LEE, THOMAS C.[1*], ALBEE, LEE D.[1], REISER, STEVEN E.[1], JENNINGS, JAMES C.[1], ASTWOOD, JAMES D.[1] AND RONALD P. LIRETTE.[1] [1]Monsanto Company, 700 Chesterfield Parkway North, St. Louis, MO 63198. **Absence of detectable CP4 EPSPS and GUS proteins and absence of detectable sugar beet DNA in refined sugar derived from Roundup Ready® sugar beet line #77.**

ABSTRACT

Monsanto Company and Syngenta Seeds have developed Roundup Ready® (RR) sugar beet line #77, which is tolerant to glyphosate, the active ingredient in Roundup® herbicide. RR sugar beet line #77 has been genetically modified to express two proteins: CP4 EPSPS and GUS. CP4 EPSPS confers tolerance to glyphosate, whereas GUS, a scorable marker, allowed for selection of transformed cells in tissue culture. This investigation sought to determine if CP4 EPSPS and GUS protein were detectable in refined sugar derived from RR sugar beet line #77 using western blot methodology and to assess the presence or absence of sugar beet DNA in refined sugar using PCR methodology.

There were four independent refined sugar samples derived from RR sugar beets and four independent control (non-transgenic) refined sugar samples used for this investigation. The refined sugar samples derived from RR sugar beet line #77 were produced from beets grown in four different countries (Germany, Denmark, United Kingdom and United States) and processed in three different countries (Germany, Technische Universität; Switzerland, RCC Ltd, Itigen; and United States, Englar Labs). The sugar samples derived from RR sugar beets were designated as follows: 1) RR sugar beet line #77 refined sugar produced by Technische Universität, Berlin, Germany in a study sponsored by Syngenta Seeds: 2) Line #77 refined sugar from European study # 95-GLY-04-DK, sponsored by Monsanto; 3) Line #77 refined sugar from European study #95-GLY-03-UK, sponsored by Monsanto and 4) Line # 77 refined sugar from US study #98-63-R-7, sponsored by Monsanto. Three of the control (non-transgenic) sugar samples were commercially-available refined sugars obtained from Sweden, Belgium and Germany. A fourth non-transgenic sugar sample was generated as a control for Monsanto studies #95-GLY-04-DK and #95-GLY-03-UK. Because the levels of protein and DNA in refined sugar were expected to be very low [see Klein *et al.*, (1998), *J. of Biotechnology*, 60: 145-153], efficient methods were developed for the concentration and recovery of CP4 EPSPS and GUS proteins, and DNA from a refined sugar matrix using centrifugal filtration devices.

The protocol for protein extraction and recovery from sugar samples was as follows: 2.5 g of each sugar sample (four RR sugar samples and one control sample) was solubilized independently in 15 mL of 25 mM Tris-Cl, pH 8.0, 0.5 mM PMSF, 1 mM EDTA, 1 mM benzamidine-HCl and 0.5 mM DTT. At this point in the procedure, 500 ng of CP4 EPSPS and GUS protein standards were spiked into selected control extracts to allow for an estimation of the recovery of these novel proteins during the sample preparation protocol. Solubilized samples were dialyzed overnight against a large excess of the solubilization buffer and ultimately concentrated via Millipore Ultrafree-15 centrifugal concentration devices to ~500 µL. A 500 µL aliquot of 1X Laemmli sample buffer was added to each extract and concentrated again to ~500 µL using a Savant Speed

Vac. The concentration of the spiked protein standards were now at 1.0 ng/μL assuming 100% recovery and each 1 μL of the concentrated extract represented 5 mg of sugar extracted. Samples in Laemmli buffer (10 μL) were subjected to SDS-PAGE (4-20% gradient mini-gels), transblotted to PVDF membrane and probed for the presence of CP4 EPSPS and GUS proteins using highly specific polyclonal antibodies produced in goats. Immunoreactive bands were ultimately visualized using the ECL detection system of Amersham. The western blot films showed that CP4 EPSPS and GUS proteins were efficiently recovered during the sample extraction and concentration protocol. Based on western blot analysis, no CP4 EPSPS or GUS proteins could be detected in any of the RR or control sugar samples analyzed. The limits of detection (LODs) for CP4 EPSPS and GUS proteins on blot films were 2 and 10 ppb, respectively, based on the lowest detectable protein standard spiked into 10 μL of control sugar matrix and analyzed concurrently with the test samples.

The protocol for DNA extraction and recovery from sugar samples was as follows: 2.5 g of each sugar sample (four RR sugar samples and four control samples) was solubilized independently in 10 mL of TE buffer (10 mM Tris-Cl, pH 8.0, and 1mM EDTA). For spike and recovery experiments, various amounts of sugar beet genomic DNA was spiked into the control sugar matrix at the start of the extraction procedure to allow for an estimation of the recovery of novel DNA during the entire sample preparation protocol. Solubilized samples were concentrated repeatedly (4 times) in Millipore Centriplus YM-10 centrifugal concentration devices to 300-400 μL final volume. A 2 μL aliquot of the retained samples were used for the PCR assays. PCR reactions were conducted in a total volume of 50 μL. Each PCR reaction mix contained 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 200 μM each dNTP, 0.2 μM each primer, 1.5-2.0 mM $MgCl_2$ and 2 units REDtaq DNA polymerase. Standard PCR assay temperature cycles were employed using a total of 38 repetitive cycles. A 40 μL aliquot of each PCR reaction was analyzed on agarose gels. PCR assays were developed for a specific portion of the large subunit of ADP-glucose pyrophosphorylase (*agp*), an endogenous gene found in the sugar beet genome, and a region of the *cp4 epsps* coding region. These PCR assays were able to amplify an ~850 bp region of the *agp* gene from 100 pg of input sugar beet genomic DNA and a 211 bp portion of the *cp4 epsps* coding region from 30 pg of RR sugar beet line #77 genomic DNA. The recovery of ng amounts of genomic sugar beet DNA isolated from RR sugar beet line #77 spiked into control sugar matrix was greater than 90%, based on PCR analysis of 2 μL of the retained samples. Based on the methods developed specifically for this investigation, no sugar beet DNA was detected in several different samples of refined sugar derived either from RR sugar beet line #77 or from the non-transgenic refined sugar samples. The LODs for the *agp* (endogenous beet gene) and *cp4 epsps* (transgene) PCR assays were 100 and 30 pg of genomic DNA template, respectively

In summary, we conclude that refined sugar from RR sugar beet line #77 lacks both detectable DNA and either of the CP4 EPSPS or GUS proteins. Furthermore, these data indicate that refined sugar derived from Roundup Ready® sugar beets is chemically equivalent to non-transgenic commercial sugar.