REVISED MANUSCRIPT

Title

A cytometry microparticle platform approach for screening tobacco microRNA changes after *Agrobacterium* delivery.

Authors

Joshua D. Powell^{*a}, Qiang Chen^b, Hugh S. Mason^b

^aChemical and Biological Signature Sciences Group, Pacific Northwest National Laboratory, Richland, WA, USA 99354

^bThe Biodesign Institute and School of Life Sciences, Arizona State University, Tempe, AZ, USA 85287

* Corresponding author at: Chemical and Biological Signature Sciences Group, Pacific Northwest National Laboratory, P.O. Box 999, MSIN P7-50 Richland, WA, USA 99354. Tel.: 1-509-375-3704. E-mail address: joshua.powell@pnnl.gov

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 were 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.

Keywords

Agrobacterium tumefaciens, Ebola, tobacco, *Nicotiana benthamiana*

Abbreviations

EF1 α	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

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 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 multi-plex 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 MgSO₄; 10 mM MES, pH 5.5] at an OD₆₀₀ 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 were 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 sec, annealing at 57°C-15 sec, extension at 72°C-15 sec. 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 was 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 *N. 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 delivered with the anti-Ebola 6D8 GP1 antibody construct harvested at day 1 post-agro (Eb6D8-d1). 4. Seven samples corresponded to anti-Ebola 6D8 GP1 harvested at day 3 post-agroinfiltration (Eb6D8-d3). It should be emphasized that each respective sample run in the assay, even within respective groups, corresponded to its own unique tobacco leaf and was not a replicate of the same harvested material. Only those RNA samples meeting strict RNA concentration and purity thresholds after purification were analyzed and therefore the reason that there are varying numbers of samples between the four described groups.

3.2. nta-mir-6149 and nta-miR-168b are the most abundant microRNAs expressed in the multiplex profile within growing N. benthamiana leaves

Table 1 summarizes the most abundant microRNAs detected based on fluorescent signal outputs for the four sample groups. The most abundant miRNAs are also depicted in Fig. 1 (orange color). Fluorescent intensity values for all 53 miRNA samples can be found in supplementary file. nta-miR166b and ath-miR396a-5p were the two most highly expressed miRNAs in all four sample groups. The high expression levels, yet lack of appreciable change in nta-miR166b and ath-miR396a-5p between groups, suggest these two miRNA's might serve as good housekeeping microRNAs for future qRT-PCR analysis and northern blot analysis in tobacco. Little is known about nta-miR166b, however a previous study looking at wounding/topping of *N. tabacum* (Tang, et al. 2012) noted significant expression for nta-miR-166b. Interestingly ath-miR396a, which also highly expressed, is a well established member of the conserved miR396 family of plant miRNAs, and is both predicted and experimentally validated to regulate cell proliferation in the leaves of Arabidopsis (Jones-Rhoades and Bartel 2004) (Rodriguez, et al. 2010).

3.3 nta-miR482d is significantly down-regulated in response to medium injection

nta-miR-482d, highlighted in Fig. 1 heat-map, appeared to be appreciably down-regulated in expression after infiltration when assessing the nanoparticle fluorescent readings. However, as shown in Fig. 2A, miRNA nanoparticle readings for this miRNA was largely attributed to infiltration LB broth solution. This finding for broth solution, causing nta-miR-482d down-regulation, is based on the findings that LB-only medium samples (white bars) showed similar trends to those that expressed the anti-Ebola antibody (grey bars and diagonal-hashed bars) when compares to healthy leaves that had not been injected with any liquid solution (black bars). Follow-up qRT-PCR validation with three of the samples from each group (Fig. 2B) were in good agreement between the nanoparticle Firefly™ assay versus qRT-PCR relative fold-change values.

