Enzyme-Linked Immunosorbent Assay for Quantitation of Neomycin Phosphotransferase II in Genetically Modified Cotton Tissue Extracts

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A double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was developed and validated to accurately quantitate neomycin phosphotransferase II (NPTII) levels in genetically modified cotton seed and leaf tissue. This assay provides a safer and more efficient alternative than the existing methods to measure NPTII levels in cotton tissues from genetically modified plants that contain NPTII as a selectable marker. Parallelism studies using purified bacterially-expressed NPTII and plant-expressed NPTII in the ELISA established that these proteins are immunologically and conformationally equivalent. Data on sensitivity, accuracy, and precision substantiated the utility of this ELISA for analysis of NPTII in genetically modified plant tissue. NPTII was extracted from cotton tissue using an aqueous buffer providing maximum recovery of NPTII and minimizing tissue interference. Expression levels of NPTII measured in three different genetically modified cotton lines ranged from 0.040 to 0.044% and from 0.009 to 0.019% of extractable protein for leaf and seed extracts, respectively.

INTRODUCTION

Neomycin phosphotransferase II (NPTII) inactivates aminoglycoside antibiotics such as neomycin and kanamycin by transferring the γ-phosphate from ATP to the respective antibiotic. This 29 000-Da enzyme was originally identified and purified from neomycin/kanamycin-resistant bacteria that contain the gene for NPTII on transposon Tn5 (Jorgenson et al., 1979; Auerswald et al., 1981). NPTII has recently found great utility in the field of plant and animal molecular biology, where it has been used in the selection process to produce genetically modified plants (Firoozabady et al., 1979; Fraley et al., 1985; Umbeck et al., 1987; Hinchee et al., 1988) and animal cells (Rosenberg et al., 1990). Current procedures to measure NPTII typically rely on the use of [γ-^{32}P]ATP. In these assays, NPTII transfers the γ-phosphate from radiolabeled ATP to the antibiotic, and the labeled antibiotic is separated from the labeled ATP by selective binding to phosphocellulose paper (McDonnell et al., 1987; Staebell et al., 1990), thin-layer chromatography (Cabanes-Bastos et al., 1989; Roy et al., 1990), or electrophoresis (Fregian et al., 1985). The amount of radiolabeled antibiotic detected by scintillation counting or autoradiography is proportional to the amount of functionally-active NPTII present. These methods have several disadvantages: the instability of NPTII enzymatic activity (Goldman et al., 1976); substantial sample interference (Henderson et al., 1991); the short half-life of [γ-^{32}P]ATP; limited sample throughput; and the use of radioactivity, which is costly and requires special disposal. We have focused our efforts on the development of a rapid, efficient, accurate, and cost effective ELISA for the quantitation of NPTII in genetically modified plant tissues.

The ELISA described herein is a sandwich procedure; antibodies immobilized on microtiter plates bind to NPTII, and horseradish peroxidase-labeled antibody is added to produce a "sandwich". The amount of NPTII present is directly proportional to the amount of peroxidase-labeled antibody bound in the sandwich. This assay has been validated to provide quantitative data for scientific analyses and to support the regulatory approval of genetically modified plants that express this protein.

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MATERIALS AND METHODS

Antisera Production and Conjugation. Antibodies specific to NPTII were produced in two New Zealand white rabbits (R372 and R373) by a modification of the multiple intradermal immunization procedure originally described by Vaitukatus (1981). The animals were immunized at monthly intervals with approximately 100 μg of NPTII (5′→3′ Inc., Boulder, CO) per rabbit in Freund's complete adjuvant (two immunizations). NPTII used for immunizations and standards was greater than 95% pure. Subsequent immunizations used 50 μg of antigen/rabbit in Freund's incomplete adjuvant. Blood was drawn from the central ear vein 7–14 days after each injection and antibody titer determined on the basis of binding of NPTII specific antibody to NPTII (200 ng) which had been adsorbed to the wells of microtiter dishes. The IgG fraction from rabbit serum from the third bleed was affinity purified on protein A–Sepharose CL4-B (Sigma, St. Louis, MO). Affinity-purified rabbit IgG was conjugated to horseradish peroxidase (Sigma) using the method described by Nakane and Kawoi (1974).

ELISA Procedure. Note: All samples and standards were diluted in 0.1% BSA, 0.05 M sodium phosphate, pH 7.4, 1% PVP-40, 0.15 M sodium chloride, and 0.05% Tween 20. Washing buffer was 0.05 M sodium phosphate, 0.15 M sodium chloride, and 0.05% Tween 20, pH 7.4.

