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Quantification of Transgene-Derived Double-Stranded RNA in Plants Using the QuantiGene Nucleic Acid Detection Platform

Toni A. Armstrong,[†] Hao Chen,[‡] Todd E. Ziegler,[†] Kelly R. Iyadurai,[†] Ai-Guo Gao,[†] Yongcheng Wang,[†] Zihong Song,[‡] Qing Tian,[‡] Qiang Zhang,[†] Jason M. Ward,[‡] Gerrit C. Segers,[†] Gregory R. Heck,[‡] and Jeffrey M. Staub*[,†]

[†]Monsanto Company, 700 Chesterfield Parkway North, St. Louis, Missouri 63017, United States

[‡]Monsanto Company, 800 North Lindbergh Boulevard, St. Louis, Missouri 63167, United States

Supporting Information

ABSTRACT: The expanding use of RNA interference (RNAi) in agricultural biotechnology necessitates tools for characterizing and quantifying double-stranded RNA (dsRNA)-containing transcripts that are expressed in transgenic plants. We sought to detect and quantify such transcripts in transgenic maize lines engineered to control western corn rootworm (*Diabrotica virgifera virgifera* LeConte) via overexpression of an inverted repeat sequence bearing a portion of the putative corn rootworm orthologue of yeast Snf7 (DvSnf7), an essential component of insect cell receptor sorting. A quantitative assay was developed to detect DvSnf7 sense strand-containing dsRNA transcripts that is based on the QuantiGene Plex 2.0 RNA assay platform from Affymetrix. The QuantiGene assay utilizes cooperative binding of multiple oligonucleotide probes with specificity for the target sequence resulting in exceptionally high assay specificity. Successful implementation of this assay required heat denaturation in the presence of the oligonucleotide probes prior to hybridization, presumably to dissociate primary transcripts carrying the duplex dsRNA structure. The dsRNA assay was validated using a strategy analogous to the rigorous enzyme-linked immunosorbent assay evaluations that are typically performed for foreign proteins expressed in transgenic plants. Validation studies indicated that the assay is sensitive (to 10 pg of dsRNA/g of fresh tissue), highly reproducible, and linear over ~2.5 logs. The assay was validated using purified RNA from multiple maize tissue types, and studies indicate that the assay is also quantitative in crude tissue lysates. To the best of our knowledge, this is the first report of a non-polymerase chain reaction-based quantitative assay for dsRNA-containing transcripts, based on the use of the QuantiGene technology platform, and will broadly facilitate characterization of dsRNA in biological and environmental samples.

KEYWORDS: *dsRNA, DvSnf7, QuantiGene, RNAi, western corn rootworm*

INTRODUCTION

Gene silencing triggered by sequence-specific double-stranded RNA (dsRNA) has been applied by biotechnologists to control a wide range of target organisms. In transgenic plants, heterologous expression of primary transcripts that carry inverted repeat sequences capable of forming dsRNA molecules that target mRNAs encoding proteins with essential functions has been shown to have efficacy against a variety of pests, including nematodes,[1] lepidopteran larvae,[2] and coleopteran larvae,[3] in addition to fungal[4] and even parasitic plant targets.[5,6] Most recently, using diet bioassays,[3,7] it has been determined that when expressed in transgenic plants, a number of dsRNAs targeting mRNAs encoding proteins with essential functions in larvae of western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte) provide effective control of this pest species. A number of factors can influence the efficacy of dsRNA-induced mortality in these insects, including dsRNA concentration and sequence length.[7,8] Attempts to dissect these parameters through diet bioassays have indicated that the LC_{50} values (the concentration of dsRNA causing 50% mortality of the insect population) can depend on the amount of dsRNA fed to the insect.[7] To facilitate accurate quantification of transgene-derived dsRNA-containing transcripts, the development of new assay types capable of specific detection in complex plant or environmental samples is necessary.

Approaches to the detection of dsRNA in biological samples include reverse transcription polymerase chain reaction (RT-PCR) or Northern blots. While denaturing Northern blots can resolve dsRNA species, this method is relatively low throughput, and the amounts of RNA can be challenging to quantify. On the other hand, RT-PCR can be high-throughput and can amplify through the secondary structure of inverted repeat-containing dsRNA transcripts if appropriately designed primers with a high annealing temperature are used. However, potential pitfalls of RT-PCR amplification of dsRNA have been described, including potential self-priming of RNA when it is expressed in both orientations that can interfere with template specificity[9] and the need for modifications to the standard RT-PCR method when strand-specific detection of dsRNA is desired.[10,11] Furthermore, for all RT-PCR applications, RNA purification is required to eliminate hybridization to cognate sequences in genomic DNA.