3.4 nta-mir398 is significantly up-regulated after agroinfiltration.

nta-miR-398, highlighted in Fig. 1, appeared to be appreciably up-regulated in expression after infiltration when assessing the nanoparticle fluorescent readings. A similar nanoparticle (Fig. 3A) versus RT-qPCR (Fig. 3B) comparison was investigated as already described in Fig. 2 findings. There appeared to be modest but significant difference in up-regulation of nta-miR-398 at day 3 versus day 1 in Ebola-6D8, however LB medium alone also lead to up-regulation of this miRNA.

3.5 Gfp experiments-

Later experiments, repeated under similar conditions, but with an Agro-vector construct containing the jellyfish green fluorescent protein (gfp) instead of 6D8 antibody also showed similar down-regulation of nta-miR-482d in both medium and the gfp-Agro construct (Fig. 4A). We can conclude from Fig. 2 and these later gfp findings (Fig. 4A) that broth medium injection leads to down-regulation of nta-miR-398 independent of the presence of Agrobacterium. In regards to nta-miR-398 it appears that the slight up-regulation in 6D8 versus LB broth medium (Fig. 3) and significant up-regulation we saw with gfp versus medium alone (Fig. 4B) indicates that the presence of an Agrobacterium and/or the expression cassette induces higher expression of nta-miR-398, however medium alone still causes an up-regulation but to a lesser degree.

4. Discussion

Presently it is quite difficult to use a bioinformatics approach for assessing *N. benthamiana* mRNA-miRNA targets, as only a draft genome sequence is available (Bombarely, et al. 2012). However, a review of the published literature and the use of online predictive tools were still explored in order to gain insight into potential effects for nta-miR-482d down-regulation and nta-miR-398 up-regulation in our screen. While most online microRNA predictive tools primarily focus on eukaryotic microRNA analysis, we were able to confirm through the use of <http://plantgrn.noble.org/psRNATarget/> -a plant small RNA target analysis server (Dai and Zhao 2011) the likely high affinity binding for nta-miR-482d with tobacco N-gene in multiple Nicotiana strains. The miR-482 plant superfamily is well documented in the literature as associating with the host N-gene that dictates the extent of the plant hypersensitive response (Shivaprasad, et al. 2012; Yin, et al. 2015; Zhu, et al. 2013). N-gene induction confers systemic resistance to pathogens such as tobacco mosaic virus through initiating a hypersensitive response. MicroRNAs that associate with N-gene trigger mRNA degradation through RNA-dependent RNA polymerase 6 (RDR6) and production of secondary small interfering RNAs (siRNAs) (Shivaprasad, et al. 2012). It is therefore not surprising that invasion of tobacco with a solution such as LB-broth containing salts buffered at pH 5.5, even without Agrobacterium, could trigger N-gene induction and subsequent down-regulation of nta-miR-482d as we find from our studies. Input of nta-miR-428d sequence in the psRNATarget database confirmed specificity to N-gene in *N. tabacum* (Fig. 5), however a similar association in *N. benthamiana* could not be determined due to lack of annotation of the draft *N. benthamiana* genome. It should be noted that a very recent published study by Baska et. al has confirmed high levels of nta-miR-482d in the leaves of *N. benthamiana* using high throughput sequencing that was later confirmed through Northern blot analysis (Baksa, et al. 2015). Furthermore, and similar to our analysis, Baska and co-workers reported no annotation for miR-482 when they used a PAREsnip pipeline approach (Folkes, et al. 2012), which we can only speculate may be due to the minimal annotation available on the *N. benthamiana* genome.