The ELISA procedure consisted of three general steps:

Step 1. Two micrograms of affinity-purified antibody in 250 μL of 0.1 M carbonate/bicarbonate buffer was adsorbed to the wells of microtiter plates overnight at 4 °C.

Step 2. Appropriately diluted standards or samples (250 μL; 5–15 μL of seed or leaf extract) were added to the plate and peroxidase-conjugated antibody (0.33 μg) and incubated overnight at 4 °C.

Step 3. TMB substrate (250 μL) was added and allowed to react for 5–10 min at room temperature (in the dark). The reaction was stopped with 100 μL of 3 M phosphoric acid. Absorbance was read on a Bio-Rad (Richmond, CA) Model 3550 microplate processor at 450 nm.

Washing Steps. Wells were washed between each step. This washing was extremely important for reducing nonspecific binding and variation. Three wash steps were used between steps 1 and 2 and four washes between steps 2 and 3. The washing was accomplished by one fast wash (adding buffer and immediately pouring it out) followed by two or three slow washes (5-min

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incubation with wash solution). All washes were performed at room temperature.

Extraction of NPTII from Seed and Leaf Tissue. NPTII was extracted from genetically modified seed and leaf samples using an extraction buffer that consisted of 100 mM Tris, 10 mM sodium borate, 5 mM magnesium chloride, 0.2% ascorbate, and 0.05% Tween 20, pH 7.8. This buffer has been used to extract other enzymes from cotton tissue and found to remove interfering substances (D. Re, unpublished results). Several experiments were performed to optimize the extraction of NPTII. To determine the optimum tissue to volume ratio, frozen leaf samples were weighed, cold (4 °C) extraction buffer (10:1 to 320:1 volume/fresh weight of tissue) was added, and samples were homogenized in a polytron (Brinkman Instruments Model PT 3000 fitted with a PT-DA 3012/2TS generator) using four 15-s bursts. Aliquots of the sample were transferred to microfuge tubes and frozen at −80 °C until ready for assay. Seeds were extracted in a similar manner but were crushed first with a hemostat. The amount of NPTII present in the tissue (at the various volume/tissue ratios) was expressed as nanograms of NPTII per gram of fresh weight of tissue.

Once the optimum tissue to volume ratio was determined, the efficiency of extraction was determined. Tissue was extracted with extraction buffer and the insoluble fraction pelleted by centrifugation (12000g, 15 min). The ELISA was used to determine the concentration of NPTII in the supernate (S1). The pellet from the first extract was re-extracted using identical conditions a second time, and the ELISA was used to determine the concentration of NPTII in the supernate (S2). A final extraction of the second pellet was performed using SDS–polyacrylamide reducing sample buffer (0.0625 M Tris-HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, pH 6.8) at approximately one-sixth of the original volume (S3). The supernates from S1, S2, and S3 were analyzed by western blotting to determine NPTII concentration and for comparison with the ELISA results. Percent extraction efficiency was calculated by determining the percent of NPTII extracted in S1 compared to the total amount of NPTII in all three extracts. Variation in the extraction method was determined by performing the extraction procedure on a pool of tissue on three separate occasions and measuring NPTII concentrations by ELISA.

Protein Assay. Protein concentrations were routinely measured in all extracts and the amounts of NPTII reported as a percent of the total soluble protein. The appropriateness of using the Bradford protein assay with BSA as a standard to estimate total soluble protein levels in cotton seed extracts was tested. Three different protein assays, dye binding (Bradford, 1976), bicinchoninic acid (BCA) (Smith et al., 1985), and total amino acid (Moore and Smith, 1963), were used. Two different proteins (bovine serum albumin and bovine γ-globulin) were compared for their suitability as standards for determining protein concentrations in cotton seed extracts. Cotton seed extracts were prepared in extraction buffer and dialyzed to remove phenolics which interfere with the measurement of protein in plant extracts (Loomis, 1974). Protein concentrations were determined on nondesalted and desalted extracts using all three methods and both protein standards. Bovine serum albumin (BSA) and bovine γ-globulin were purchased from Sigma. Dye-binding reagent was purchased from Bio-Rad, and the BCA reagent was obtained from Pierce (Rockford, IL).

