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Commentary

From Pandemic Preparedness to Biofuel Production: Tobacco Finds Its Biotechnology Niche in North America

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Abstract: In 2012 scientists funded by the United States Defense Advanced Research Projects Agency (DARPA) produced 10 million doses of influenza vaccine in tobacco in a milestone deadline of one month. Recently the experimental antibody cocktail Zmapp™, also produced in tobacco, has shown promise as an emergency intervention therapeutic against Ebola virus. These two examples showcase how collaborative efforts between government, private industry and academia are applying plant biotechnology to combat pathogenic agents. Opportunities now exist repurposing tobacco expression systems for exciting new applications in synthetic biology, biofuels production and industrial enzyme production. As plant-produced biotherapeutics become more mainstream, government funding agencies need to be cognizant of the idea that many plant-produced biologicals are often safer, cheaper, and just as efficacious as traditionally used expression systems.

Keywords: Agrobacterium; *Nicotiana benthamiana*; tobacco; Zmapp

1. Introduction

For almost two decades, plants of the family *Solanaceae* and *Brassicaceae* have been attractive for use in expressing a range of biotherapeutics, the most notable products being antibodies and vaccine-stimulating antigens. The use of green plants, particularly tobacco (*Nicotiana benthamiana* and *Nicotiana tabacum*), to express products to combat infectious bacterial and viral agents has also been the subject of hundreds of primary publications and numerous review articles [1,2]. To produce biotherapeutics in tobacco, *Agrobacterium tumefaciens* containing a cloned recombinant DNA plasmid are introduced into the plant tissue, but in some instances plant viruses can alternatively be engineered

for delivery of the gene construct of interest. Various plants including cabbage, turnips and mustard are amendable for expressing a given RNA that is delivered by a plasmid encoded within the *Agrobacterium* vector. However, the robust growth of tobacco and the large surface area of tobacco leaves are considered ideal for injecting *Agrobacterium* broth solutions with high efficiency. The diversity of *Agrobacterium*-related products such as vaccine antigens, cancer-preventing agents and even human growth factors that have been delivered and subsequently produced in tobacco is briefly summarized in Table 1. Through a process referred to as agroinfiltration plant-produced hormones, cytokinins, auxin and opines interact with virulence components encoded by the bacteria that allow bacterial transfer and expression of the cloned gene within the tobacco leaf. Additionally, unlike many other herbaceous plant species, tobacco is preferred for these processes as the leaves have favorable stomata structures that "suck up" bacteria and retain the target of interest for expression over the course of three to five days. Lastly, the DNA plasmid constructs engineered by researchers to be transferred from bacteria into plants have historically incorporated or built upon genetic elements of tobacco mosaic virus (TMV) or other plant viruses that replicate in tobacco leaves, therefore further justifying the use of tobacco as a plant host for expression of these target biotherapeutics.

Table 1. Representative publications using *Nicotiana* (tobacco)-produced therapies with corresponding (first author, year published: PubMed database identifier).

