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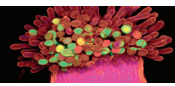*USDA-ARS National Peanut Research Laboratory*

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RESEARCH PAPER

Detection of exogenous double-stranded RNA movement in *in vitro* peanut plants

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ABSTRACT

- New technologies are needed to eliminate mycotoxins and/or fungal pathogens from agricultural products. RNA interference (RNAi) has shown potential to control fungi associated with crops. In RNAi, double-stranded RNA (dsRNA) targets homologous mRNA for cleavage, and can reach the mRNA of pathogens in contact with the plant. The key element in this process is the movement of RNA signals cell-to-cell and over long distances within the plant, and between host plants and parasites.

- In this study, we selected a regulatory gene in the aflatoxin biosynthesis pathway, *aflS/aflR*, necessary for the production of aflatoxins in *Aspergillus* spp. We designed a Dicer-substrate RNA (DsiRNA) to study the movement and stability of the duplex over time in *in vitro* peanut plants using stem-loop primers and RT-PCR for DsiRNA detection.

- The preliminary results demonstrated that DsiRNA was absorbed and moved away from the point of application, spread systemically and was transported rapidly, most likely through the phloem of the shoot, to the sink tissues, such as new auxiliary shoots, flowers and newly formed pegs. The DsiRNA remained detectable for at least 30 days after treatment.

- This is the first time that movement of exogenous DsiRNA in *in vitro* peanut plants has been described. Since DsiRNA was detectable in the pegs 15 days after treatment, aflatoxin reduction may be possible if the duplexes containing part of the aflatoxin biosynthesis pathogen gene induce silencing in the peanut seeds colonised by *Aspergillus* spp. The application of small RNAs could be a non-transformative option for mycotoxin contamination control.

INTRODUCTION

Aflatoxins, carcinogenic chemicals produced by *Aspergillus* spp., cause heavy losses in different crops around the United States and throughout the world (Zain 2011; Leidner 2012). The Food and Agriculture Organisation of the United Nations has expressed concern about how the agriculture sector will address the future increase in global food production demands (James 2015; Brookes & Barfoot 2016; FAO 2017). Even though plant diseases are not considered the main yield reducers, harvest quality and safety have been impacted seriously in many crops (Savary *et al.* 2012). Peanut is not an exception, and the susceptibility of most cultivars to toxin contamination during pre- and post-harvest is a cause for concern among growers (Diao *et al.* 2015).

New technologies are needed to eliminate mycotoxins and/or fungal pathogens from agricultural products. RNA interference (RNAi) technology continues to be one of the most valuable tools to reduce expression of targeted genes in plant breeding programmes (Emani & Hunter 2013; Saurabh *et al.* 2014; Younis *et al.* 2014; Lombardo *et al.* 2016; Ricroch & Hénard-Damave 2016; Majumdar *et al.* 2017). Reviewing the complex mechanism of RNA silencing, double-stranded RNA (dsRNA) plays an important role as a regulator of fundamental processes that conclude in the cleavage of the mRNA with

which it has homology (Tinoco *et al.* 2010). After Fire *et al.* (1998) were able to study gene function in the nematode *Caenorhabditis elegans* using dsRNA injections and soaking, new applications were investigated, not only to identify genes but also to study different kinds of strategies to protect plants against insects and pathogens. Research looking at the systemic movement of dsRNAs from cell-to-cell and over long distances within the plant (Scott *et al.* 2013; Zotti & Smagghe 2015), from host plant across the haustorium to the parasite (Tomilov *et al.* 2008), and from egg and pollen to progeny plants (Moriyama *et al.* 1996), allowed for further experiments to address questions related to the broad applications of dsRNA as a management technique (Baum *et al.* 2007; Gordon & Waterhouse 2007; Baum & Roberts 2014; Zotti & Smagghe 2015; Joga *et al.* 2016; Ricaño-Rodríguez *et al.* 2016). In the case of fungal toxins, transgenic maize was obtained using host-induced gene silencing (HIGS) where the targeted aflC *Aspergillus* transcript was knocked down and aflatoxins were not detected in any of the transgenic lines (Thakare *et al.* 2017).

