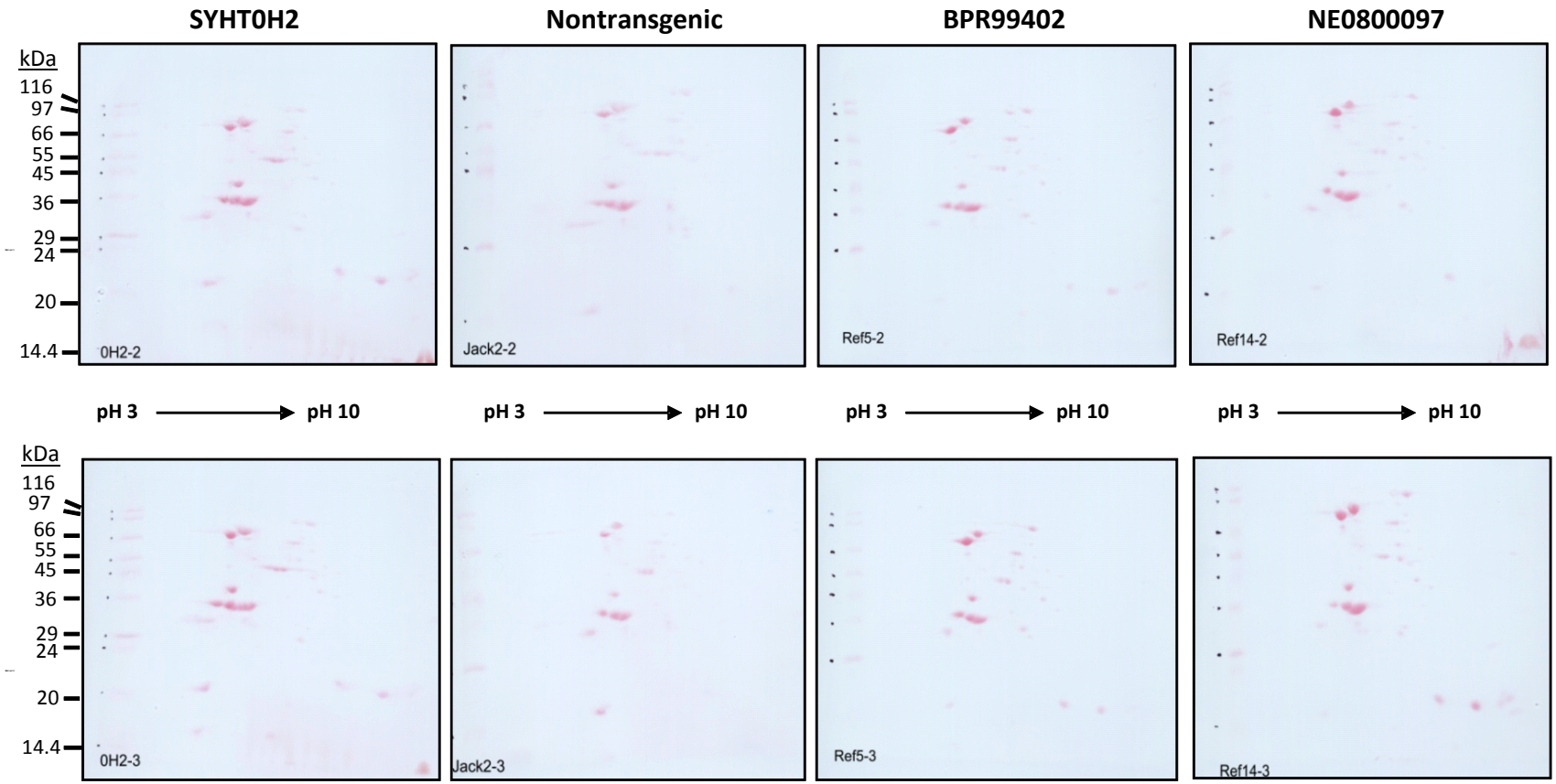


FIGURE 1 Series of Ponceau S stained blotting membrane sets (Continued)