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We sought to identify an assay technology that could be used for quantitative strand-specific detection of transgene-derived dsRNA from complex plant matrices, including crude tissue lysates that contain contaminating DNA. Detection and quantification of transgene-derived dsRNA-containing primary and processed transcripts are important for safety and environmental assessments that precede and follow the release of transgenic plants. The QuantiGene technology (Affymetrix) was chosen as it circumvents the need for PCR amplification and can be performed using crude tissue lysates without the need for RNA purification. Furthermore, The QuantiGene assay has been compared to multiple quantitative gene expression platforms and shown to have a high level of concordance with those other methods of RNA quantification, including its detection sensitivity, coefficient of variation, and change in the level of RNA expression.[12−14]

The QuantiGene technology measures RNA directly via a nucleic acid hybridization platform in which target RNAs are captured through cooperative binding of multiple oligonucleotide probes that are conjugated to magnetic microbeads. Cooperative binding of the multiple oligonucleotide probes with specificity for the target sequence results in exceptionally high assay specificity.[15] Detection of this oligonucleotide complex occurs through amplification of a branched DNA amplifier and fluorescent signal, which is counted digitally by high-throughput flow cytometry sorting of the microbead.[15] The QuantiGene technology has been shown to be capable of highly sensitive detection of single-stranded RNA but has not previously been used for detection of dsRNA-containing transcripts.

The QuantiGene-based assay was designed against a portion of the sense strand of the WCR Snf7 orthologue (DvSnf7) expressed in transgenic maize plants, which encodes an essential component of the ESCRT-III complex required for sorting of insect cell membrane receptors.[16] Upon consumption of the plant-produced transgene-derived dsRNA by the insect, suppression of DvSnf7 results in WCR larval stunting and lethality.[3,7] Because no available QuantiGene assay methods were available for dsRNA, we designed our assay validation strategy to be analogous to the rigorous enzyme-linked immunosorbent assay evaluations that are typically performed for proteins expressed in transgenic plant products, which includes experimental determination of assay specificity, sensitivity, accuracy, and precision.[17,18] We validated this assay for quantification of DvSnf7 dsRNA expression using purified total cellular RNA derived from multiple transgenic tissue types and successful use of this assay for direct detection of DvSnf7 dsRNA from crude transgenic leaf lysates, thus providing a new tool for quantitative evaluation of transgenic dsRNAs.

MATERIALS AND METHODS

dsRNA DvSnf7 Expression Construct and Transgenic Plant Growth Conditions. Transgenic MON87411 maize events used in this study were previously described[3] and carry a single copy of a T-DNA construct produced by *Agrobacterium*-mediated transformation, as determined by PCR and Southern blot analysis. The T-DNA insert in these plants includes a plant expression cassette that includes two copies of a 240 bp segment of the *D. virgifera* gene orthologous to yeast Snf7 (DvSnf7). A neutral 150 bp spacer sequence (described in ref 19) separates the DvSnf7 inverted repeats and was designed to facilitate cloning and subsequent sequence confirmation of the inverted repeat construct. DvSnf7 transgene expression is driven by the CaMV 35S promoter and leader,[20] with a downstream maize DnaK intron 1[21] shown previously to enhance mRNA expression.[22] The 3′-untranslated region is from the ribulose 1,5-bisphosphate carboxylase small subunit E9 (RbcS2 E9) gene transcript from *Pisum sativum*.[23] The plants also carry a single copy each of a plant selectable marker gene that confers tolerance to glyphosate (CP4 EPSPS[24,25]) and a modified *Bacillus thuringiensis* protein (Cry3Bb2[26,27]).

Plants were grown in the greenhouse under 16 h day/8 h night cycles at a relative humidity between 60 and 80%. Leaf and root tissues were collected at the V6−V8 development stage, and forage was collected at the R5 stage, silk at the R1 stage, and pollen at the VT stage. Samples were frozen on dry ice before being stored at −80 °C.

***In Vitro*-Transcribed (IVT) DvSnf7 RNA.** *In vitro* T7 RNA polymerase-transcribed 968-nucleotide DvSnf7 RNA (IVT DvSnf7 RNA) was used as the reference standard for the QuantiGene Plex 2.0 assay. This transcript accumulates abundantly in MON87411 plants and was determined by cDNA sequencing to include the 240-nucleotide inverted repeat region and adjacent 5′- and 3′-sequences. The 968-nucleotide DvSnf7 fragment was amplified by PCR from the original plant transformation vector and cloned downstream of a synthetic T7 promoter sequence (TAATACGACTCACTATAGGG) in the pUC19 plasmid. The identity of the recombinant pUC plasmid template used to produce the IVT DvSnf7 RNA was verified by DNA sequencing. For *in vitro* RNA synthesis, the pUC plasmid was linearized by *Bgl*II restriction digestion and incubated with nucleoside triphosphates (8 mM each, Sigma) and T7 RNA polymerase in transcription buffer overnight at 37 °C. The reaction mixture was then treated with DNase I (100 units/mL, Ambion) and extracted with a phenol/chloroform mixture [1:1 (v:v)]. The size of the IVT DvSnf7 RNA was confirmed by agarose gel electrophoresis, and its concentration was determined using a NanoDrop-8000 (Thermo Scientific, Wilmington, DE), according to the manufacturer's instructions. Aliquots of the IVT DvSnf7 RNA sample were stored in a −80 °C freezer.