Real-Time PCR (RT-PCR) technology has become one of the most frequently used methods for quantification of gene expression levels (Schefe *et al.* 2006). Since the early 2000s, dsRNAs have been degraded using Dicer (Bernstein *et al.* 2001), and the resulting small RNAs are shorter than the minimum target length ($\geq$40 nucleotides –nt-) required for

standard RT-PCR methods (Kramer 2011). Chen *et al.* (2005) designed an efficient amplification method to detect small RNAs using a highly stable stem-loop primer to lengthen the first strand cDNA from its original size to >60 nt, and a universal reverse primer that is complementary to a sequence within the RT stem-loop primer. This method allowed identification and monitoring of microRNAs in tissues or cultured cells.

Non-transformative RNAi strategies employing exogenous dsRNA have been used successfully to control insect pests (Scott *et al.* 2013; Baum & Roberts 2014; de Andrade & Hunter 2016; Joga *et al.* 2016), but these strategies still depend on a number of different factors. Choice of the right gene, definition of the length and concentration of the dsRNA and delivery method are a few of the variables that must be considered when a new conceptual experiment is designed. In this study, we designed a synthetic Dicer-substrate RNA (DsiRNA), also called a duplex, from an important regulatory gene, *aflS/aflR*, necessary for the production of aflatoxins in *Aspergillus* spp. (Woloshuk *et al.* 1994; Yu *et al.* 2004). The movement of the duplexes and their stability was examined over time in *in vitro* peanut plant cultures using stem-loop primers and RT-PCR for their detection. Although RNA mobility within an organism has been demonstrated (Moriyama *et al.* 1996; Tomilov *et al.* 2008; Scott *et al.* 2013), this is the first time that movement of exogenous DsiRNA in *in vitro* peanut plants has been described. Pegs and pods containing peanut seeds grow in direct contact with the soil population of aflatoxigenic fungi, so invasions have the opportunity to occur (Horn 2005). Duplexes containing part of the aflatoxin biosynthesis pathogen gene move toward the pegs. Once there, DsiRNA could come into contact with *Aspergillus* growing in the seed, enabling aflatoxin gene silencing. These preliminary results will provide information about the factors necessary to define, prior to RNAi silencing studies, and expand opportunities for new aflatoxin contamination control.

MATERIAL AND METHODS

Plant tissue culture

Seeds of peanut variety Georgia-06G were surface sterilised in 75% ethanol for 5 min followed by immersion in 10% Clorox (55 g·l^{-1} active chlorine) on a shaker for 15 min. They were rinsed three times with sterile deionised water and transferred to tubes containing MSO medium consisting of Murashige & Skoog (1962) salts, 3% sucrose and 0.8% agar (Sigma, St. Louis, MO, USA). The pH was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. To induce flowering, the shoots were transferred to MS supplemented with 1 mg·l^{-1} BAP (6-benzylaminopurine; Caisson Laboratories, UT, USA) for 3–5 months. After the plants started to bloom, they were transferred to MS supplemented with 1 mg·l^{-1} BAP and 0.5 mg·l^{-1} GA3 (gibberellic acid A3; RPI, Chicago, IL, USA) until they started to peg. Then they were transferred to MSO and the surface of the medium was covered with a layer of MSO with 1 g·l^{-1} activated charcoal (Sigma) to block light transmission. Every month, the elongated shoots were cut between three nodes for cloning and transferred to fresh medium. Seed germination and cloning occurred in a growth chamber (Percival Scientific, Model CU41L4C8, Perry, IA, USA) at 26 ± 2 °C under a 16 h/8 h day/night photoperiod.