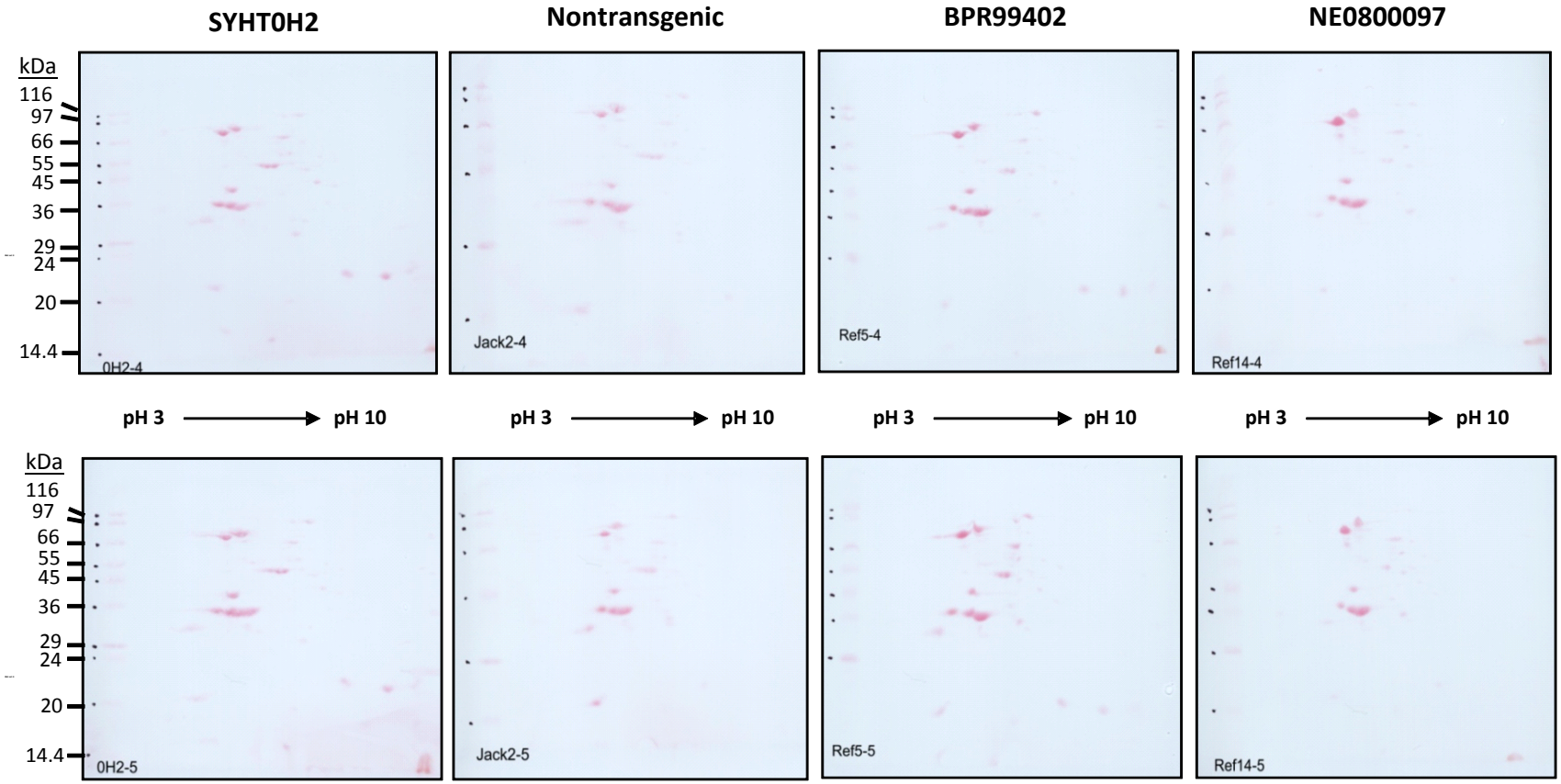


FIGURE 1 Series of Ponceau S stained blotting membrane sets (Continued)