ELISA Validation: Recovery, Accuracy, Sensitivity, Precision. The recovery of NPTII was determined by spiking radiolabeled NPTII into cotton seed and leaf extracts and incubating the radiolabeled NPTII with the extracts on a horizontal shaker for 4 h at 4 °C. The insoluble fraction of the extract was pelleted by centrifugation, and the amounts of labeled NPTII in the supernates and remaining in the pellet were determined. NPTII was labeled with ^{125}I by the Bolten and Hunter (1973) method to a specific activity of 25 μCi/μg. Attempts at labeling NPTII with ^{125}I using the chloramine T method described by Hunter and Greenwood (1962) produced labeled NPTII that was nonimmunoreactive. Bolten–Hunter-radiolabeled NPTII was 60% immunoreactive on the basis of the ability of anti-NPTII antisera to bind to the labeled molecule and the complex to be immunoprecipitated with goat anti-rabbit IgG and poly(ethylene glycol).

Accuracy was determined by spiking known amounts of NPTII into cotton seed and leaf extracts and determining the amount of NPTII recovered in the ELISA (percent recovery). A parallelism study was conducted to determine whether bacterially-expressed NPTII was immunologically and conformationally equivalent to plant-expressed NPTII and to determine if the assay was free from matrix effects. Dilutions of *Escherichia coli* NPTII and cotton seed or leaf extracts were prepared in ELISA assay buffer. *E. coli* NPTII was used in the calibration curve to determine the concentration of the enzyme in leaf or seed extracts. Volumes from 1 to 15 μL of extract were assayed in triplicate in the ELISA. The concentration in individual replicates was determined. The log of the nanograms observed at each dilution was plotted vs the log of the volume assayed and a linear regression performed using selected volumes. The upper and lower confidence intervals were calculated for the regression line derived from the volumes selected.

Sensitivity (lowest detectable level) was defined as the amount of NPTII that could be measured by an absorbance reading of 2 standard deviations above background absorbance. Specificity was determined by assaying nontransformed plant extracts in the ELISA.

Intra-assay precision was based on the pooled variation derived from absorbance values in standards from 10 independent assays performed on different days. Interassay precision was derived from the analysis of aliquots of frozen leaf extracts prepared from a pool of genetically modified cotton leaves which was used for interassay controls. These data were obtained from 32 separate assays performed on different days over a period of 4 months when the assay was in routine use.

Analysis of Genetically Modified Cotton Plants. Genetically modified cotton plants which express a *Bacillus thuringiensis* var. kurstaki insect control protein and NPTII protein were produced as described by Perlak et al. (1990). Leaf tissue was obtained from plants grown under greenhouse conditions. Five young leaves were removed from four plants/line (three genetically modified lines and one nonmodified line) that were beginning to set bolls. The tissue was immediately frozen on dry ice, and individual extracts were prepared from the leaf tissue of each plant. Seed tissue from the same lines was harvested from plants grown in the field. For the four lines, cotton seed was harvested from six different plots/line within the same field. The cotton seed was ginned and acid delinted, and six seeds were randomly chosen from each sample and used to prepare extracts. The leaf and seed extracts from the four different lines were assayed for NPTII content and protein concentration. Analysis of variance was used to evaluate NPTII expression levels in the four lines.

RESULTS

Characterization of NPTII Antisera. Antibodies specific for NPTII were produced after the first immunization of rabbits with NPTII antisera (Figure 1A). The antisera titer peaked at day 49 and declined thereafter. Horseradish peroxidase-conjugated antisera from both rabbits readily bound to NPTII adsorbed to the surface of microtiter plates, and both conjugated antisera produced superimposable titration curves (Figure 1B). Horseradish peroxidase-conjugated antiserum from R373, however, had higher nonspecific binding levels and was slightly less sensitive when used in the sandwich ELISA. Therefore, antiserum from R372 was selected for the development of the ELISA. The optimum IgG concentrations were 2 μg/well for the coating antibody and 0.33 μg/well for conjugated antibody based on the highest response at low antigen concentrations. Increased concentrations of either the coating antibody or the peroxidase-conjugated antibody produced linear dose–response curves but nonspecific binding also increased, while decreased concentrations of either antisera produced flatter, less sensitive dose–response curves (data not shown). Affinity-purified antisera from R372 were also conjugated to alkaline phosphatase. However, the conjugated antisera did not bind