Multiple *in vitro* plants taller than 3 cm were air-stressed for 15 min in a laminar airflow work station (NU-S301-536; NuAire, Plymouth, MN, USA). The apical leaf was cut, and after 10 min one drop containing DsiRNA at the desired concentration was applied. The magenta box was closed immediately to allow the plant to recover and absorb the drop. After 30 min, the plants were transferred to a growth chamber (26 ± 2 °C; 16 h/8 h day/night photoperiod). At each sampling time, one whole plant was sectioned into 1-cm pieces, noting which part of the plant the section came from, *i.e.* stem, leaf, auxiliary shoot, leaf from auxiliary shoot, root, flower and peg. The samples were then immediately frozen at −80 °C until processed for total RNA extraction from each section.

Dicer-substrate RNA (DsiRNA)

Dicer-substrate short interfering RNA (DsiRNA) was designed as 27mer duplex RNA, with a single 2-base 3'-overhang on the antisense strand and a blunt end modified with DNA bases (IDT 2011) using the RNAi design tool available at www.idtdna.com (IDT, Coralville, IA, USA). The DsiRNA (ds_aflR) was designed to target the *aflS/aflR* gene from the aflatoxin biosynthesis gene cluster (AFLA_139360; 1335 bp). The ds_Rib was designed using the 60S ribosomal protein L7 mRNA sequence (NCBI Reference Sequence: XP_002377633.1) to be an unrelated aflatoxin DsiRNA control.

Stem-Loop RT-PCR forward and reverse primers, for detection of both sense and antisense strands of the DsiRNA, were designed following the protocol published by Kramer (2011), using the online tool of Czimmerer *et al.* (2013). Synthetic DsiRNA and stem-loop RT-PCR forward and reverse primers were purchased from Integrated DNA Technologies (IDT; Table 1).

Analysis with RT-PCR

Frozen *in vitro* peanut samples were pulverised with 2 ml lysing matrix D tubes (MP Biomedicals, CA, USA) using the Omni Bead Ruptor 24 (Kennesaw, GA, USA). Total RNA was extracted using the Direct-Zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA), and a supplemental protocol that incorporates Proteinase K treatment prior to extraction to digest unwanted proteins. RNA concentration was determined using the Nanodrop 2000c spectrophotomer (Thermo Scientific, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesised by reverse transcription using equal amounts of oligo dT and random hexamers, and Superscript III reverse transcriptase (Invitrogen, Life Technologies, CA, USA). cDNA for DsiRNA detection by stem-loop RT-PCR was synthesized from 1 μg total RNA according to Invitrogen instructions. The RT-PCR reaction was performed using a QuantStudio 7 Flex Real-Time PCR System (Life Technologies). The 24 μl reaction contained 5 μl cDNA diluted four-fold, 12 μl SYBR Green Mastermix (Qiagen) and 7 μl of the mix of stem-loop (SL) forward (F) primer and universal stem-loop reverse (R) primer (ds_Universal_RT_R; 0.5 μM each; Table 1). The conditions for amplification were as described in Arias *et al.* (2014). Each experiment was performed using two biological replicates and three technical repetitions. The comparative C$_T$ (ΔΔC$_T$) method was used to determine the presence of the target DsiRNA in different samples. Measurements were normalised

Table 1. Sequences of DsiRNA (ds_aflR, ds_Rib), stem-loop (SL) and RT-PCR oligonucleotide primers (forward "F" and reverse "R") used in the study.

	CCU UCA GCC AGG UCG GAA CAG GGA C	$5' \rightarrow 3'$
ds_aflR	AA GGA AGU CGG UCC AGC CUU GUC CCU G	$3' \rightarrow 5'$
ds_aflR_RT_Sense_SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTCCCT	
ds_aflR_RT_AntiSense_SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCCTT	
ds_aflR_RT_Sense_F	GCGGCGGCCTTCAGCCAGGTCG	
ds_aflR_RT_AntiSense_F	GCGGCGGGTCCCTGTTCCGACC	
	GCA GCU CCU CCG UCU GCU CCA GAU C	$5' \rightarrow 3'$
ds_Rib	GA CGU CGA GGA GGC AGA CGA GGU CUA G	$3' \rightarrow 5'$
ds_Rib_RT_Sense_SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATCTG	
ds_Rib_RT_AntiSense_SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGCAG	
ds_Rib_RT_Sense_F	GCGGCGGGCAGCTCCTCCGTCTG	
ds_Rib_RT_AntiSense_F	GCGGCGGGATCTGGAGCAGACG	
ds_Universal_RT_R	ATCCAGTGCAGGGTCCGAGG	
Actin_RT_F	CACATGCCATCCTTCGATTG	
Actin_RT_R	CCAAGGCAACATATGCAAGCT	