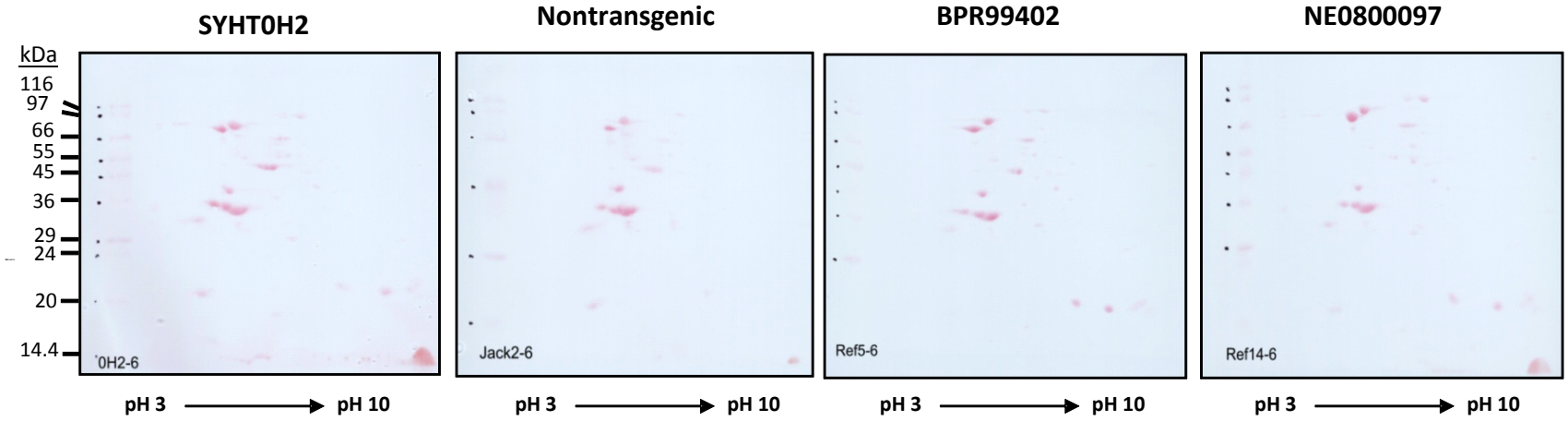


FIGURE 2 **Series of 2-dimensional Western blots with SYHT0H2, a nontransgenic control and two reference soybean varieties**