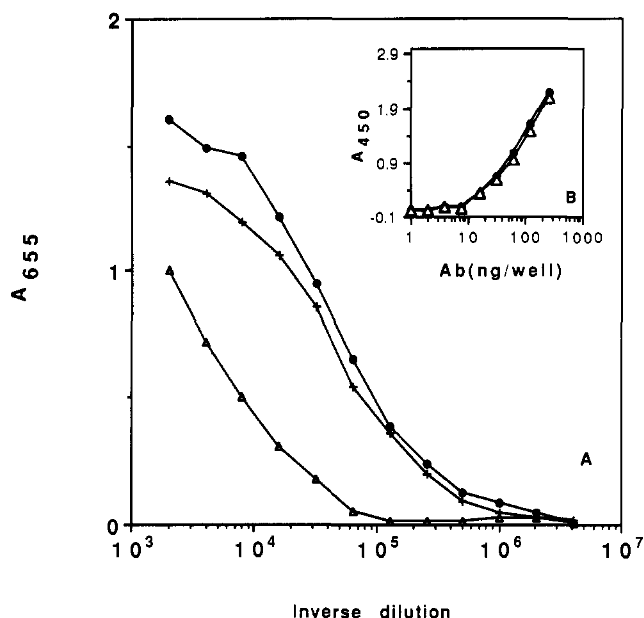

Figure 1. Titration (microliters per milliliter) of peroxidase-labeled and -unlabeled NPTII antisera. (A) NPTII (200 ng in 0.1 M carbonate/bicarbonate buffer, pH 9.6) was adsorbed to microtiter plates and incubated with dilutions of rabbit 372 NPTII antisera. IgG bound to NPTII was detected with peroxidase-labeled anti-rabbit IgG. Days post initial immunization: 21 (▵); 49 (●); 136 (+). (B) Dilutions of peroxidase-conjugated anti-NPTII from rabbits 372 (●) and 373 (▵) were incubated in microtiter plates that had been coated with 200 ng of NPTII/well.

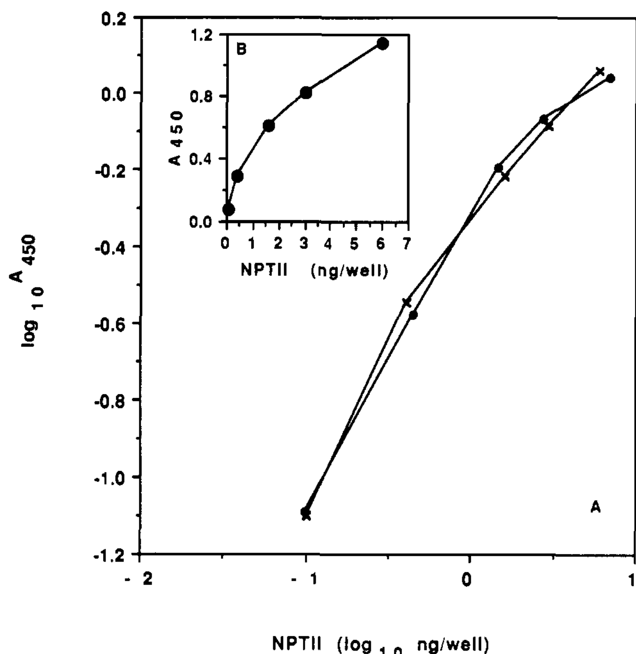

Figure 2. Standard curve transformation. The NPTII ELISA was performed as described in the text. (A) (×) log-log transformation; (●) line of best fit. (B) Plot of absorbance vs nanograms of NPTII/well produces a nonlinear dose-response curve.

to NPTII when used in the double antibody sandwich procedure (W. Kaniewski, personal communication).

Standard Curve Transformation. The standard curve was curvilinear when absorbance was plotted vs nanograms of NPTII/well (Figure 2B). Several transformations were evaluated such as the log-logit method and three-parameter polynomial and log-log fit (Fackrell et al., 1985). However, a log absorbance vs log nanograms/well transformation using quadratic regression was used

Table I. Comparison of Protein Assays for the Measurement of Total Soluble Protein in Cotton Seed Extracts

assay	standard	protein, mg/mL
dye binding	BSA	0.17
	γ-globulin	0.35
BCA	BSA	0.33
	γ-globulin	0.27
total amino acid		0.16

Table II. Extraction Efficiency of NPTII from Cotton Seed and Cotton Leaf Tissue

extract	tissue	total, ng	efficiency,[b] %
S1	seed	2230	71.2
S2	seed	0	
S3	seed[a]	900	
S1	leaf	3526	85.5
S2	leaf	300	
S3	leaf[a]	300	

[a] Quantitated by western blot. [b] % efficiency = (S1/(S1 + S2 + S3)) × 100.

for data reduction (Figure 2A). All data reductions were performed using either a Microsoft Excell spreadsheet or the Bio-Rad Microplate Manager program on a Macintosh SE computer.