Biotherapy Target	**Publication (First Author, Year: PubMed Identifier [a])**
Influenza [3–11]	[Shoji, 2015: 25483524], [Chichester, 2012: 23202523], [Shoji, 2008: 18440103], [Le Mauff, 2015: 25523794], [Ward, 2014: 25240757], [D'Aoust, 2010: 20199612], [D'Aoust, 2008: 19076615], [Mallajosyula, 2014: 24378714], [Kalthoff, 2010: 20810729], [Kanagarajan, 2012: 22442675]
Ebola virus [12–15]	[Phoolcharoen, 2011: 21281425], [Phoolcharoen, 2011: 22143779], [Huang, 2010, 20047189], [Castilho, 2011: 22039433]
Dengue [16–20]	[Kim, 2015: 25728317], [Conconi-Linares, 2013: 23499580], [Saejung, 2007: 17659815], [Martinez, 2010: 20213522], [Martinez, 2012: 22480936]
Norovirus [21–25]	[Mathew, 2014: 24949472], [Santi, 2008: 18325641], [Lai, 2012: 22134876], [Mason, 1996: 8643575], [Herbst-Kralovetz, 2010: 20218858]
HIV-AIDS [26–32]	[Mantoba, 2004: 15347807], [Kessens, 2013: 23506331], [Rosenberg, 2015: 25807114], [Sainsbury, 2010: 21103044], [Ma, 2015: 26147010], [O'Keefe, 2009: 19332801], [Sack, 2015: 26214282]
Hepatitis B virus [33–35]	[Huang, 2009: 19309755], [Huang, 2006: 16417953], [Triguero, 2011: 21819534]
Coronavirus SARS-CoV [36]	[Zheng, 2009: 19523911]
Y. pestis (plague) [37–40]	[Santi, 2006: 16410352], [Chichester, 2009: 19200825], [Mett, 2007: 17287055], [Del Prete, 2009: 19309560]
Respiratory syncytial virus [41]	[Yusibov, 2005: 15755607]
Malaria [42–48]	[Jones, 2015: 25483525], [Farrance, 2011: 21715576], [Feller, 2013: 24278216], [Kapelski, 2015: 25651860], [Voepel, 2014: 25200253], [Clemente, 2012: 22911156], [Beiss, 2015: 25615702],
West Nile Virus [49–51]	[Chen, 2015: 25676782],[Lai, 2014: 24975464], [He, 2014: 24675995]
Bacillus anthracis (anthrax) [52–56]	[Mett, 2011: 21270531], [Roy, 2010: 20673747], [Arzola, 2011: 21954339], [Chichester, 2007: 17280756], [Wycoff, 2011: 20956592]
Human papilloma viruses [57–61]	[Venuti, 2009: 19200826], [Regnard, 2010: 19929900], [Maclean, 2007: 17412974], [Pineo, 2013: 23924054], [Massa, 2007: 17280752]
Bluetongue virus [62]	[Thuenemann, 2013: 23647743]
Toxoplasma gondii [63]	[Albarracin, 2015: 25823559]
Hepatitis C virus [64,65]	[Mohammadzadech, 2015: 25990925], [Nemchinov, 2000: 11205105]

Table 1. *Cont.*

Biotherapy Target	Publication (First Author, Year: PubMed Identifier [a])
Human metapneumovirus [66]	[Marquez-Escobar, 2015: 25828350]
Streptococcus pneumoniae [67,68]	[Starkevič, 2015: 25744664], [Smith, 2014: 24498433]
Tuberculosis [69]	[Pepponi, 2014: 24629003]
Rabies [70–72]	[van Dolleweerd, 2014: 24511101], [Yusibov, 2002: 12163267], [Lee, 2013: 23967055]
Topical microbicides [73,74]	[Fuqua, 2015: 25887919], [O'Keefe, 2009, 19332801]
Hepatitis A virus [75]	[Chung, 2011: 21442402],
Nerve Agents [76–78]	[Geyer, 2015: 20353404], [Schneider, 2014: 24618259], [Larrimore, 2013: 23000451]
Hookworm [79,80]	[Seid, 2015: 25905574], [Pearson, 2015: 26018444]
Cholera [81–83]	[Levinson, 2015: 25865265], [Hamorksy, 2015: 25614217], [Yuki, 2013: 23601492],
Tularemia [84]	[Banik, 2015: 26098553]
Livestock/animal diseases [85–89]	[Nelson, 2012: 22554468], [Pérez, 2004: 15338319], [Monger, 2006: 17309733], [Gellért, 2012: 23285149], [Love, 2012: 22718313]
Venom anti-toxin [90]	[Richard, 2013: 23894495]
Asthma/allergins [91–93]	[Li, 2013: 23354320], [Marconi, 2012: 21904913], [Krebitz, 2000: 10877820]
Cancer treatment [94–98]	[McCormick, 2008: 18645180], [Marusic, 2015: 25879373], [Grohs, 2010: 20799692], [Komarova, 2011: 21390232], [Jobsri, 2015: 25692288]
Cocaine addiction [78]	[Larrimore, 2013: 23000451]
Atherosclerosis [99]	[Salazar-Gonzalez, 2014: 25143122]
Wound-healing factors [100,101]	[Feng, 2014: 24783215], Abdelghani, 2015: 25984768]
Human growth factors [100,102]	[Deepa, 2013: 23955346], [Feng, 2014, 24783215]

[a] PMID-PubMed identifier from the *N*ational Center for Biotechnology Information (NCBI) online database [103].