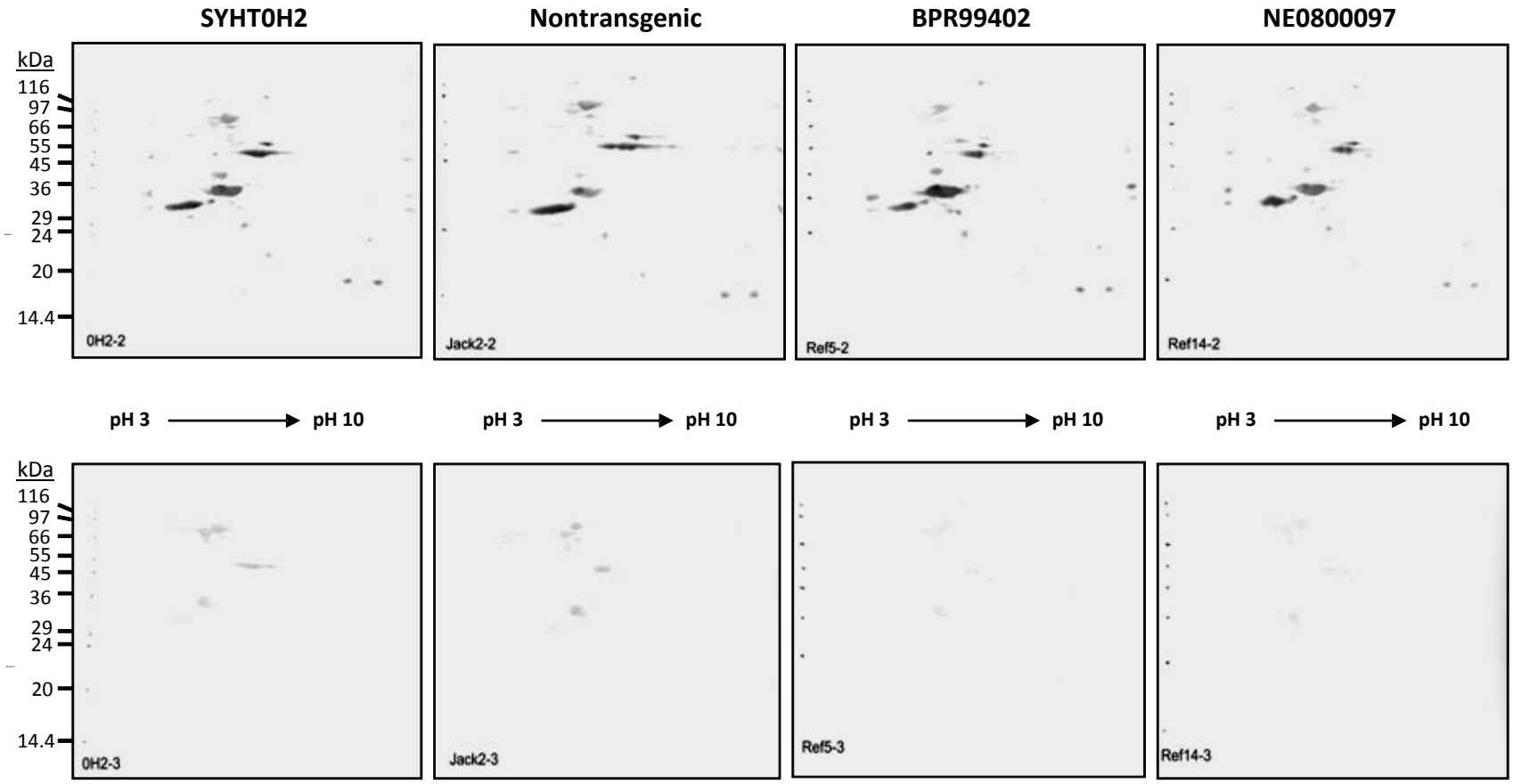


FIGURE 2 **Series of 2-dimensional Western blots with SYHT0H2, a nontransgenic control and two reference soybean varieties (Continued)**