Protein Assay. The dye-binding assay using BSA as the standard provided the best estimate of the absolute protein concentration as determined by total amino acid analysis (Table I). Furthermore, estimates of protein concentration in cotton seed extracts using the Bradford assay and a BSA standard before and after desalting were similar, suggesting low interference with the assay by the extracts. These results support the appropriateness of the Bradford assay using BSA as a standard to estimate total protein levels in cotton seed extracts.

Extraction of NPTII from Cotton Tissue. Approximately 71% of immunoreactive NPTII was extracted from cotton seed in the first extract (S1) (Table II). NPTII was not detectable (by ELISA) in the re-extracted pellets (S2) obtained from the first extraction. An estimate of the amount of NPTII remaining in the pellet (S3) was determined by western blotting. Immunoreactive bands could be visualized on the nitrocellulose, and the amount of NPTII remaining in the pellet was estimated to be 29% of the original amount. The extraction efficiency from leaf was slightly higher (Table II). Eighty-five percent of NPTII was extracted from leaf tissue in the first extract (S1). In contrast to seed, NPTII was detected in the pellet from the first extract (S2). This is most likely due to the higher expression of NPTII in leaf tissue as compared to seed. The amount of NPTII remaining in the S3 pellet was 7.3% on the basis of quantitation by western blot.

The maximum amount of NPTII was extracted from seed at a 1:40 ratio and nearly equivalent amounts extracted up to a 1:160 ratio (Figure 3). Below a 1:40 ratio the extraction efficiency decreases. Concentrations of NPTII in seed extracted above a 1:160 ratio were greatly decreased. This may be due to degradation of the protein or nonspecific binding of target protein during extraction.

The optimum tissue to volume ratio for extraction of NPTII from leaf tissue was above 1:50 (Figure 4). Roughly equivalent amounts of NPTII were extracted from tissue from 1:50 to 1:320. Below 1:50 the amount of NPTII extracted decreased, suggesting that the buffer was saturated. In general, a plateau was observed for both seed and leaf volume to tissue ratios. If volume to tissue ratios below the plateau are used, the amount of NPTII

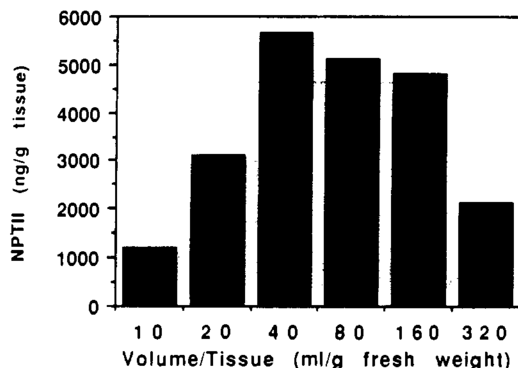

Figure 3. Seed volume to tissue ratio. Various volumes of cold extraction buffer were added to cotton seed, and NPTII was extracted as described in the test. The amount of NPTII extracted per gram of tissue is plotted vs the volume of buffer added. The NPTII ELISA was performed as described in the text.

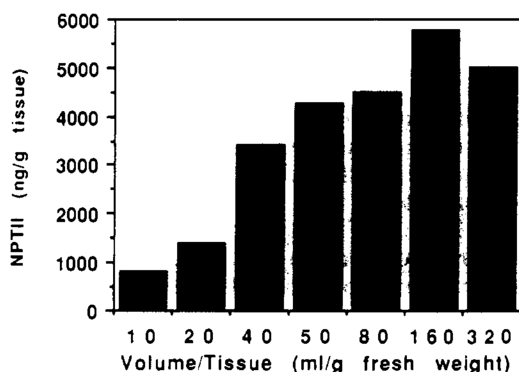

Figure 4. Leaf volume to tissue ratio. Various volumes of cold extraction buffer were added to cotton leaf, and NPTII was extracted as described in the text. The amount of NPTII extracted per gram of tissue is plotted vs the volume of buffer added. The NPTII ELISA was performed as described in the text.

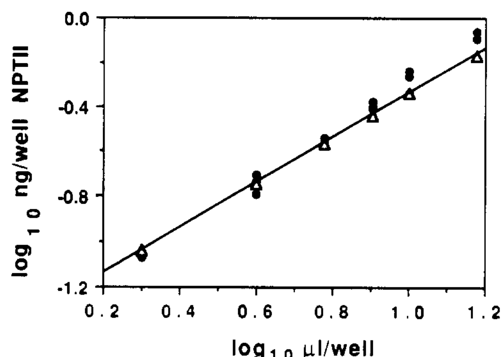

Figure 5. NPTII ELISA parallelism test. Cotton seed tissue was extracted at a 60:1 ratio (buffer/tissue fresh weight) and 2–15 μL of extract assayed in triplicate for NPTII. (△) Predicted line with a slope of 1; (●) NPTII concentration in individual replicates.