Plant RNA Extraction. For the purification of total cellular RNA, frozen plant tissues were processed to a fine powder in milling tubes chilled with liquid nitrogen. For leaf, root, and silk tissues, RNA was extracted from ∼0.1 g of tissue using the TRIzol (Ambion) method from Chomczynski and Sacchi.[28] For forage (entire above-ground plant at the R5 development stage), pollen, and grain tissues, RNA was extracted using an acid phenol method. Briefly, 0.5 mL of RNA extraction buffer [25 mM Tris-HCl (pH 8.0), 25 mM EDTA, 75 mM NaCl, 1% SDS, and 1% β-mercaptoethanol] was added to ∼0.1 g of powdered tissue and the solution mixed by vortexing, and then 0.5 mL of an acid phenol/chloroform mixture (2:1) was added and the solution vortexed again. After centrifugation at 12000*g* and 4 °C for 15 min, the upper aqueous phase was transferred to a new tube and extracted with an equal volume of an acid phenol/chloroform mixture (1:1). The upper aqueous phase was then mixed with $^1/_2$ volume of 15 M LiCl and incubated in a −20 °C freezer for approximately 30 min. RNA was pelleted by centrifugation at 20000*g* and 4 °C for 30 min. The RNA pellet was washed with 70% ethanol and dissolved in nuclease-free water.

Northern Blot Analysis. Total RNA was isolated from ground leaf tissue using TRIzol reagent (Ambion), according to the manufacturer's recommendations. For RNase treatment to digest single-stranded RNA, 10 μg of RNA was mixed with 25 units of RNase I_f (New England Biolabs) and the mixture incubated at 37 °C for 10 min. The reaction was stopped by addition of 0.5 μL of 0.5 M EDTA and 1 μL of β-mercaptoethanol, followed by heat inactivation for 20 min at 70 °C. RNA was heat denatured at 70 °C for 10 min and loaded onto a 1.5% agarose, 6.5% formaldehyde denaturing gel and electrophoresed using MESA buffer, and RNA was subsequently transferred using a Turbo-blotter (MidSci) onto a Highbond-N nylon membrane (Amersham). The 240 bp DNA fragment used as a probe was labeled by PCR amplification using [^{32}P]dATP using a Random Prime kit (Invitrogen). The probe was subsequently purified using a Roche G-50 spin column and denatured by being boiled prior to hybridization of the Northern blot. The Northern blot was hybridized overnight at 55 °C in a 250 mM sodium phosphate solution (pH 7.2) including 0.1% SDS. Blots were washed multiple times at 55 °C in 0.1× SSC with 0.1% SDS prior to film exposure (Kodak Biomax MS) at −80 °C.

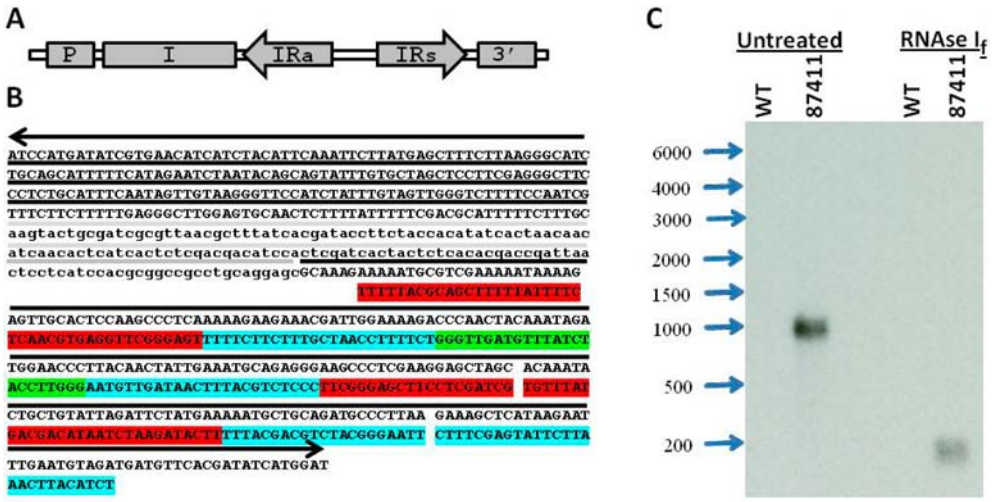

Figure 1. Maize transgene encoding a 240-nucleotide region in an inverted repeat orientation of the *D. virgifera virgifera* DvSnf7 coding region that is targeted by the QuantiGene assay. (A) The transgene expression cassette is driven by the CaMV 35S promoter and leader sequences (P) and includes the downstream DnaK intron 1 (I) that enhances expression and the 3′-untranslated region from the pea RbcS E9 gene. The DvSnf7 inverted repeat regions (IRa, antisense orientation; IRs, sense orientation) are separated by a neutral spacer region to facilitate double-strand RNA duplex formation. (B) The DvSnf7 inverted repeat sequences (uppercase letters and black arrows) separated by a neutral spacer region (lowercase letters and gray line) are shown, including the multiple oligonucleotide probes designed by the QuantiGene platform provider to recognize the sense DvSnf7 strand (colored). Capture extender probes (blue) recognize the Dv49Snf7 sense strand sequence, while blocking probes (green) prevent nonspecific RNA hybridization. Label extender probes (red) initiate amplification of a branched DNA amplifier complex that carries the fluorescent signal. (C) The Northern blot was loaded with equal amounts (10 μg) of untreated wild-type (WT) or MON87411 maize leaf total cellular RNA or RNA that was treated with RNase I_f to digest single-stranded RNAs. The blot was probed with the 240 bp DvSnf7 fragment. Note high-molecular weight RNA and its disappearance in the RNase I_f-treated samples resulting in accumulation of the 240 bp dsRNA. A DNA size ladder is shown (arrows) for the approximate molecular size only.