In regards to nta-miR-398 up-regulation (Fig. 3,4), little miRNA-mRNA associations have been reported in tobacco. The already-mentioned Baska et. al publication has also confirmed expression of miR-398 in *N. benthamiana* through sequencing and northern blot analysis. In the Baska et. al study PAREsnip

analysis identified Umecyanin (comp100607_c0_seq1 102_557) a family of plant-produced cupredoxins as a potential host target. In light of these previous findings we performed a psRNAtarget analysis with input of multiple *Nicotiana* genomes and nta-miR-398 sequence and found no strong predicted associations for this miRNA with either *N. benthamiana* or *N. tabacum*. However, nta-miR-398 sequence in the tobacco strain *N. sylvestris* may have a plausible association with a pollen-specific SF3-like protein from the psRNAtarget analysis (Fig. 5). The role for down-regulating a pollen-specific protein through miR-398 expression after agroinfiltration is however uncharacterized. Finally, it should be noted that Arabidopsis ath-miR398b is identical in 20 of 21 nucleotide positions to that of nta-miR-398 and has been characterized to a greater degree (Beauclair, et al. 2010). ath-miR-398 over-expression in transgenic Arabidopsis plants leads to increased bacterial susceptibility by *P. syringae* and was found to negatively regulate Pathogen-associated molecular patterns (PAMPs)-triggered immunity when induced (Sunkar, et al. 2012) (Zhu, et al. 2011), therefore is it conceivable that up-regulation of miR-398 in *N. benthamiana* may be a stress-related response induced by the host to limit damage to the whole plant. The presence of Agrobacterium in our 6D8 and gfp constructs (Fig. 3,4) may have enhanced this effect more so than the medium.

Further studies will need to be explored as to the effects of silencing and/or artificially expressing nta-miR-398 and nta-miR-482d in *N. benthamiana* as it relates to the yield of an Agrobacterium-based biotherapeutic antibody. The finding that both of these microRNA are altered by the medium solution suggests that alternative medium delivery methods may prove beneficial in limiting this miRNA response. It should be mentioned that nta-mir-6154a, nta-mir6159, sly-mir4376, stu-mir4376-5p were all significantly up regulated at day 3 post-agroinfiltration versus healthy leaves (see supplementary file). Unfortunately these four miRNAs were not pursued through RT-qPCR as these specific miRNAs from healthy leaf controls were at or below the limit of detection that we felt warranted an accurate fold-change calculation (Ct values < 38). Nonetheless, this screen does prove useful in determining which miRNA sequences are present in *N. benthamiana* since there is little in the way of literature characterizing miRNA expression in tobacco used for the purpose of expressing biotherapeutics. This screen also proves useful in confirming the reliability of using a microparticle cytometry approach to initially screen a panel of miRNA targets as the follow-up RT-qPCR values were in good agreement with fluorescent nanoparticle assay values. While next-generation sequencing (NGS) is the most informative platform for identifying novel small RNAs, this

approach tends to be labor intensive and more expensive for profiling miRNA expression. In our experiments sequencing and characterizing 20 samples (Fig. 1) through NGS would be a substantial undertaking. In conclusion, the Firefly™ multiplex assay represents an appropriate platform for screening miRNA expression in plants before using more labor and cost-intensive approaches (i.e. Illumina NGS).

Acknowledgements

Pacific Northwest National Laboratory is operated by Battelle Memorial Institute for the United States Department of Energy under contract DE-AC06-76RLO. Pacific Northwest National Laboratory 2015 NSD Innovation award SA95470-N38540 was used too offset the cost of this project.

Figure Legends

Figure 1. Heat-map of relative abundance of 53 miRNA fluorescent probes for 20 samples. Heat map of differentially expressed tobacco miRNAs in healthy mock infection sample (healthy-, n=4), tobacco infiltrated with LB-infiltration broth alone (LB broth-,n=3), tobacco infiltrated with LB-infiltration broth supplemented with an Agrobacterium expressing an anti-Ebola antibody construct harvest either at day 1 (d1Eb-, n=6) or day 3 (d3Eb-, n=7) post-infiltration. Heat map represent fold-change values For detailed explanation of software analysis the reader is directed to the Firefly™ multiplex miRNA software analysis guide freely available at: <http://www.abcam.com/kits/firefly-analysis-workbench-software-for-multiplex-mirna-assays>. Setting used were: Analysis-cluster, Metric-RMS, Method-Average, Branch Law-1.0. The reader can find raw numerical values used in this analysis in supplementary file. Highlighted miRNA's (black boxes) emphasize the expression of nta-mir482d and nta-mir398, both of which are described in more detail in Fig. 2-4.