using actin expression as endogenous control (NCBI accession no. KJ186103; Table 1). The software determined the relative quantity (RQ) of the target in each sample by comparing normalised C_T (ΔC_T) in each sample to normalised C_T (ΔC_T) in the reference sample (untreated control). RT-PCR does not allow measuremnt of 'no transcript', therefore, the limit of detection in our experiment was defined as 1, meaning that any RQ value above 1 will be considered as 'negative' DsiRNA detection. The $2^{-\Delta\Delta CT}$ was calculated using the method of Livak & Schmittgen (2001) and the results were displayed in Log_{10} graph type.

RESULTS

Using an *in vitro* system, exogenous DsiRNA was applied on an apical cut in ≥3 cm peanut plants to study the movement throughout them. Figure 1 shows the detection of DsiRNA (ds_aflR) with stem-loop RT-PCR. Figure 1a shows how, at a 25 nM concentration, the ds_aflR moved 1, 2, 3, 4, 15, 30 and 60 days after treatment (dat) and Fig. 1b shows movement 15, 30 and 60 dat at a higher concentration (50 nM). As early as 2 days after treatment, we were able to detect the duplexes at 3 cm distance from the application site, and DsiRNA was still detectable at least 60 days post-treatment. As the plants began to grow, a decrease, and in some cases a lack, of the DsiRNA in the main shoot was observed, while the auxiliary shoots contained DsiRNA at a higher concentration. This could be observed 15 or more days after treatment. Flowers and pegs were sampled and both were positive for DsiRNA. Increasing the duplex concentration to 50 nM produced a wider systemic spread of the DsiRNA that could be detected in roots; however, it still could not be detected in most of the leaves sampled (Fig. 1b).

In the next experiment, we used plants pegging in *in vitro* conditions to specifically monitor the movement of the ds_aflR in the direction of the pegs. At 15 dat, the DsiRNA was detected in sense and antisense directions, suggesting that the movement toward the pegs probably was in the DsiRNA form and remained detectable for at least 30 dat (Fig. 2).

The movement of another DsiRNA (ds_Rib) designed from a non-aflatoxin related gene was evaluated as a control. This DsiRNA showed a similar movement pattern to ds_aflR (Table 2, Table S1). The DsiRNA in all the plants moved from top to bottom, suggesting that transport may occur *via* the phloem.

DISCUSSION

With this study, we show that it is possible to detect long-distance movement of DsiRNA in *in vitro* peanut plants using the stem-loop RT-PCR method. These preliminary results demonstrate that DsiRNA was absorbed by the plant and moved from the point of application, spread systemically, and was transported rapidly, most likely through the phloem of the shoot to sink tissues, such as new auxiliary shoots, flowers and newly formed pegs within approximately 15 days after treatment (Fig. 3). The stem-loop RT-PCR method was useful and sensitive for the detection of both sense and antisense strands of the 27mer-duplex RNA (DsiRNA), as reported by Kramer (2011).