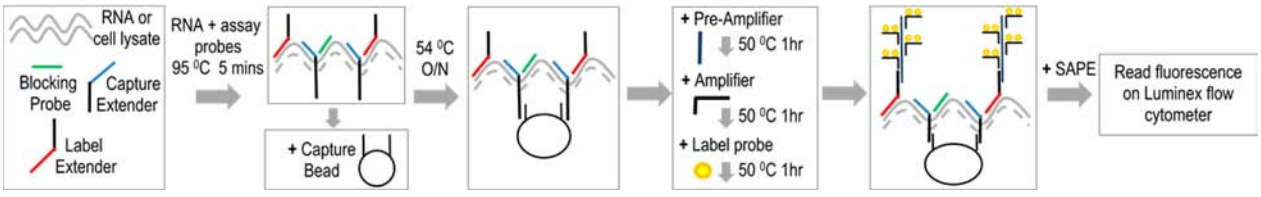

Figure 2. Schematic of the QuantiGene assay protocol described in detail in Materials and Methods. Purified RNA or tissue lysate was first mixed with bifunctional capture extender (blue and black) and label extender (red and black) probes, and blocking probes (green), prior to heat denaturation (at 95 °C for 5 min). The addition of magnetic capture beads resulted in a hybridization complex after overnight (O/N) hybridization at 54 °C. Note that the dashed line represents the denatured strand of the dsRNA. Subsequent addition and hybridization (at 50 °C for 1 h each) of the pre-amplifier, amplifier, and label probes resulted in an amplified oligonucleotide complex that was detected by fluorescence of SAPE (streptavidin phycoerythrin) on a Luminex flow cytometer.

Preparation of Plant Lysates. Frozen leaf powder (20 mg) was added to a 1.4 mL matrix tube on dry ice, followed by addition of two stainless steel beads (ABCO metal 5/32 in. STN 302) to each tube. Extraction buffer consisted of the QuantiGene homogenization solution supplemented with 1 μL of 50 μg/μL proteinase K per 100 μL of solution. Samples were extracted at a tissue:buffer ratio of 1 mg of tissue in 20 μL of extraction buffer for 2 min at 800−1000 rpm in a homemade bead beater machine. The samples were then incubated at 65 °C for 30 min, while being shaken for 1 min every 10 min during the incubation. After centrifugation at 4000*g* for 10 min, the supernatant was collected for the QuantiGene assay.

Purification of Genomic DNA. Frozen leaf powder (1.0 g) was extracted by vortexing in 15 mL of a CTAB solution [75 mM Tris-HCl (pH 8.0), 1.5% (w/v) CTAB, 100 mM EDTA, 1.05 M NaCl, and 0.75% (w/v) 40K PVP] supplemented with 100 μg of RNase A to remove single-stranded RNA. Samples were incubated at 65 °C for 50 min with periodic mixing by inversion. After centrifugation at 10000*g* for 5 min, the supernatant was extracted three times with 15 mL of chloroform. The aqueous phase was mixed with 15 mL of cold 100% ethanol and DNA precipitated at −20 °C for several hours. DNA was recovered by centrifugation at 5100*g* for 10 min, followed by washing of the DNA pellet with 70% ethanol. The final DNA pellet was air-dried and resuspended in 500 μL of TE buffer [10 mM Tris-HCl and 1 mM EDTA (pH 8.0)]. The yield of genomic DNA was >0.25 mg of DNA/g of leaf tissue. To remove double-stranded RNA, purified genomic DNA was incubated with 10 units of dsRNA-specific endoribonuclease RNase III in reaction buffer (Life Technologies, catalog no. AM2290) at 37 °C for 1 h.

QuantiGene Assay. Purified RNA based on the concentration or tissue lysates based on the tissue weight were used in equal amounts for each tissue type that was assayed. The QuantiGene Plex 2.0 oligonucleotide probes were designed against the sense strand sequence of the 240-nucleotide DvSnf7 (Figure 1), and all solutions used for the assay were supplied by Affymetrix. The QuantiGene assay was performed according to the manufacturer's instructions (Affymetrix, Inc., User Manual, 2010) except that a heat denaturation step was included at the time of oligonucleotide addition and prior to overnight incubation, as described below. All liquid handling was performed using a Biomek FX machine except for the magnetic bead washes, which were performed using a Bio Tek ELx405 Select CW machine.

To begin the assay (shown in Figure 2), 20 μL of purified RNA or tissue lysate was first mixed with 5 μL of the QuantiGene 2.0 probe set, containing capture extender, label extender, and blocking probes, in 96-well plates. The mixture was then heated to 95 °C for 5 min in a thermocycler and subsequently held at 46 °C. A premixed solution of 38.7 μL of nuclease-free water, 2 μL of blocking reagent, 33.3 μL of lysis mixture, and 1 μL of magnetic capture beads was then added to the mixture and hybridized overnight at 54 °C in a shaking incubator.

After overnight hybridization, the samples were washed three times with washing buffer with aspiration at each step. To the washed beads were then added 100 μL each of 2.0 pre-amplifier probe solution, amplifier probe solution, and label probe solution, each with a 1 h shaking incubation at 50 °C and three washes with wash buffer at room temperature between probe additions. After the last washes, 100 μL of SAPE (streptavidin phycoerythrin) working reagent was added and the mixture incubated for 30 min at room temperature and then washed three times with SAPE wash buffer. The bead/RNA/probe hybridization mixture was resuspended in 120 μL of SAPE wash buffer. The median fluorescence intensity (MFI) for each sample was captured on a Luminex 200 machine (Luminex Corp.).