Figure 2. (A) Nanoparticle fluorescent reading versus follow-up (B) qRT-PCR analysis for nta-miR-482d. Sample identifier notes which samples from nanoparticle fluorescent assay (left panels) were used for qRT-PCR analysis (right panels). Standard deviation for each of the three representative samples within the qRT-PCR group were compiled from quadruplicate Ct values. Fold-change is shown relative to healthy mock leaf samples 1,2,3 normalized to the elongation factor 1-alpha (EF1 α). For clarity 1/fold change is shown to reflect the down-regulation in this respective miRNA.

Figure 3. (A) Nanoparticle fluorescent reading versus follow-up (B) qRT-PCR analysis for nta- nta-miR-398. Sample identifier notes which samples from nanoparticle fluorescent assay (left panels) were used for qRT-PCR analysis (right panels). Standard deviation for each of the three representative samples within the qRT-PCR group were compiled from quadruplicate Ct values. Fold-change is shown relative to healthy mock leaf samples 1,2,3 normalized to the elongation factor 1-alpha (EF1 α).

Figure 4. qRT-PCR analysis of (A) nta-miR-482d and (B) nta-398d after day 1 and day 3 infiltration with a gfp-expressing construct. Samples were analyzed in triplicate as shown. Standard deviation for each of the three representative samples within the qRT-PCR group were compiled from quadruplicate Ct values. Fold-change is shown relative to uninfected healthy samples normalized to the elongation factor 1-alpha (EF1 α). (C) day 3 leaf discs viewed at 10x magnification to confirm gfp expression of samples.

Figure 5. Representative host mRNA targets through analysis using the online plant small RNA Target Analysis Server (psRNATarget) <http://plantgrn.noble.org/psRNATarget/> (Dai and Zhao 2011) with miRNA input sequence for nta-miR-482d (top) and nta-miR-398 (bottom).

References

Baksa I, Nagy T, Barta E, Havelda Z, Varallyay E, Silhavy D, Burgyan J, Szittyá G (2015) Identification of *Nicotiana benthamiana* microRNAs and their targets using high throughput sequencing and degradome analysis. *BMC genomics* 16:1025

Beauclair L, Yu A, Bouche N (2010) microRNA-directed cleavage and translational repression of the copper chaperone for superoxide dismutase mRNA in *Arabidopsis*. *Plant J* 62:454-462

Bombarely A, Rosli HG, Vrebalov J, Moffett P, Mueller LA, Martin GB (2012) A draft genome sequence of *Nicotiana benthamiana* to enhance molecular plant-microbe biology research. *Molecular plant-microbe interactions* : MPMI 25:1523-1530

Chapin SC, Appleyard DC, Pregibon DC, Doyle PS (2011) Rapid microRNA profiling on encoded gel microparticles. *Angewandte Chemie* 50:2289-2293

Chapin SC, Doyle PS (2011) Ultrasensitive multiplexed microRNA quantification on encoded gel microparticles using rolling circle amplification. *Analytical chemistry* 83:7179-7185

Chen Q, He J, Phoolcharoen W, Mason HS (2011) Geminiviral vectors based on bean yellow dwarf virus for production of vaccine antigens and monoclonal antibodies in plants. *Human vaccines* 7:331-338

Dai X, Zhao PX (2011) psRNATarget: a plant small RNA target analysis server. *Nucleic acids research* 39:W155-159

Diamos AG, Rosenthal SH, Mason HS (2016) 5' and 3' untranslated regions strongly enhance performance of geminiviral replicons in *Nicotiana benthamiana* leaves. *Frontiers in Plant Science* 7:

