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A cytometry microparticle platform approach for screening tobacco microRNA changes after agrobacterium delivery

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ABSTRACT

MicroRNAs are a class of non-coding regulatory RNAs that can modulate development as well as alter innate antiviral defenses in plants. In this study we explored changes in *Nicotiana benthamiana* tobacco microRNA expression as it relates to expression of a recombinant anti-Ebola GP1 antibody. The antibody was delivered to tobacco leaves through a bacterial *Agrobacterium tumefaciens* "agroinfiltration" expression strategy. A multiplex microparticle-based cytometry assay tracked the expression changes of 53 host tobacco microRNAs. Our results revealed that the most abundant microRNAs in actively growing leaves corresponded to nanoparticle probes specific to nta-mir-6149 and nta-miR-168b. After agroinfiltration, probes specific for nta-mir-398, and nta-mir-482d were significantly altered in their respective expression levels, however changes were partially attributed to the infiltration broth medium used in the antibody delivery process. Confirmation of nta-mir-398 and nta-mir-482d expression changes was also verified through RT-qPCR. To our knowledge this study is the first to profile medium and Agrobacterium injection at the microRNA level through a multiplex microparticle approach.

1. Introduction

The tobacco species *Nicotiana benthamiana* is often used to express monoclonal antibodies and vaccine antigens. This expression is possible through the delivery of engineered genetic constructs from *Agrobacterium tumefaciens* bacteria (Powell, 2015; Rybicki, 2014). The delivery of *A. tumefaciens* cultured in bacterial broth medium into tobacco is referred to as agroinfiltration. Small-scale agroinfiltration can be performed through injecting Agrobacterium into the backside of well-watered tobacco leaves using a blunt-end syringe, or on a large scale through vacuum infiltration. Over the course of one to five days post-agroinfiltration the product of interest is expressed transiently in tobacco leaves and is subsequently purified after homogenization. While numerous Agrobacterium-vector expression systems have been designed to deliver constructs encoding biotherapeutics, a detailed understanding of host plant genetic responses to the agroinfiltration process is lacking.

The aim of this study was to analyze the microRNA (miRNA) responses within actively growing *N.* benthamiana leaves after agroinfiltration. The agroinfiltrated product corresponds to an anti-Ebola antibody encoded on a plasmid expressed within *A. tumefaciens*. The anti-Ebola antibody of interest (referred to as 6D8) has potent neutralizing activity against the GP1 region of Ebola, and the synthesis of 6D8 in tobacco (Chen et al., 2011), along with 6D8's effectiveness in animals (Olinger et al., 2012; Phoolcharoen et al., 2011) has been well established. As miRNAs can modulate gene expression in plants, determining host miRNA expression changes after agroinfiltration may lead to more tailored experiments in an attempt to increase the yield of a product in tobacco leaves. As an example, if expression of a specific host miRNA can be associated with increasing or decreasing expression of an antibody construct, targeted experiments to either silence or artificially express this miRNA may lead to novel ways to increase antibody yield in tobacco. Of note, bacterial Luria broth (LB) medium was also delivered without *A. tumefaciens* to study miRNA responses. LB medium is widely considered to be the most common medium used when injecting *A. tumefaciens* into green plant leaves, therefore we wanted to know how miRNA responses would be altered after this injection process.

In order to screen for host miRNAs, a microparticle-miRNA technology, referred to as the Firefly™ assay was used. Microparticle hydrogel particles within the Firefly™ assay function as a fluorescent reporter with a unique barcode that can be run within a conventional flow

Abbreviations: EF1α, elongation factor 1-alpha; gfp, green fluorescent protein; GP1, glycoprotein 1; LB, Luria broth; miRNA, micro-RNA; mRNA, messenger RNA; NGS, Next-generation sequencing; PAMPs, pathogen-associated molecular patterns; qRT-PCR, quantitative real-time polymerase chain reaction; siRNAs, small interfering RNAs.

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cytometer. This cytometry-based approach can quantify miRNA levels with sensitivity comparable to RT-qPCR, can be multi-plexed with 70 miRNA targets in a single well, and this technology is well documented in the published literature (Chapin et al., 2011; Chapin and Doyle, 2011; Hoss et al., 2015; Le et al., 2014). To our knowledge we report the use of this Firefly™ technology for the first time to study plant miRNAs. The expression of 53 miRNA tobacco targets using a microparticle multiplex screen is of focus in the results and discussion sections.

2. Materials and methods

2.1. Agrobacterium culturing and infection

Plasmid pBYR29e-6D8 was constructed using gemini-viral elements derived from pBY-HL(6D8). R and expressed within *A. tumefaciens* strain GV3101 (Huang et al., 2010). The expression cassettes for heavy and light chains of monoclonal antibody 6D8 were modified to contain the tobacco mosaic virus 5′ untranslated region, the tobacco extensin gene 3′ terminator and the high-copy colE1 origin of replication (Diamos et al., 2016). An expression cassette for RNAi suppressor p19 was also incorporated (Chen et al., 2011). The sequence of pBYR29e-6D8 is available upon request. pBYR293-gfp was constructed in a similar fashion with the difference being that jellyfish green fluorescent protein (gfp) was expressed instead of 6D8 (Huang et al., 2010). The sequence of pBYR29e-gfp is available upon request.