A standard curve using the IVT DvSnf7 RNA standard was included on every assay plate. IVT DvSnf7 RNA was diluted to the appropriate concentration in nuclease-free water containing purified total RNA (6.25 ng/μL) derived from wild-type maize leaf tissue as a carrier.

A nuclease-free water blank (for total RNA assay) or a buffer blank (for tissue lysate assay), a negative control (QC−) using maize wild-type RNA, and a positive control (QC+) containing the appropriate concentration of the IVT DvSnf7 RNA standard were also included on every assay plate. All samples were run in triplicate.

RESULTS AND DISCUSSION

Expression of DvSnf7 Double-Stranded RNA in Plants. MON87411 transgenic maize plants are the product of *Agrobacterium*-mediated transformation and carry a single copy of the transforming T-DNA insert, as described previously.[3] These transgenic plants carry a constitutively expressed transgene (Figure 1A) that includes two copies in inverted repeat orientation of a 240 bp segment from *D. virgifera* that is orthologous to the yeast Snf7 gene (DvSnf7),[7] separated by a neutral 150 bp spacer sequence. The two copies of the DvSnf7 fragment align identically to each other in reverse complement fashion (Figure 1B).

Transcription of the DvSnf7 transgene is expected to produce transcripts that include the DvSnf7 sequences as a double-stranded duplex. Northern blot analysis of extracts of tissue samples taken from MON87411 plants was used to confirm expression of the DvSnf7 transgene and the production of double-stranded RNA-containing transcripts. As shown in Figure 1C, a single abundant transcript was observed in RNA extracted from the untreated MON84711 leaf sample. The size (968 nucleotides) of the full-length transcript (excluding the polyA tail) was confirmed by cDNA sequencing of mRNA from these transgenic plants. Upon digestion of total cellular RNA from MON87411 plants with RNase I_b, which digests only single-stranded RNA and leaves double-stranded RNA intact, only the 240 bp DvSnf7 duplex RNA remains. In contrast, nontransformed wild-type plants have no RNA species that are cross reactive with the 240 bp DvSnf7 probe, as expected. These results indicate that the majority of the transgene-derived transcripts in MON87411 plants carry DvSnf7 sequences in double-stranded form. However, we cannot rule out the possibility that intermediate forms of DvSnf7 sequences may exist in single-stranded form, though these would be very low-abundance transcripts.

Design and Use of the DvSnf7 QuantiGene Assay. The QuantiGene Plex 2.0 assay relies on a series of hybridizing oligonucleotide probes that specifically recognize the distal 240 bp copy (sense strand) of the DvSnf7 inverted repeat target sequence (Figure 1A). DvSnf7-containing RNA is first hybridized to the bifunctional capture extender probe that also recognizes an oligonucleotide conjugated to the capture bead used in subsequent flow cytometry counting. Blocking probes are included to prevent nonspecific hybridization to unrelated cellular RNAs. The bifunctional label extender probe is designed to allow detection of the DvSnf7 hybridization complex by fluorescent detection of a set of self-amplifying branched DNA probes that are subsequently hybridized to the complex (Figure 2). As PCR is not used for target RNA amplification, the fluorescence intensity of the hybridization mixture is directly proportional to the amount of RNA hybridized in the plant sample, as read directly from the detection apparatus. For each assay plate, a standard curve using *in vitro*-transcribed 968-nucleotide DvSnf7 RNA (IVT DvSnf7 RNA) is included on the same plate to allow quantification of target RNA in the plant samples.

To detect DvSnf7 transcripts in MON87411 plants, it was important to heat denature purified total cellular RNA or tissue lysates at 95 °C in the presence of the hybridizing oligonucleotide probes prior to overnight incubation. Heat denaturation of the RNA in the presence of the oligonucleotide probes was required for an optimal response in the assay, and no fluorescence signals above background were detected in the absence of heat denaturation or if RNA was first heat denatured and subsequently incubated overnight with the oligonucleotide probes. These results suggest that access of the oligonucleotide probes is significantly inhibited by the duplex DvSnf7 RNA structure, which apparently re-anneals rapidly in the absence of the probes.

The Source Tissue Matrix Has No Effect on DvSnf7 Standard Curve Generation. To test the use of the QuantiGene-based assay for *in planta*-derived dsRNA DvSnf7 detection, we first utilized purified total cellular RNA derived from multiple tissue types of transgenic plants. For accurate quantification of DvSnf7 RNA species, an *in vitro*-transcribed RNA (IVT DvSnf7 RNA) that carries the 240 bp dsRNA target was used as a standard. Standard curves were generated using the IVT DvSnf7 RNA spiked into purified total cellular RNA extracted from six different nontransgenic maize tissue types. The standard curve from the six tissue types across eight IVT DvSnf7 RNA concentrations is shown in Figure 3.