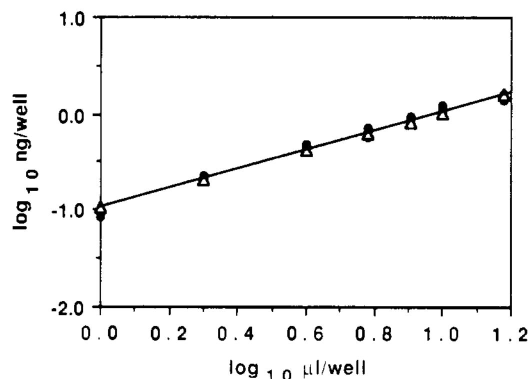

Figure 6. NPTII ELISA parallelism test. Cotton leaf tissue was extracted at a 50:1 ratio (buffer/tissue fresh weight) and 1–15 μL of extract assayed in triplicate for NPTII. (△) Predicted line with a slope of 1; (●) NPTII concentration in individual replicates.

(nanograms per gram of tissue fresh weight) is underestimated. At volume to tissue ratios above the plateau, roughly equivalent concentrations are obtained. Cotton leaf was extracted at a 1:50 ratio and seed at a 1:60 ratio.

The variation in NPTII concentrations for leaf ranged from +25 to −20% from the observed mean. In seed extracts variation ranged from +12.8 to −18.9%.

The correlation in the levels of NPTII detected by the ELISA and by the western blot was very good for leaf and seed extracts. For instance, the concentration of NPTII in S1 seed extracts determined by ELISA was 121 ng/mL vs 70 ng/mL from western quantitation. Leaf S1 levels were 139 ng/mL by ELISA and 128 ng/mL by western. The S2 seed extracts yielded values of 3.5 ng/mL (western) and were undetectable by ELISA. Leaf S2 extracts were 11.5 and 12.1 ng/mL for western and ELISA, respectively. The differences seen between the two systems are most likely due to the inaccuracy associated with the western. The quantitation of this assay was based on a visual estimate of the concentration of NPTII in plant extracts. This estimate was based on the intensity and thickness of the plant NPTII band compared to the band produced by known concentrations of NPTII which were spiked into wild-type extracts and electrophoresed on the same gel.

Assay Validation. Typical intra-assay percent coefficient of variation (% CV) (based on absorbance values in triplicates in the buffer standard curve) were less than 5% for concentrations of NPTII between 0.1 and 6 ng/well. Ninety-six percent of labeled NPTII was recovered when spiked into the supernatant of seed extracts, whereas 72% was recovered when spiked into leaf extracts. These results demonstrate that the NPTII does not partition with the pellet and provides an independent measure of the recovery of the enzyme. Mean percent recoveries (accuracy) of NPTII spiked into seed and leaf extracts were 112 and 103%, respectively.

According to Rodbard et al. (1978), a linear regression performed using the log microliters assayed vs log nanograms observed should yield a slope of 1 if parallelism exists. Linear regression performed on the log–log transformed data from seed extract assayed from 6 (2.74 μg) to 15 μL (6.84 μg) satisfied the parallelism test (Figure 5). The confidence intervals for the slope generated from these data ranged from 0.9400 to 1.2974 with a correlation coefficient (R^2) of 0.95. The slope of the log–log regression line was not different from 1 for volumes of leaf tissue from 2 (0.17 μg) to 15 μL (1.26 μg) (Figure 6). The confidence intervals for the slope generated from these data ranged from 0.9255 to 1.0596 with a correlation coefficient of 0.98. These data establish that bacterially-expressed standard is immunologically equivalent to NPTII expressed in genetically modified cotton seed and leaf and that the seed and leaf extracts do not interfere with the ELISA. In addition, the plant-expressed protein and the NPTII standard are indistinguishable when analyzed by western blot analysis (data not shown).