Folkes L, Moxon S, Woolfenden HC, Stocks MB, Szittyá G, Dalmay T, Moulton V (2012) PAREsnip: a tool for rapid genome-wide discovery of small RNA/target interactions evidenced through degradome sequencing. *Nucleic Acids Res* 40:e103

Hoss AG, Labadorf A, Latourelle JC, Kartha VK, Hadzi TC, Gusella JF, MacDonald ME, Chen JF, Akbarian S, Weng Z, Vonsattel JP, Myers RH (2015) miR-10b-5p expression in Huntington's disease brain relates to age of onset and the extent of striatal involvement. *BMC medical genomics* 8:10

- 352 Huang Z, Phoolcharoen W, Lai H, Piensook K, Cardineau G, Zeitlin L, Whaley KJ, Arntzen CJ, Mason
353 HS, Chen Q (2010) High-level rapid production of full-size monoclonal antibodies in plants by a single-
354 vector DNA replicon system. *Biotechnology and bioengineering* 106:9-17
- 355 Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant microRNAs and their targets,
356 including a stress-induced miRNA. *Molecular cell* 14:787-799
- 357 Le MT, Hamar P, Guo C, Basar E, Perdigao-Henriques R, Balaj L, Lieberman J (2014) miR-200-
358 containing extracellular vesicles promote breast cancer cell metastasis. *The Journal of clinical investigation*
359 124:5109-5128
- 360 Leuzinger K, Dent M, Hurtado J, Stahnke J, Lai H, Zhou X, Chen Q (2013) Efficient agroinfiltration of
361 plants for high-level transient expression of recombinant proteins. *Journal of visualized experiments : JoVE*
- 362 Liu D, Shi L, Han C, Yu J, Li D, Zhang Y (2012) Validation of reference genes for gene expression studies
363 in virus-infected *Nicotiana benthamiana* using quantitative real-time PCR. *PloS one* 7:e46451
- 364 Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, Vandesompele J (2009)
365 A novel and universal method for microRNA RT-qPCR data normalization. *Genome biology* 10:R64
- 366 Olinger GG, Jr., Pettitt J, Kim D, Working C, Bohorov O, Bratcher B, Hiatt E, Hume SD, Johnson AK,
367 Morton J, Pauly M, Whaley KJ, Lear CM, Biggins JE, Scully C, Hensley L, Zeitlin L (2012) Delayed
368 treatment of Ebola virus infection with plant-derived monoclonal antibodies provides protection in rhesus
369 macaques. *Proceedings of the National Academy of Sciences of the United States of America* 109:18030-
370 18035
- 371 Phoolcharoen W, Dye JM, Kilbourne J, Piensook K, Pratt WD, Arntzen CJ, Chen Q, Mason HS, Herbst-
372 Kralovetz MM (2011) A nonreplicating subunit vaccine protects mice against lethal Ebola virus challenge.
373 *Proceedings of the National Academy of Sciences of the United States of America* 108:20695-20700
- 374 Powell J (2015) From Pandemic Preparedness to Biofuel Production: Tobacco Finds Its Biotechnology
375 Niche in North America. *Agriculture* 5:901
- 376 Rodriguez RE, Mecchia MA, Debernardi JM, Schommer C, Weigel D, Palatnik JF (2010) Control of cell
377 proliferation in *Arabidopsis thaliana* by microRNA miR396. *Development* 137:103-112
- 378 Rybicki EP (2014) Plant-based vaccines against viruses. *Virology journal* 11:205
- 379 Shivaprasad PV, Chen HM, Patel K, Bond DM, Santos BA, Baulcombe DC (2012) A microRNA
380 superfamily regulates nucleotide binding site-leucine-rich repeats and other mRNAs. *Plant Cell* 24:859-874
- 381 Sunkar R, Li YF, Jagadeeswaran G (2012) Functions of microRNAs in plant stress responses. *Trends Plant*
382 *Sci* 17:196-203