The standard curves for DvSnf7 RNA derived from all six plant tissue types (leaf, root, silk, pollen, grain, and forage) tested exhibit nearly identical and linear responses ($r^2 > 0.99$ for all curves) to the spiked standard DvSnf7 RNA across an ~285-fold range, from 0.0022 to 0.63 ng/mL. These results indicate that there is no significant effect of the plant RNA matrix from any tissue type on the QuantiGene assay for DvSnf7, suggesting that further assay validation could be performed using RNA from a single tissue type.

DvSnf7 Assay Precision, Limits of Detection, and Quantification. The precision of the assay could be determined both within a run (intra-assay precision of technical replicates) and across runs (inter-assay precision of assay plates over a period of time). To determine the precision of the QuantiGene platform across the entire working range of the assay, the eight-point standard curve of IVT DvSnf7 RNA (at concentrations listed in Table 1) and a QC+ sample (at 0.07 ng/mL) were assayed in triplicate wells over 20 assay runs conducted on 10 different days by two different analysts over a

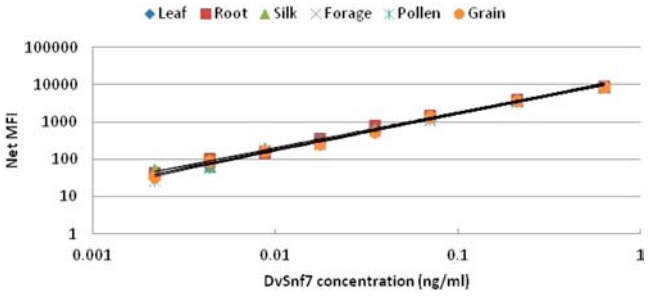

Figure 3. Total RNA derived from different tissue types has no effect on DvSnf7 detection. Independent standard curves using the IVT RNA standard spiked into total RNA isolated from six maize tissue types derived from nontransgenic plants are shown. Note that the standard curve for each matrix type is linear and nearly identical across all RNA concentrations and tissue types, from 0.0022 to 0.63 ng/mL. Data were generated from three replicates of each standard on each of two plates. MFI is the median fluorescence intensity.

Table 1. DvSnf7 QuantiGene Plex 2.0 Assay Dose-Dependent Intra-run and Inter-run Precision[a]

	%CV	
IVT DvSnf7 (ng/mL)	intra-run	inter-run
0.63	1.0	
0.21	1.6	
0.07 (QC+)	3.3	
0.035	3.1	
0.0175	3.7	
0.00875	4.3	
0.004375	6.1	
0.0021875	7.7	
QC+	1.4	2.3

[a]Eight standard points and the QC+ sample were included on each QuantiGene Plex 2.0 assay plate in triplicate wells. The coefficient of variation (CV) was calculated on the basis of the log 2 of net MFI of the standards and the QC+ sample observed over all QuantiGene runs. Data are averaged over triplicate replicates from 20 runs of the QuantiGene assay. Note that the CV increases as the IVT DvSnf7 standard concentration decreases.

period of 3 weeks. Assay precision was calculated as the coefficient of variation (CV) using the values of the QC+ samples and the standards observed over all assay runs.

As shown in Table 1, the CV for all points on the standard curve was well below 10%. As expected, the CV increases slightly as the concentration of the standard becomes smaller, from a minimum CV of only 1% at the highest concentration (0.63 ng/mL) of the standard curve to a maximal CV of 7.7% for the lowest concentration of the standard curve (0.0022 ng/mL). On the other hand, the CV for the QC+ sample across runs (inter-run) was only 2.3%, while the intra-assay precision for the QC+ sample was slightly lower (1.4%). The limit of quantification (LOQ) for DvSnf7 was defined as the lowest concentration on the standard curve with a CV of ≤15% and was determined to be 0.0022 ng/mL.

For the accurate detection of transgenic RNA in plant samples, it was also important to determine the limit of detection (LOD) to verify the lowest DvSnf7 RNA concentration that can reliably be detected above a background of nontransgenic plant RNA. For this determination, total cellular RNA independently extracted from 14 nontransgenic maize leaf samples was assayed over two independent runs by two different analysts. The DvSnf7 assay was performed in the absence of spike-in IVT DvSnf7 RNA. The LOD was defined as the average concentration of DvSnf7 that could be back-calculated from the background fluorescence observed in the nontransgenic leaf samples plus three standard deviations of the concentration. By these criteria, the LOD for DvSnf7 in the leaf samples was determined to be 0.0005 ng/mL, which we estimate represents ∼19000 transcripts (see the Supporting Information), similar to the mRNA transcript detection sensitivity previously reported for the QuantiGene assay.[15] Because no matrix effects were observed in the standard curve derived from any of the multiple tissue types shown in Figure 3, the DvSnf7 LOD determined from leaf is considered to apply to all tissue types (Table 1 of the Supporting Information).

Accurate Dilutional Parallelism Using Transgenic Plant Samples. In addition to assay precision using a standard curve and QC+ samples derived from IVT DvSnf7 RNA, it is important to confirm that the DvSnf7 assay is accurate across a range of expression levels in samples from transgenic plants. Dilutional parallelism assesses the QuantiGene assay across a range of sample RNA dilutions, with the concordant back-calculated assay values as the measure of accuracy. For this test, total RNA from four transgenic leaf samples was first extracted, and on the basis of the total RNA concentration, each sample was diluted over a 16-fold range to concentrations of 4, 16, and 64 ng/μL. The DvSnf7 assay of the diluted samples was then repeated over two independent runs, and the amount of RNA in the leaf samples was quantified at each dilution by back-calculation against the standard curve derived from IVT DvSnf7 RNA. The average recovery of DvSnf7 across the multiple dilutions of transgenic leaf RNA ranged from 91 to 114% (Table 2), indicating that this assay can accurately detect transgenic RNA purified from leaf samples across a range of working concentrations.