Concentration of NPTII in Cotton Seed and Leaf Extracts. The levels of NPTII expressed as a percent of total soluble protein in seed and leaf extracts for three independent genetically modified cotton lines are pre-

Table III. NPTII Levels (Percent of Soluble Protein) in Cotton Leaf and Seed Extracts

line	sample	mean value[a]	SD[b]
WT[c]	leaf	0.000	
81	leaf	0.040a	0.004
247	leaf	0.042a	0.014
249	leaf	0.044a	0.011
WT	seed	0.000	
81	seed	0.009a	0.004
247	seed	0.009a	0.005
249	seed	0.019b	0.007

[a] Means with different letters are significantly different. Mean values were derived from at least four individual plants/line. [b] SD, standard deviation. [c] Wild type.

sented in Table III. Mean expression levels of NPTII ranged from 0.040 to 0.044% of total extractable protein for leaf tissue and from 0.009 to 0.019% in seed tissue. No NPTII was detected in nontransformed plants.

DISCUSSION

We report the development and validation of an ELISA for NPTII, a selectable marker protein routinely used in the construction of genetically modified plants and animals. We initially attempted to develop a radioimmunoassay. Unfortunately, the sensitivity, accuracy, and reproducibility were unacceptable. NPTII has been detected using functional assays but quantitation is difficult due to the presence of inhibitors of activity requiring special sample preparation (Henderson et al., 1991; Staebell et al., 1990) and reducing sample throughput. Furthermore, these assays only measure functionally active enzyme.

During development of the assay we noticed that recovery of unlabeled NPTII spiked into cotton seed and leaf extracts was dependent upon the amount of extract (protein) added to the microtiter wells. Percent recoveries were highly variable when greater than 30 μg of seed or leaf extract was added to the well. This does not affect the ability of the assay to detect the enzyme but does affect the ability to accurately quantitate NPTII.

NPTII expression levels determined in the leaf tissue from the greenhouse-grown genetically modified cotton leaves (81, 247, 249) were remarkably similar and correlated with expression levels in the field (data not shown). Seed expression of NPTII was approximately half that of leaf tissue and more variable from line to line.

We have optimized and validated this ELISA for NPTII analysis in cotton tissue and have confirmed the expression levels observed for NPTII in the ELISA in leaf and seed extracts using both a modified version of an NPTII functional assay (McDonnell et al., 1987) and a western blot analysis. Because of the accuracy, reliability, and throughput of this ELISA, we have used this assay extensively to detect NPTII in other genetically modified crops including tomato, tobacco, corn, and potato (data not shown). Accurate quantitation of the enzyme for each crop must be established. This assay is superior to current published procedures for measurement of NPTII because this assay does not rely on the use of radioisotopes and does not require a functionally active NPTII enzyme. On the basis of these parameters, this ELISA is and will continue to be an important tool in the development of genetically modified plants since NPTII has been used extensively as a selectable marker for *Agrobacterium tumefaciens*-mediated plant transformation.

ABBREVIATIONS USED

BSA, bovine serum albumin; PVPP, polyvinylpolypyrrolidone; ATP, adenosine triphosphate; TMB, 3,3′,5,5′-tetramethylbenzidene.

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LITERATURE CITED

Auerswald, E. A.; Ludwig, G.; Schaller, H. Structural analysis of Tn5. *Cold Spring Harbor Symp. Quant. Biol.* **1981**, *45*, 107–113.

Bolton, A. E.; Hunter, W. M. The Labelling of Proteins to High Specific Radioactivities by Conjugation to a ^{125}I-Containing Acylating Agent. *Biochem. J.* **1973**, *133*, 529–538.

Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein Dye Binding. *Anal. Biochem.* **1976**, *72*, 248.

Cabanes-Bastos, E.; Day, A. G.; Lichenstein, C. P. A Sensitive and Simple Assay for Neomycin Phosphotransferase II Activity in Transgenic Tissue. *Gene* **1989**, *77*, 169–176.

Fackrell, H. B.; Surujballi, O. P.; Burgess, S. R. ELISA Data Reduction: a Review. *J. Clin. Immunoassay* **1985**, *8*, 213–219.

Firoozabady, E.; DeBoer, D. L.; Merlo, D. J.; Halk, E. L.; Amerson, L. N.; Rashka, K. E.; Murray, E. E. Transformation of Cotton (*Gossypium hirsutum* L.) by *Agrobacterium tumefaciens* and Regeneration of Transgenic Plants. *Plant Mol. Biol.* **1987**, *10*, 105–116.

Fraley, R. T.; Rogers, S. G.; Horsch, R. B.; Eichholtz, D. A.; Flick, J. S.; Fink, C. L.; Hoffman, N. L.; Sanders, P. R. The SEV System: A New Disarmed TI Plasmid Vector System for Plant Transformation. *Bio/Technology* **1985**, *3*, 629–635.

Fregian, N.; Davidson, N. Quantitative In-Situ Gel Electrophoretic Assay for Neomycin Phosphotransferase Activity in Mammalian Cell Lysates. *Anal. Biochem.* **1985**, *148*, 101–104.