On a pilot project level, *Agrobacterium* can be injected into the backside of well-watered tobacco leaves using a blunt-end syringe. On a large-scale level, tobacco plants are often subjected to vacuum infiltration with *Agrobacterium*, which ensures the entire leaf is infiltrated as a result of the plant being lowered into a bacterial solution. While the yield of the product of interest in tobacco after bacterial delivery can vary based on the target sequence and the expression plasmid, it is not unreasonable to expect milligram quantities of a protein product for each gram of tobacco tissue after some genetic optimization is performed. Technical aspects and recent advances in *Agrobacterium* vector engineering can be found in published reviews [104–106]. Additionally, highly informative scientific online videos showing the *Agrobacterium* delivery and expression process are available through the *Journal of Visualized Experiments* (JoVE) [107,108]. The remaining sections focus on the following: 1. Production of biotherapeutics in tobacco: the major players. 2. Why use tobacco to express vaccines? 3. Future directions and opportunities. 4. The need for United States (U.S.) government investments in tobacco research. 5. Perceptions related to tobacco biotechnology

2. Production of Biotherapeutics in Tobacco: The Major Players

Decades of plant biology research is now coming to fruition as the Canadian company Medicago, Inc., recently announced that a $ 245 million tobacco vaccine production plant will be built and fully operational by 2019 [109]. Once completed Medicago's facility should have the capacity to produce 40–50 million doses of quadrivalent seasonal flu vaccine annually. Medicago's success can be largely attributed to the company's track record of being able to rapidly produce millions of pandemic vaccine doses, which was largely proven through a $ 21 million dollar United States Defense Advanced Research Projects Agency (DARPA) investment. DARPA funding of Medicago has allowed the

construction of the company's existing 97,000 square feet tobacco facility in North Carolina, which produces vaccines under current good manufacturing practice (cGMP)-grade standards. It should be noted that Medicago was the first to produce a virus-like particle (VLP) vaccine candidate for H7N9 avian influenza shortly after the outbreak in China using its tobacco expression system, and also the first to obtain preclinical data on H7N9 [110].

Kentucky Bioprocessing (KBP) LLC of Owensboro, Kentucky U.S.A. has been the major producer of Zmapp™ anti-Ebola virus antibody in tobacco and has experience producing numerous tobacco-based products through agroinfiltration. In 2009 KBP successfully purified 100,000 functional doses of the HIV-1 entry inhibitor Griffithsin, a topical microbicide that was produced from 9300 tobacco plants [74]. In 2012 through a $ 2.7 million contract from DARPA, KBP successfully expressed and purified butyrylcholinesterase from tobacco, which can be used as an emergency countermeasure to combat the harmful effects of nerve agents. It should be noted that butyrylcholinesterase from tobacco extracts has been calculated to be significantly less expensive to purify on a large scale *vs.* butyrylcholinesterase purified from plasma [111]. Caliber Biotherapeutics in Texas U.S.A. and the Fraunhofer U.S.A. Center of Molecular Biotechnology in Delaware U.S.A. also have excellent track records for producing plant-produced biotherapeutics. Caliber has a state-of-the-art LED growth facility capable of supporting 2.2 million plants, while Fraunhofer has an advertised automated facility that can produce 300 kg of tobacco biomass each month. Fraunhofer has also been the recipient of multiple Gates Foundation grants that have used tobacco-based therapeutics to combat malaria, influenza and to produce a vaccine against African trypanosomiasis. Collectively Caliber, Fraunhofer and Medicago have all had separate partnerships with iBio Inc. a plant biotechnology company that specializes in plant expression vectors for use in vaccine manufacturing.

In the academic sector it can be argued that the Biodesign Institute at Arizona State University U.S.A. has more experience in engineering, expressing and purifying biotherapies in green plants than any other academic entity in North America. Multiple tobacco-based products listed in Table 1 have at one time or another been studied at the Biodesign institute. The use of immune-stimulating edible vaccines has also been an avenue of interest for Biodesign researchers and is highlighted by the work done with their immune-stimulating Norwalk virus vaccine that is orally delivered through the consumption of potatoes [32]. It should be noted that significant advances in plant-based biopharmaceutical expression technologies have come through the work of George Lomonossoff's lab at the John Innes Centre in the United Kingdom and Ed Rybicki's lab at the University of Cape Town in South Africa.

3. Why Use Tobacco to Express Vaccines?

The aforementioned plant biology companies have been largely successful as they offer an expression system that is cheap and relies upon two simple input systems: light and water. When one compares plant *vs.* egg-based vaccine production systems, the cost of housing, feeding, and dealing with the biological waste of both eggs and the egg-laying hens represents a significant cost to production. While plant-based systems will not overtake the well-establish egg-based vaccine platforms or even newer mammalian cell culture platforms any time soon, tobacco does represent an attractive system to at least supplement part of our global influenza vaccine supply. Additionally,

