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A novel method of demonstrating the molecular and functional equivalence between *in vitro* and plant-produced double-stranded RNA

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ABSTRACT

A biotechnology-derived corn variety, MON 87411, containing a suppression cassette that expresses an inverted repeat sequence that matches the sequence of western corn rootworm (WCR; *Diabrotica virgifera virgifera*) has been developed. The expression of the cassette results in the formation of a double-stranded RNA (dsRNA) transcript containing a 240 bp fragment of the WCR *Snf7* gene (DvSnf7) that confers resistance to corn rootworm by suppressing levels of DvSnf7 mRNA in WCR after root feeding. Internationally accepted guidelines for the assessment of genetically modified crop products have been developed to ensure that these plants are as safe for food, feed, and environmental release as their non-modified counterparts (Codex, 2009). As part of these assessments MON 87411 must undergo an extensive environmental assessment that requires large quantities of DvSnf7 dsRNA that was produced by *in vitro* transcription (IVT). To determine if the IVT dsRNA is a suitable surrogate for the MON 87411-produced DvSnf7 dsRNA in regulatory studies, the nucleotide sequence, secondary structure, and functional activity of each were characterized and demonstrated to be comparable. This comprehensive characterization indicates that the IVT DvSnf7 dsRNA is equivalent to the MON 87411-produced DvSnf7 dsRNA and it is a suitable surrogate for regulatory studies.

1. Introduction

RNA interference (RNAi) is a gene silencing mechanism triggered by double-stranded RNA (dsRNA; (Fire et al., 1998)) and is a vital part of the immune response to viruses and the regulation of gene transcription in eukaryotes (Sharp, 2001). In addition to regulating endogenous gene expression, dsRNA has been shown to be an effective tool for insect control (Mao et al., 2007; Whyard et al., 2009; Zhu et al., 2012), including western corn rootworm (WCR, *Diabrotica virgifera virgifera*) and southern corn rootworm (SCR, *Diabrotica undecimpunctata howardi* (Baum et al., 2007; Bolognesi et al., 2012). One such dsRNA, DvSnf7, induces mortality in WCR (Bolognesi et al., 2012) by suppressing DvSnf7, a component of the ESCRT-III complex (Endosomal Sorting Complex Required for Transport-III) that is essential for biological processes including the sorting of cell membrane receptors (Kim et al., 2011; Sweeney et al., 2006; Vaccari et al., 2009). Shortly after oral exposure to DvSnf7 dsRNA, there is significant suppression of DvSnf7 mRNA in the midgut and other tissues in the insect followed by a reduction in DvSNF7 protein levels (Bolognesi et al., 2012). In turn, the degradation of DvSNF7 protein leads to the accumulation of ubiquitinated proteins, disruption of autophagy and ultimately mortality in WCR larvae (Koci et al., 2014; Ramaseshadri et al., 2013).

Prior to commercialization of a genetically modified crop an extensive assessment of the inserted gene product and whole crop is performed. The assessment of a pesticidal gene product includes several types of studies (e.g., non-target organism testing, environmental fate and characterization of the mechanism of action) that require large amounts of purified product from the inserted gene. Historically the product from the inserted gene has been a protein that is present in amounts that are too low to feasibly isolate in sufficient quantities to conduct the full range of studies. As such, these assessments typically use protein purified from a bacterial expression system. Before the bacterial-expressed protein

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can be used in analyses, it is necessary to demonstrate that the bacterial protein is a suitable surrogate (structurally and functionally)for the plant-produced protein from the inserted gene. A number of comparisons including mass, sequence, immunereactivity, glycosylation status, and functional activity between the bacterial-produced and plant-produced proteins are made to demonstrate they are equivalent (Raybould et al., 2013).

Unlike other genetically modified crops that have insecticidal activity, MON 87411 is unique in that its product from the inserted DvSnf7 gene is a dsRNA rather than a protein. What is not unique is the large amounts of the gene product that is needed to be produced to complete the required environmental assessments of MON 87411's dsRNA. In order to support the environmental assessments the *in vitro* (IVT) produced DvSnf7 dsRNA must be demonstrated to be a suitable surrogate, in both structure and function, to that of the plant-produced DvSnf7 dsRNA. In this paper we describe the isolation and quantification of DvSnf7 dsRNA in MON 87411 and the subsequent generation and purification of DvSnf7 dsRNA by *in vitro* transcription in amounts sufficient to complete the environmental assessments regulatory studies. We also describe the studies completed to demonstrate the structural and functional equivalence between the IVT and MON 87411-produced DvSnf7 dsRNA. To our knowledge, this is the first published demonstration of equivalence between an IVT-produced dsRNA and plant-produced dsRNA for the purpose of an assessment.