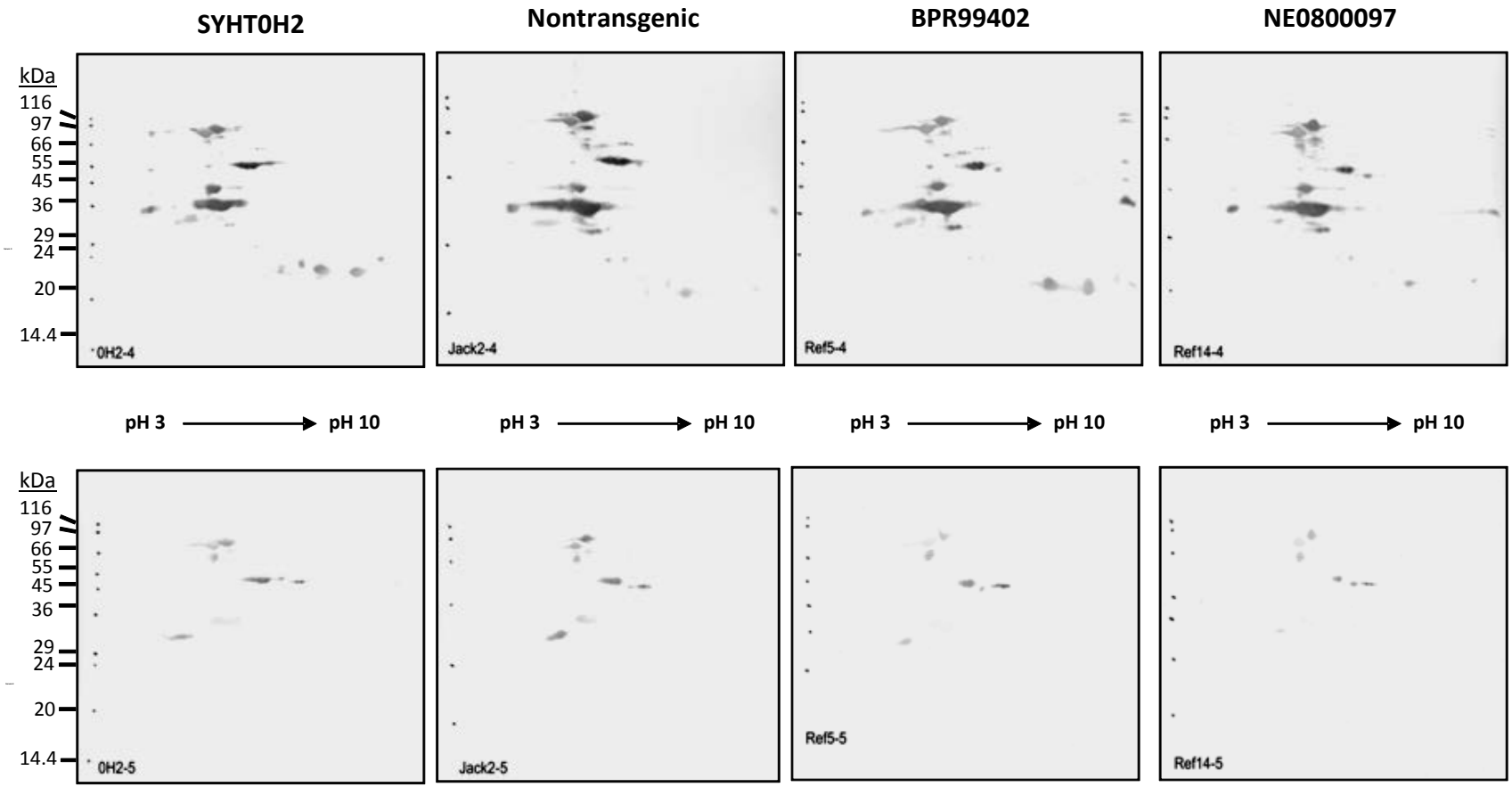


FIGURE 2 Series of 2-dimensional Western blots with SYHT0H2, a nontransgenic control and two reference soybean varieties (Continued)

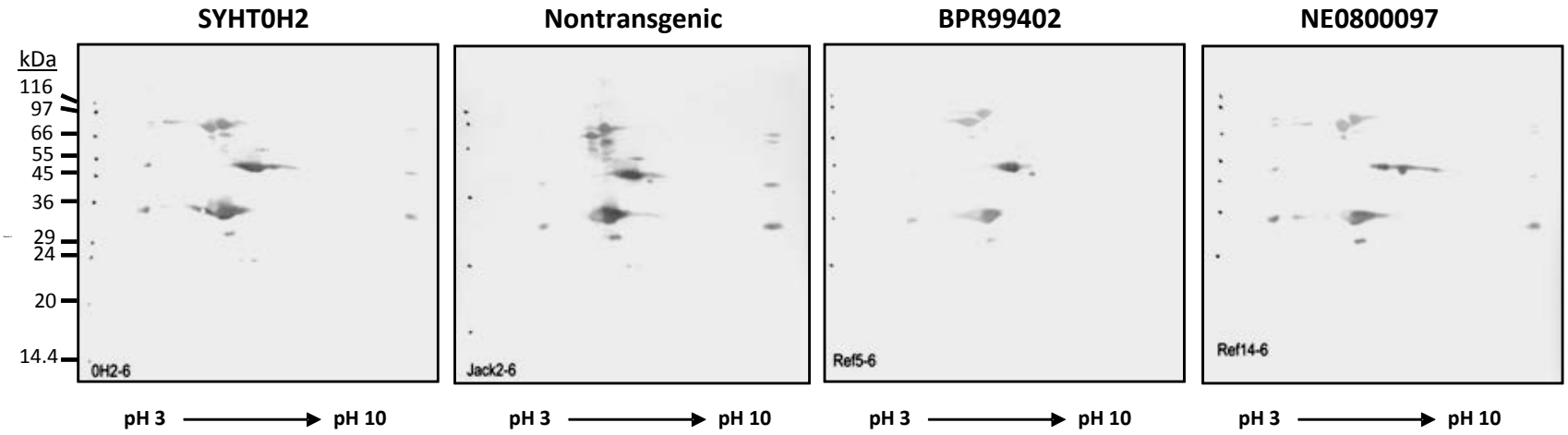


FIGURE 3 **Ponceau S and control serum Western blots with SYHT0H2, a nontransgenic control and two reference soybean varieties.**