- 383 Tang S, Wang Y, Li Z, Gui Y, Xiao B, Xie J, Zhu QH, Fan L (2012) Identification of wounding and
384 topping responsive small RNAs in tobacco (*Nicotiana tabacum*). *BMC plant biology* 12:28
- 385 Yin K, Tang Y, Zhao J (2015) Genome-Wide Characterization of miRNAs Involved in N Gene-Mediated
386 Immunity in Response to Tobacco Mosaic Virus in *Nicotiana benthamiana*. *Evol Bioinform Online* 11:1-11
- 387 Zhu C, Ding Y, Liu H (2011) MiR398 and plant stress responses. *Physiol Plant* 143:1-9
- 388 Zhu QH, Fan L, Liu Y, Xu H, Llewellyn D, Wilson I (2013) miR482 regulation of NBS-LRR defense
389 genes during fungal pathogen infection in cotton. *PLoS One* 8:e84390
390
391
392

Figure1

Figure2

Figure3

Figure4

Figure5

22	CACCAUACCCCCCUCAGCCCUU	1	nta-miR-482d
	: : : : : : : : : : : : : : : : : :		
621	GGGGAAUGGGGGGAGUCGGUAA	642	<i>N. tabacum</i> N-protein [AB120513.1]
758	GGGGAAUGGGGGGAGUCGGUAA	779	<i>N. tabacum</i> ACRE4 [AF211528.1]

20	UCCCCGCUGGACUCUUGUGU	1	nta-miR-398
	: : : : : . : : : : : : : : : : : :		
319	AGGGGUGAC-UGAGAACACA	337	<i>N. sylvestris</i> pollen-specific protein SF3-like (LOC104248891) [XM_009805241.1]

Table 1: The top 8 most abundant miRNAs for each group along with standard deviation for mock infected, Luria broth alone at day 1 post-agroinfiltration expression (LB-d1) and anti-Ebola GP1 6D8 antibody expressed at day 1 (Eb6D8-d1) and day 3 (Eb6D8-d3) post-agroinfiltration respectively.

Rank	mock (n=4)	LB-d1(n=3)	Eb6D8-d1 (n=6)	Eb6D8-d3 (n=7)
1	nta-mir166b [455.9 ± 108.2]	ath-mir396a-5p [346.7 ± 67.8]	ath-mir396a-5p [444.8 ± 209.0]	nta-mir166b [467.9 ± 104.6]
2	ath-mir396a-5p [277.3 ± 117.6]	nta-mir166b [338.2 ± 80.1]	nta-mir166b [378.3 ± 27.5]	ath-mir396a-5p [380.0 ± 195]
3	nta-mir6149b [234.8 ± 81.0]	nta-mir6149b [306.9 ± 69.1]	nta-mir6149b [254.1 ± 47.0]	nta-mir398 [336.3 ± 312.2]
4	stu-mir166b [232.6 ± 52.0]	nta-mir167a [243.5 ± 35.1]	nta-mir167a [189.4 ± 21.5]	nta-mir6149b [302.9 ± 78.1]
5	ant-mir393a-5p [173.7 ± 22.0]	stu-mir166b [160.3 ± 17.6]	ant-mir393a-5p [172.7 ± 14.9]	stu-mir166b [206.0 ± 56.4]
6	nta-mir167a [162.8 ± 43.7]	ant-mir393a-5p [153.8 ± 21.4]	stu-mir166b [170.8 ± 22.7]	nta-mir167a [185.9 ± 28.8]
7	ath-mir157a-5p [115.1 ± 69.2]	ath-mir157a-5p [107.0 ± 16.8]	ath-mir157a-5p [118.4 ± 34.4]	ant-mir393a-5p [146.1 ± 16.6]
8	ath-mir319a [80.7 ± 21.4]	nta-mir398 [104.6 ± 55.2]	nta-mir172d [104.7 ± 24.6]	nta-mir172d [125.3 ± 33.2]