Goldman, P. R.; Northrup, D. B. Purification and Spectrophotometric Assay of Neomycin Phosphotransferase II. *Biophys. Res. Commun.* **1976**, *69*, 230–236.

Henderson, L.; Rao, A. G.; Howard, J. An Immunoaffinity Immobolized Enzyme Assay for Neomycin Phosphotransferase II in Crude Cell Extracts. *Anal. Biochem.* **1991**, *194*, 64–68.

Hinchee, M. A.; Connor-Ward, D. V.; Newell, C. A.; McDonnell, R. E.; Sato, S. J.; Gasser, C. S.; Fischhoff, D. A.; Re, D. B.; Fraley, R. T.; Horsch, R. B. Production of Transgenic Soybean Plants using *Agrobacterium*-Mediated DNA Transfer. *Bio/Technology* **1988**, *6*, 915–922.

Hunter, W. M.; Greenwood, F. C. Preparation of Iodine-131 Labelled Human Growth Hormone of High Specific Activity. *Nature* **1962**, *194*, 495–496.

Jorgenson, R. A.; Rothstein, S. J.; Reznikoff, W. S. A Restriction Enzyme Cleavage Map of Tn5 and Location of a Region Encoding Neomycin Resistance. *Mol. Gen. Genet.* **1979**, *177*, 65–72.

Loomis, W. D. Overcoming Problems of Phenolics and Quinones in the Isolation of Plant Enzymes and Organelles. *Methods Enzymol.* **1974**, *31*, 528–544.

McDonnell, R. E.; Clark, R. D.; Smith, W. A.; Hinchee, M. A. A Simplified Method for the Detection of Neomycin Phosphotransferase Activity in Transformed Tissue. *Plant Mol. Biol. Rep.* **1987**, *5*, 380–386.

Moore, S.; Stein, W. H. Chromatographic Determination of Amino Acids by the Use of Automatic Recording Equipment. *Methods Enzymol.* **1963**, *6*, 819–831.

Nakane, P. K.; Kawoi, A. Peroxidase-Labelled Antibody. A New Method of Conjugation. *J. Histochem. Cytochem.* **1974**, *22*, 1084–1091.

Perlak, F. J.; Deaton, R. W.; Armstrong, T. A.; Fuchs, R. L.; Sims, S. R.; Greenplate, J. T.; Fischhoff, D. A. Insect Resistant Cotton Plants. *Bio/Technology* **1990**, *8*, 939–943.

Rodbard, D.; Munson, P.; DeLean, A. Improved Curve Fitting, Parallelism Testing, Characterization of Sensitivity and Specificity, Validation, and Optimization for Radioligand Assays. In *Radioimmunoassay and Related Procedures in Medicine*; IAEA: Vienna, 1978; Vol. 1, pp 469–504.

Rosenberg, S. A.; Aebersold, P.; Cornetta, K.; Kasid, A.; Morgan, R. A.; Moen, R.; Karson, E. M.; Lotze, M. T.; Yang, J. C.; Topalian, S. L.; Merino, M. J.; Culver, K.; Miller, A. D.; Blaese, M.; Anderson, W. F. Gene Transfer into Humans—Immunotherapy of Patients with Advanced Melanoma, Using Tumor-Infiltrating Lymphocytes Modified by Retroviral Gene Transduction. *N. Engl. J. Med.* **1990**, *323*, 570–578.

Roy, P.; Sahasradbudhe, N. A Sensitive and Simple Paper Chromatographic Procedure for Detecting Neomycin Phosphotransferase II (NPT-II) Gene Expression. *Plant Mol. Biol. Rep.* **1990**, *14*, 873–876.

Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of Protein Using Bicinchoninic Acid. *Anal. Biochem.* **1985**, *150*, 76–85.

Staebell, M.; Tomes, D.; Weissinger, A.; Maddock, S.; Marsch, W.; Huffman, G.; Bauer, R.; Ross, M.; Howard, J.; A Quantitative Assay for Neomycin Phosphotransferase Activity in Plants. *Anal. Biochem.* **1990**, *185*, 319–323.

Umbeck, P.; Johnson, G.; Barton, K.; Swain, W. Genetically Transformed Cotton (*Gossypium hirsutum* L.) plants. *Bio/Technology* **1987**, *5*, 263–266.

Vaitukatus, J. L. Production of Antisera with Small Doses of Immunogen: Multiple Intradermal Injections. *Methods Enzymol.* **1981**, *73*, 46–52.

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