The movement and stability of DsiRNA in the plants over time was followed. RNA molecules travel long distances in plants to different organs, similar to the way in which virus RNA spreads through the plasmodesmata to enter the vascular system (Leisner & Howell 1993; Lucas & Wolf 1993). Phloem has been related to movement of the silencing signal more than xylem, which is responsible for water and ion RNA-free transportation (Buhtz *et al.* 2008). Previous studies using a variety of delivery methods (topical sprays, root drench, trunk injections; Xie & Gu 2006) found that dsRNA and other nucleic acids move between cells through plasmodesmata and over long distances *via* the phloem (Ruiz-Medrano *et al.* 1999; Mlotshwa *et al.* 2002; Molnar *et al.* 2011). Other studies using topically applied sprays and soil applications have shown that the dsRNA is absorbed and then moves systemically through the plant's xylem and phloem (Susi *et al.* 2004; Yu *et al.* 2013; Li *et al.* 2015; Zotti & Smagghe 2015; de Andrade & Hunter 2016).

In our study, a droplet containing the DsiRNA was applied to the cut where both phloem and xylem were exposed. The DsiRNA did not remain at the application point, so we were able to amplify DsiRNA from a shoot segment at 2 cm distance from the application site 1 day after treatment. DsiRNA was detectable at 3 cm from the cut 2 days after treatment and at
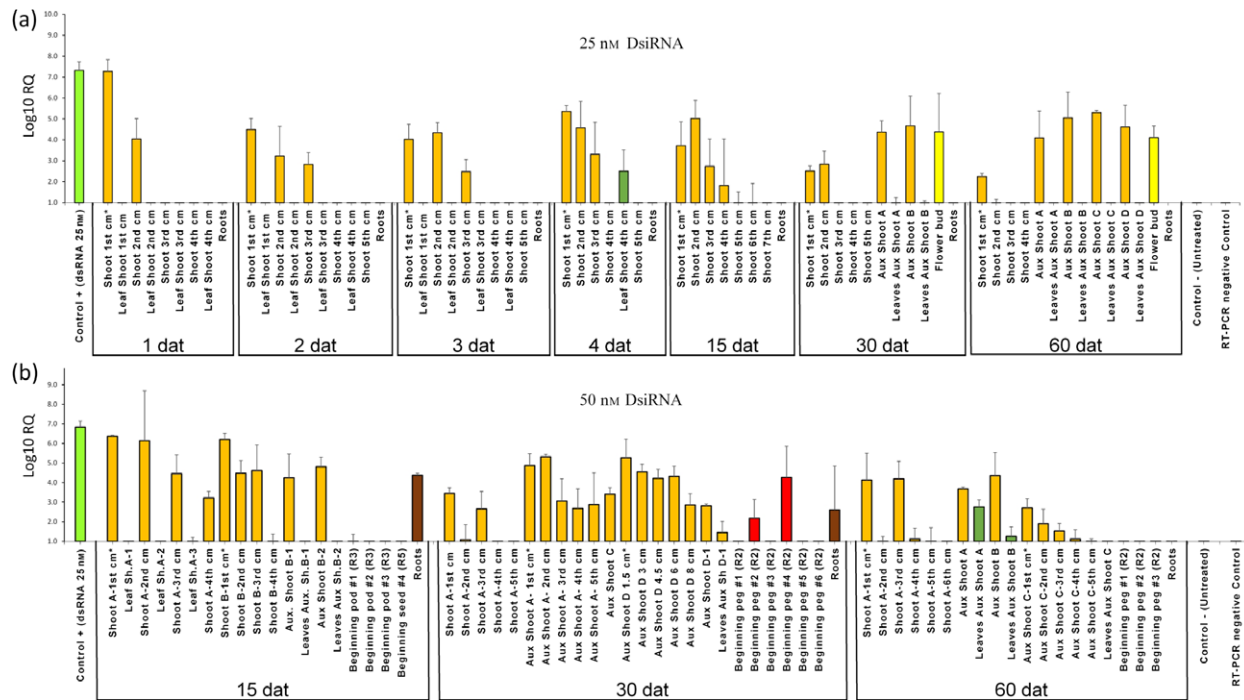
Fig. 1. RT-PCR detection of exogenous DsiRNA in *in vitro* peanut plants. a: Detection in antisense direction of ds_aflR applied at 25 nM in different tissues from the whole peanut plant 1, 2, 3, 4, 15, 30 and 60 days after treatment (dat). b: Detection of ds_aflR applied at 50 nM in different tissues from the whole peanut plant over 15, 30 and 60 dat.
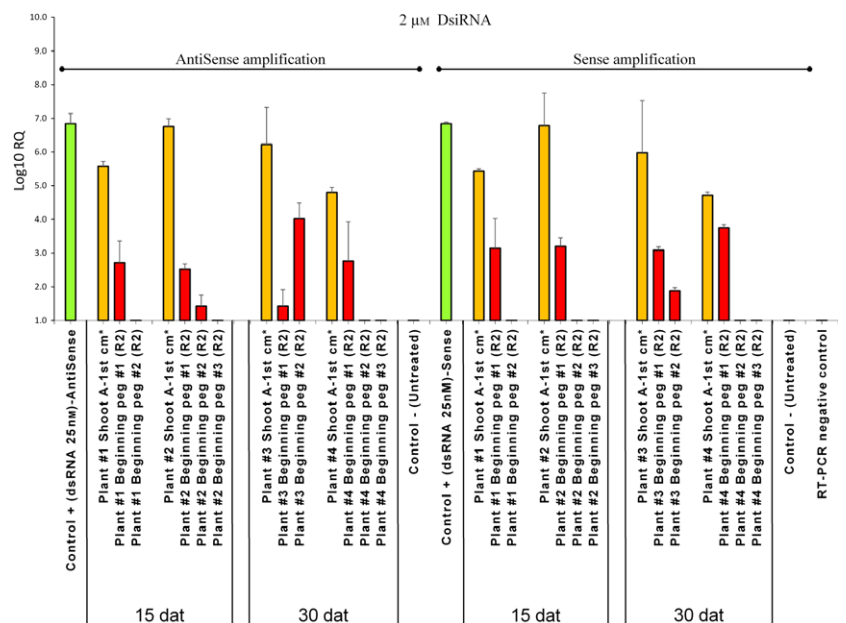