Spike and Recovery of IVT DvSnf7 RNA. Detection and quantification of DvSnf7 may be affected by the extent of

Table 2. DvSnf7 QuantiGene Plex 2.0 Assay Dilutional Parallelism[a]

sample number	ng/μL	dilutional parallelism (%, run 1)	dilutional parallelism (%, run 2)	average dilutional parallelism (%)
1	64	71	86	91
	16	99	95	95
	4	130	118	114
2	64	91	101	
	16	99	98	
	4	110	101	
3	64	84	96	
	16	101	80	
	4	115	123	
4	64	98	101	
	16	92	98	
	4	110	101	

[a]RNA was extracted from four transgenic leaf samples. Each sample of RNA was diluted to the concentrations shown and assayed in two independent QuantiGene runs (1 and 2) with quantification of the diluted samples calculated on the basis of the standard curve using IVT DvSnf7 RNA. Dilutional parallelism is the percent concordance of the back-calculated sample values at each dilution vs the theoretical 100% accuracy at each dilution. The average dilutional parallelism takes into account all individual results at each dilution from all samples.

recovery of RNA in different tissue types during RNA extraction and purification. To evaluate the recovery of DvSnf7 RNA from different tissue types, RNA extraction buffer was first added into pools of nontransgenic powdered tissue derived from the same plant tissues as described in the legend of Figure 3, and IVT DvSnf7 RNA was then spiked immediately into the sample. Three different amounts of the IVT DvSnf7 RNA standard were spiked (in duplicate) into the pooled nontransgenic tissue for each tissue type to cover a range of target concentrations. Duplicate samples for each amount of spike were used, and extraction was performed by two different analysts. Each extract was assayed in two independent runs performed by two different analysts. The recovery of DvSnf7 RNA was calculated as a percentage of the IVT DvSnf7 RNA recovered from the extraction over the total amount spiked prior to RNA extraction.

The TRIzol extraction method was used for leaf, root, and silk tissues and yielded a recovery of 87−92% for these tissue types (Table 3). Surprisingly, the recovery of IVT DvSnf7 RNA from maize tissue samples with high polysaccharide contents, including forage, pollen, and grain, was <20% when using the TRIzol extraction method (data not shown). This result illustrates the value of testing extraction conditions when different tissue types are to be assayed. In an attempt to more quantitatively recover RNA from those maize tissue types, a more harsh extraction method employing acid phenol was used for these tissue types. As shown in Table 3, the average recoveries of DvSnf7 at each spike amount in forage, grain, and pollen were ∼95, ∼89, and ∼67%, respectively.

Table 3. Recoveries of IVT DvSnf7 RNA (input level in nanograms per milliliter) after RNA Extraction and Purification from Multiple Tissue Types[a]

tissue	IVT DvSnf7 input amount (ng)	average % recovery at each input amount	overall average % recovery
leaf	0.9	92	92
	0.3	97	
	0.1	88	
root	0.3	79	87
	0.1	88	
	0.025	93	
silk	0.6	89	87
	0.2	95	
	0.05	76	
forage	0.6	99	95
	0.2	94	
	0.05	93	
pollen	0.75	75	67
	0.25	63	
	0.1	64	
grain	0.3	95	89
	0.1	80	
	0.025	93	

[a]Samples were assayed in duplicate over two independent runs performed by two different analysts. The recovery of IVT DvSnf7 RNA, or a nontransformed control sample (data not shown), over three input RNA levels is shown. Spike and recovery was calculated as the percentage of back-calculated concentration to theoretical spiked concentration.

Extraction Efficiency. To fully understand the quantification of transgene-derived DvSnf7 *in vivo* and to optimize quantitative assay detection methods, the efficiency of its extraction from transgenic plant tissues must be determined. For this evaluation, total cellular RNA from transgenic plant tissues was extracted using the TRIzol (leaf, root, and silk) or acid phenol (forage, pollen, and grain) method. For each tissue sample, the cellular debris that results from the first RNA extraction attempt was centrifuged and the remaining tissue pellet was subjected to four additional sequential re-extractions, with RNA precipitation performed at each of the five extraction steps. Precipitated samples were then assayed for DvSnf7 RNA, and the percent extraction efficiency for each sample was determined by dividing the RNA concentration measured in the first extract by the sum of the RNA concentrations measured in all five extractions.

As shown in Table 4, the extraction efficiency of the transgenic DvSnf7 in leaf, root, silk, and forage tissues ranged from ∼81 to 89%. Because of very low transgene expression levels in pollen and grain tissues (data not shown), the extraction efficiency was not determined for those tissue types.