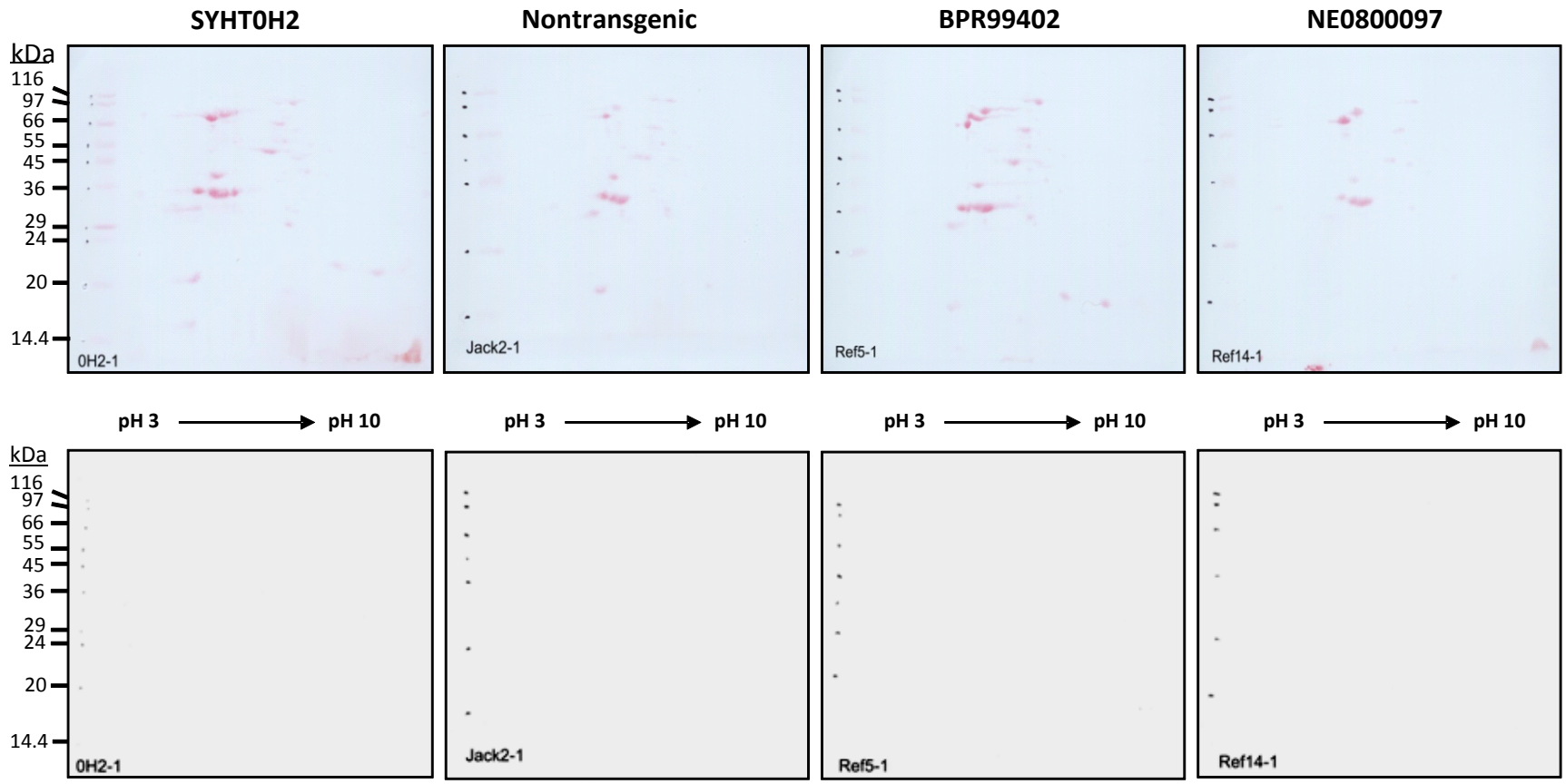
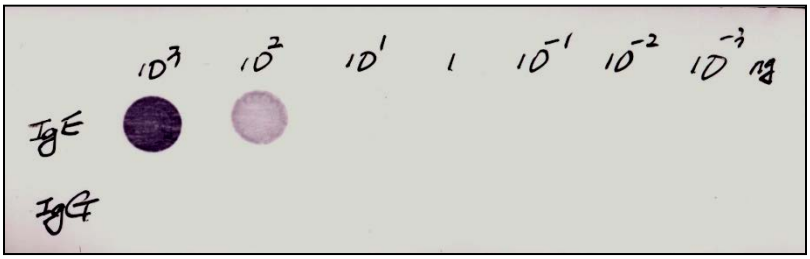


FIGURE 4 Quality control evaluation of the anti-human IgE detection antibody used in the Western blot analysis.

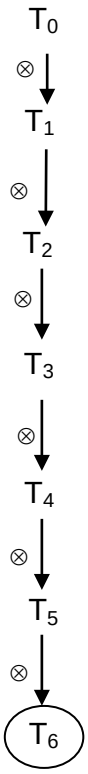
There was no cross-reactivity of the anti-human IgE antibody with purified human IgG protein. IgE and IgG were loaded as spots at the indicated level of ng of purified antibody protein.

Purified human IgE protein (top row)
Purified human IgG (bottom row)



APPENDICES SECTION

APPENDIX A Pedigree Chart of SYHT0H2 Soybean Seed Illustrating the Production of the Test Substance Used in this Study



Transformation recipient line is 'Jack' soybean variety

T_0 = original transformant.
⊗ = self-pollination.
The generation of test material, SYHT0H2, used in this study is denoted with a circle.