2. Materials and methods

2.1. Transformation and DvSnf7 dsRNA expression cassette

MON 87411 was developed through *Agrobacterium*-mediated transformation of immature corn embryos (Sidorov and Duncan, 2009). The R_0 plants (the first transformed generation) were selected on medium containing glyphosate. After self pollination and the identification of homozygous seed, plants were subjected to further molecular and phenotypic assessments (Data not shown). MON 87411 was selected based on its ability to confer resistance to WCR and its agronomic, phenotypic, and molecular characteristics. In brief the T-DNA insert consists of two copies of a 240 bp segment of the *D. virgifera* gene orthologous to yeast Snf7 (sequence reported in Bolognesi et al., 2012), the repeats are separated by a 150 bp spacer sequence and the transgene expression is driven by the CaMV 35S promoter and leader. A more complete description of the T-DNA insert was previously described (Armstrong et al., 2013).

2.2. DvSnf7 dsRNA quantification from MON 87411

MON 87411 leaf, root, whole plant, and grain were collected from field trials conducted at five sites in Argentina during the 2011–2012 growing season. At each site, four replicated plots were planted using a randomized complete block field design and plant materials were collected from each replicated plot at each field site. Leaf, root, and whole plant were collected and placed on dry ice at development stage vegetative 3–4 (V3–V4: Ritchie et al., 1997). Total RNA was extracted from a known amount of corn tissue using the method described by Armstrong et al. (2013) and normalized to an appropriate concentration. DvSnf7 dsRNA was quantified using a QuantiGene Assay method (Affymetrix Inc. 2010; Armstrong et al., 2013). The concentration of DvSnf7 dsRNA from the assay was converted to concentration in fresh weight (fw) tissue (ng/g fw) based on the total RNA/tissue ratio and total RNA dilution factor.

2.3. Synthesis of DvSnf7 dsRNA

The IVT dsRNA was synthesized *in vitro* using an *E. coli* T7 polymerase produced by expression in BL21 (Rosetta2) competent cells and purified using His-SELECT-HF (Sigma, St. Louis, MO). A plasmid containing the DvSnf7 sequence was linearized with a restriction enzyme that cut at the end of the DvSnf7 transcript and used in an *in vitro* transcription reaction containing 32 mM $MgCl_2$, 10 mM DTT, 200 mM each ATP, CTP, GTP and UTP, 10 U/ml IPP, 0.05 mg/ml T7 polymerase and 5% DMSO to produce a 968 nucleotide RNA. Following transcription, the RNA was heated to ~70 °C and then allowed to cool at room temperature ensuring the inverted repeats contained within the RNA were annealed to each other and form a hairpin loop. This dsRNA was treated with DNase to remove any remaining plasmid and purified using phenol:-chloroform. The pellet was resuspended in UltraPure Water (Life Technologies, Grand Island, NY), quantified using a NanoDrop spectrophotometer, and stored at − 80 °C.

2.4. RNA isolation and mRNA enrichment

Corn leaves (V6 growth stage) from greenhouse grown plants were collected and subsequently ground to a fine powder in liquid nitrogen. RNA was extracted using E.Z.N.A. total RNA Kit II (Omega Bio-tek Norcross, USA). The mRNA was enriched in the samples through the use of Omega bio-tek's Mag-Bind mRNA enrichment kit. The following changes were made to increase mRNA capture: (1) The bead-to-sample ratio was increased from 0.5:1 to 1:1. (2) The supernatant from the first mRNA capture step was rehybridized in a second pass to increase the amount of mRNA captured. (3) Washing steps were reduced from 3 times to 1. (4) The sample was heated to 80 °C for 2 min during the elution step. The mRNA was subsequently concentrated using ethanol and sodium acetate to precipitate the mRNA. The mRNA was resuspended in water, quantified using a NanoDrop spectrophotometer, and stored at −80 °C.

2.5. cDNA synthesis

MON 87411 enriched mRNA was reverse transcribed using the Omniscript Reverse Transcription kit (Qiagen, Valencia, CA). An anchored oligo dT primer that is a random nucleotide sequence that causes the oligo dT primer to anneal to the start of the poly (A) tail rather than a position further downstream was used to produce cDNA. The manufacturer's specifications were followed with the exception of the incubation time (reaction was run for 30 min instead of 60 min).

2.6. PCR amplification

Two overlapping PCR products (Product A (609 base pairs) and Product B (425 base pairs)) were generated that span the dsRNA portion of DvSnf7 RNA sequence in MON 87411.

The PCR amplifications of Product A and B were performed on MON 87411 DvSnf7 cDNA. The primer sequences for Product Awere: 5′-TAACAACATCAACACTCATCACTC-3′ and 5′-TTTGAGAA TGAACAAAAGGACCATATCATTC-3′. The primer sequences fro Product B were: 5′-ACACGCTGAACCGTCTTC-3′ and 5′-TAATCG GTCGTGTGAGAGTAGTGA-3′

2.7. RNA i_f treatment

IVT DvSNf7 dsRNA and MON 87411 RNA were digested with RNase I_f (New England Biolabs, Ipswich, MA) using the manufacturer's suggested reaction conditions. Following the 5 min