Table 4. Extraction Efficiencies of Transgene-Derived DvSnf7 from Multiple Transgenic Tissue Types[a]

tissue	sample number	extraction efficiency (%, run 1)	extraction efficiency (%, run 2)	average extraction efficiency (%)
leaf	1	89	91	89
	2	88	89	
	3	90	89	
	4	88	86	
root	1	78	76	85
	2	83	88	
	3	90	89	
	4	86	88	
silk	1	83	80	85
	2	86	82	
	3	87	83	
	4	85	94	
forage	1	68	87	81
	2	86	85	
	3	78	86	
	4	72	85	

[a]Four replicates of each tissue sample was subjected to five repeated extractions utilizing the same extraction conditions and method. The percent extraction efficiency for each sample was determined by dividing the RNA concentration measured in the first extract by the sum of RNA concentrations measured in the five repetitive extracts. Each isolated RNA sample was assayed in two independent QuantiGene runs.

Quantification of DvSnf7 Directly from Tissue Lysates. In an attempt to increase the speed and decrease the costs associated with extraction and purification of RNA from complex tissues, we also evaluated the quantification of DvSnf7 RNA in tissue lysates. The lack of RNA purification in tissue lysates may change or enhance any potential matrix effects on the QuantiGene assay. Furthermore, the presence of a single copy of the DvSnf7 transgenic DNA integrated into the plant genome, which may be recognized by the QuantiGene assay probes, could affect quantification of the DvSnf7 RNA. For these experiments, transgenic leaf discs were homogenized using metal beads, the crude lysate was clarified by centrifugation, and the supernatant was used directly in the QuantiGene assay.

Figure 4 shows the results of the DvSnf7 assay in leaf lysates derived from two independent MON87411 transgenic lines,

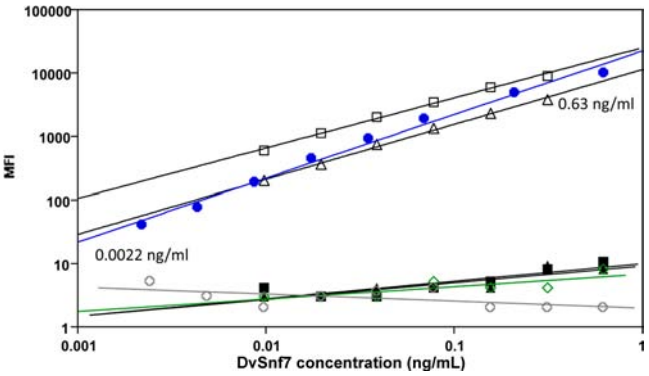

Figure 4. Detection of DvSnf7 RNA in crude lysates is not affected by the presence of genomic DNA. The IVT DvSnf7 standard curve (blue circles) is shown along with crude lysates from two independent transgenic maize lines (empty squares and triangles) that were serially diluted 2-fold over the range of the assay (from 0.0022 to 0.63 ng/mL). The linear response indicates accurate detection and dilutional parallelism of the QuantiGene assay in transgenic lysates. On the other hand, purified transgenic DNA from either MON87411 event (filled squares and triangles), nontransgenic DNA (diamonds), and buffer blank (empty circles) that is serially diluted 2-fold shows only background activity in the assay. MFI is the mean fluorescence intensity.

using the IVT DvSnf7 RNA to generate the standard curve. Twofold serial dilutions of the leaf lysates were assayed to test for the linearity of the assay response over the range of the standard curve. As can be seen in Figure 4, assay signals are readily observed in both transgenic events, and in both cases, the assay response is linear with expected dilutional parallelism through multiple points along the standard curve. In contrast, leaf lysates generated from nontransgenic plants show only a background assay signal, similar to buffer controls. These results suggest that the QuantiGene assay for DvSnf7 is accurate when it is conducted directly with tissue lysates.

Multiple controls were utilized to determine the potential assay signal, if any, contributed by the genomic DvSnf7 transgene relative to the DvSnf7 RNA present in the sample. For example, removal of cellular RNA by RNase digestion of the lysates should reveal any assay signal contributed by DvSnf7 genomic DNA. However, we found that digestion of DvSnf7 RNA in the crude lysates using a combination of RNase A and RNase III (for digestion of ssRNA and dsRNA, respectively) was not completely effective (data not shown). Therefore, to directly measure the contribution of the DvSnf7 transgene to the assay, we extracted and purified genomic DNA from the same amount of transgenic leaf tissue that was used in the lysates assayed for DvSnf7 RNA. The purified genomic DNA was further treated with RNase A and RNase III to ensure removal of any contaminating DvSnf7 RNA. Figure 4 clearly shows that the purified genomic DNA extracted from MON87411 plants also has only a background signal in the DvSnf7 assay, like nontransgenic leaf lysates and buffer controls. These results indicate that the single copy of the genomic transgene in transgenic plants is not present at a sufficiently high copy number to register any signal in the QuantiGene assay or that the genomic DNA is not accessible to the QuantiGene assay probes.

While leaf lysates were utilized in these experiments because they usually have minimal matrix effects in most assays, additional testing of the lysate approach in the remaining tissue types used here is ongoing. Furthermore, the assay should be able to be extended to other evaluations in which quantification of the specific dsRNA is needed, such as the persistence in the environment (e.g., soil matrices).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

IVT DvSnf7 transcript number calculation and DvSnf7 QuantiGene Plex 2.0 assay sensitivity (Table 1). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jeffrey.m.staub@monsanto.com.

Author Contributions

T.A.A., H.C., and T.E.Z. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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