Fig. 2. RT-PCR detection of exogenous DsiRNA in peanut pegs develop in *in vitro* conditions. Detection of ds_aflR in sense and antisense direction, applied at 2 μM, 1 cm distant from the application and in new pegs at 15 and 30 dat.

15 dat in the new pegs, remaining there for at least 30 dat. The movement of a non-related aflatoxin DsiRNA control (ds_Rib) also showed a similar pattern to ds_aflR. These results agree with those of de Andrade & Hunter (2016), where dsRNAs applied to the roots of citrus trees, moved systemically throughout a 2.5-m tall citrus tree and were detected at least 57 days post-treatment. These authors also reported that topically applied sprays and soil applications to citrus trees and grapevines in field conditions resulted in detection for up to 42 days post-treatment (Hunter *et al.* 2012).

Dicer is the enzyme that recognises and cleaves dsRNA in eukaryotic cells. Both strands can be detected but only the guide strand (antisense strand) is preserved, while the passenger (sense strand) is degraded (Fruscoloni *et al.* 2003). Here, the detection of both sense and antisense DsiRNA strands was possible, and we attributed this to the stability of the 27-mer Dicer-substrate duplex design (IDT 2011), in agreement with the observation of Kim *et al.* (2005) on the longer duration of the 27-mer dsRNA compared with 21+2 siRNAs, which translates to higher efficiency to trigger RNAi.

Table 2. Comparison of the movement of two different kinds of DsiRNA, ds_aflR and ds_Rib, in *in vitro* peanut plants 15 days after treatment by RT-PCR. See Table S1 for raw data.

| | DsiRNA detection by RT-PCR[a] | | | | | | | |
| | ds_aflR | | ds_Rib | | Untreated[b] | | Untreated[c] | |
	Plant #3	Plant #4	Plant #5	Plant #6	Plant #1	Plant #2	Plant #1	Plant #2
Shoot 1st cm	+	+	+	+	−	−	−	−
Shoot 2nd cm	+	+	+	+	−	−	−	−
Shoot 3rd cm	−	+	+	+	−	−	−	−
Leaf #1	−	−	−	−	−	−	nd	nd
Leaf #2	−	−	−	−	−	−	−	−
Roots	−	−	−	−	−	−	−	−