Detailed procedures for delivering Agrobacterium to *N. benthamiana* has been described and were followed (Leuzinger et al., 2013). Briefly, the backside of well-watered tobacco leaves were infiltrated with Agrobacterium suspended in infiltration buffer [Luria Broth (LB) solution containing 10 mM $MgSO_4$; 10 mM MES, pH 5.5] at an OD_{600} value of 0.1 using a 1 ml blunt-end syringe for injection. Alternatively tobacco was injected with infiltration buffer alone without Agrobacterium and this solution is referred to as "LB" or "LB broth" in results.

2.2. RNA extraction

2 cm leaf discs were harvested from uninfected or agroinfiltrated tobacco leaves at either 1 or 3 days post infection. miRVana™ total RNA isolation kit (Thermo Fisher) in combination with the use of the Ambion® Plant RNA Isolation Aid (Thermo Fischer) were used according to manufacturers recommendations. After RNA extraction, samples were eluted in ultrapure RNase-free water. Those samples with RNA concentrations above 35 ng/ul with 260/280 absorbance ratios within 1.9 and 2.1 were analyzed further.

2.3. Microparticle microRNA assay

The AbCam-Firefly multiplex cellular assay was used to analyze RNA samples. A detailed technical workflow that has previously validated specificity, sensitivity and reproducibility can be found at the AbCam-Firefly BioWorks website: https://www.fireflybio.com/node/9 with technical aspects discussed previously (Chapin et al., 2011). Briefly, the Firefly microRNA assay utilizes a post-hybridization ligation-based approach that fluorescently labels a bound miRNA target sequence to a hydrogel particle through a fluorescent reporter-expression system. This barcoded ligation approach allows miRNA detection in a flow cytometer in a multiplex manner. Three distinct functional regions each separated from the other by inert spacer regions make up the sequencing components of the particle. The central analyte quantification region contains probes that capture target miRNAs. Separate ends of the particle function as two halves of a barcode to distinguish different particles. Oligonucleotide sequences bound to particles, raw signal counts from cytometry readings, along with normalized data values are available in the supplementary file. The global mean normalization strategy (Mestdagh et al., 2009) was used for normalization in the heat map depicted in Fig. 1.

2.4. qPCR analysis of nta-miR-398 and nta-miR-482d

200 ng of total RNA from three representative samples used in the microparticle assay was also used to analyze miRNA expression by qPCR. The NCode™ EXPRESS SYBR ® GreenER™ miRNA qRT-PCR Kit (Thermo Fischer) was used for cDNA and qPCR analysis according to manufacturers recommendations. Forward qPCR primer corresponding to the microRNA sequence of interest (see supplementary file) with the proprietary universal reverse primer provided by the NCode™ kit was used. Ct values for each sample were obtained from quadruplicate wells for each sample using an input of 8 ng of cDNA in each reaction. All qPCR reactions were carried out in an Applied Biosystems 7500 real-time qPCR instrument with an initial 5 min denaturation, and 40 cycles of: denaturation at 95 °C-15 s, annealing at 57 °C-15 s, extension at 72 °C-15 s. The house keeping gene elongation factor 1-alpha (EF1α) primers served as an internal control to deduce fold-change expression. EF1α primer set was chosen based on previous studies confirming specificity and stability of this reference gene in *N. benthamiana* using the primer sequence previously described (Liu et al., 2012).

2.5. Software and statistical analysis

The Firefly™ analysis workbench software was used for analysis and was downloaded at: http://www.abcam.com/kits/firefly-analysis-workbench-software-for-multiplex-mirna-assays. GraphPad Prism 5.0 software was used to generate bar graphs and calculate standard deviations.

3. Results

3.1. In total 37 of 53 microRNA targets were detectable in the multiplex assay

Information in the literature, in combination with miRNA sequences previously deposited in the miRBase v21 database (www.mirbase.org) was used to pre-design 53 potential plant microRNA microparticle targets in the initial Firefly™ screen. While there are no *N. benthamiana* sequences yet deposited by researchers in miRBase, we attempted to choose highly expressed miRNA from related Solanaceae members that were available in the hopes they would also be present in *N. benthamiana*. Of note, a recent publication has identified a handful of unique microRNAs through a deep sequencing approach within *N. benthamiana* that have not been identified to date in the closely-related *Nicotiana tabacum* tobacco species, therefore these nanoparticle candidates were also appealing for analysis (Baksa et al., 2015). In total six unique miRNAs from this recent *N. benthamiana* study (nbe-mir) were chosen for analysis along with 33 of the most abundantly expressed microRNAs for *N. tabacum* (nta-mir) found in miRBase v21. Five highly expressed microRNAs for *Solanum tuberosum* (stu-mir) and two highly expressed microRNAs for *Solanum lycopersicum* (sly-mir) from miRBase v21 were also chosen as both of these species, like tobacco, are also within the Solanaceae family. Of note, an additional seven highly expressed microRNAs from the well-studied plant species *Arabidopsis thaliana* (ath-mir) were included with the intent to see if there was conservation in microRNA expression between tobacco and Arabidopsis.

The microparticle assay screen of the 53 miRNA-nanoparticle sequences was sensitive enough to detect the expression of 37 targets, while the remaining 16 miRNA targets fell below the detectable threshold in one or more of the 20 samples (Fig. 1, right blue cluster). The 20 samples subjected to the miRNA multiplex screen represented the following four categories- 1. Four samples were from actively growing non-injected leaves (healthy: 1–4), 2. Three mock-infected samples were infiltrated with LB infiltration broth medium alone with no agrobacterium or construct and harvested at day 1 post-agroinfiltration (LB-d1: 1–3). 3. Six samples were from post-agroinfiltrated leaves