[a]+ = RT-PCR antisense amplification signal present; − = RT-PCR antisense amplification signal absent; nd = no data.
[b]RT-PCR amplification with ds_aflR antisense stem-loop primer.
[c]RT-PCR amplification with ds_Rib antisense stem-loop primer.
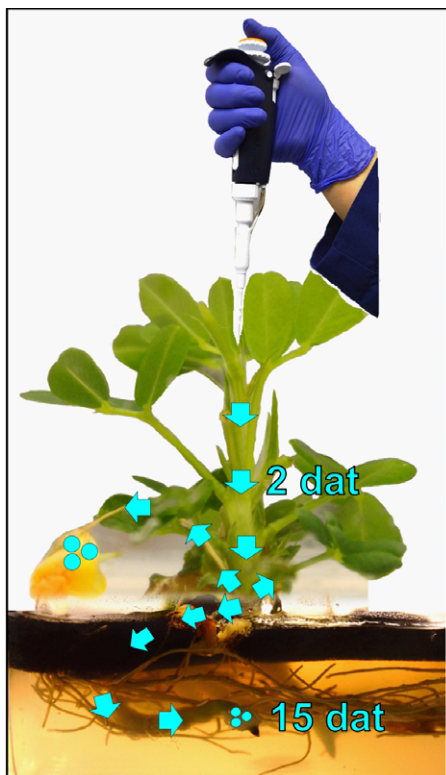

Fig. 3. Schematic movement of DsiRNA in *in vitro* peanut plants. The movement and stability of DsiRNA in the *in vitro* peanut plants over time was observed. DsiRNA (ds_aflR) was applied to an apical cut on *in vitro* peanut plants to study the movement throughout plants. At each sampling time, one whole plant was sectioned into 1-cm pieces, noting which part of the plant the section came from, *i.e.* stem, leaf, auxiliary shoot, flower, peg, etc. Total RNA was extracted from each 1-cm section. Detection of the DsiRNA present in each section was performed using stem-loop RT-PCR primers and a RT-PCR system. DsiRNA was absorbed and moved from the point of application throughout the plant, and transported rapidly, most likely through the phloem of the shoot to the sink tissues, such as new auxiliary shoots, flowers and newly formed pegs, in ca. 15 days. DsiRNA in the roots could only be detected when applied at a higher concentration (50 nM). However, it still could not be detected in most of the leaves sampled.

The mobility of RNA within an organism has been demonstrated (Scott *et al.* 2013) and, combined with the fact that RNAi molecules can move between host plant and parasite (Tinoco *et al.* 2010), demonstrate that opportunities for pathogen control exist. The design of small RNAs to down-regulate the expression of aflatoxin genes has the potential to reduce toxin contamination, as demonstrated by Abdel-Hadi *et al.* (2011). This is the first time that movement of exogenous DsiRNA in *in vitro* peanut plants has been studied. Since DsiRNA was detectable in the pegs at 15 dat, aflatoxin reduction may be possible if the duplexes containing part of the aflatoxin biosynthesis pathogen gene induce significant silencing in *Aspergillus* spp. colonising peanut seeds. However, further studies will need to determine: (i) the optimal DsiRNA concentration required for silencing, (ii) the most convenient strategy to deliver the DsiRNA, (iii) the efficacy of DsiRNA suppression of aflatoxin in *Aspergillus* spp., and (iv) the stability of the DsiRNA in mature seeds during harvest to prevent contamination. Emerging biotechnologies like RNAi, CRISPR-Cas9 and antisense oligonucleotides, provide new and safer tools for more focused management strategies to effectively and efficiently control pathogens in crop plants.

ACKNOWLEDGEMENTS

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Comparison of the movement of two different kinds of DsiRNA, ds_aflR and ds_Rib, in *in vitro* peanut plants 15 days after treatment by RT-PCR. Raw data used for Table 2.

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