unlike eggs, it is rather easy to increase tobacco production by planting seeds that are ready for infiltration in three weeks' time if an unexpected pandemic were to arise. Furthermore some flu strains do not propagate well in eggs, as was the circumstance for the 2009 with H1N1 swine flu vaccine in which there were well-publicized vaccine shortages. It should also be noted that unlike egg-laying hens, tobacco is impervious to avian flu infection, which has resulted in either the death or culling of over 45 million birds in the U.S.A. in 2015 alone. Also, tobacco poses little concern for microbial contamination, which has the potential to bring down a large-scale vaccine production process using a mammalian cell-culture system. Finally, there is a level of flexibility in using tobacco-*Agrobacterium* systems for the delivery of an antigen, as expression can be triggered at the juvenile plant-growth stage or weeks later when the plant is much more mature.

4. Future Directions and Opportunities

While it's easy to envision multiple large-scale greenhouses one day producing the bulk of the world supply of seasonal influenza vaccine, the cost-savings for producing an unproven experimental trial drug may not always justify the use of tobacco-based expression systems. For example, producing the amount of anti-Ebola virus Zmapp™ cocktail in tobacco needed on a global scale that consists of three separate recombinant antibodies would be difficult. Zmapp™ was originally intended for expression at levels sufficient for animal trial use, even though a limited stockpile has proven rather effective in select human emergency interventions. In its current form Zmapp™ therapy requires multiple doses of highly pure antibody to be delivered directly into the blood stream and as a result it would be difficult to meet the antibody demand for widespread use in Ebola virus-stricken African countries. However, multi-million dollar government contracts have been recently awarded to scale up Zmapp™ production and clinical trials are ongoing which would prove valuable in justifying the creation of a strategic stockpile of the antibody cocktail [112–114].

While multiple plants are sometimes required to produce a single monoclonal antibody dose, immune-stimulating antigens such as seasonal influenza often can be expressed at fairly high yields in the leaf tissue. Influenza antigen is attractive for production in plants since there is a multi-million-dose demand annually that would justify the cost-savings of using tobacco instead of eggs. Biosimilars and biobetters derived from established in-demand drugs also represent an attractive option for expression in tobacco. As an example, a biosimilar version of the multi-billion dollar cancer drug trastuzumab (Herceptin) that had patent expiration in Europe in 2014 and will soon have patent expiration in North America has already been produced in tobacco [96,115]. The Canadian biotech corporation PlantForm is expecting human trials with their plant-based trastuzumab biosimilar to begin next year. On their website PlantForm claims their production system should also lower the existing manufacturing costs of trastuzumab by as much as 90% [116]. In the biofuels sector, a $ 4.9 million project funded by United States Department of Energy's Advanced Research Projects Agency-Energy (ARPA-E) has explored ways to engineer tobacco to produce alkanes and isoprenoid hydrocarbons [117]. Of note, Boeing and South African Airways (SAA) through a partnership with SkyNRG [118] are using the seeds and flowers of the nicotine-free tobacco strain Solaris for biofuel production [119]. Although this Boeing-SAA tobacco jet fuel project is only on a pilot-project scale,

there are plans for a scale-up and use of the remaining parts of the Solaris plant to feed livestock in arid locations where soil would otherwise go unutilized [120].

As innovative ways to repurpose tobacco continue to be established one can envision multiple products produced within the same tobacco plant. For example, a vaccine clarified and purified through column chromatography would still leave much of the leftover plant biomass available for extracting hydrocarbons. It's not unreasonable to imagine that the electricity needed to purify a vaccine could come from the tobacco itself, therefore making the growth and purification of a given product at least partially self-sustaining. A potential game-changer in *Agrobacterium* delivery that would increase the efficiency of tobacco-expressed biotherapeutics even further is a recent technology produced by Nomad Bioscience of Germany. Lead by Yuri Gleba, Nomad has developed a fully scalable *Agrobacterium* spray-based process for manufacturing cellulases and other cost-sensitive proteins in plants [121]. This spray-based technology would allow for a convenient means within a multi-acre field setting to produce a product of interest without having to invert the potted-plants into bacterial solutions or grow the plants in an enclosed greenhouse space that requires electricity.

5. The Need for U.S. Government Investments in Tobacco Research

As newer bioinformatics and next-generation sequencing tools become cheaper for scientists to use, understanding tobacco host responses that are altered during *Agrobacterium*-based expression will help researchers discover new ways to increase the yield of products in the tobacco leaf tissue. Government investments in both plant virology and *Agrobacterium* research in the 1980's and 1990's resulted in the plasmids, expression cassettes and *Agrobacterium* strains now used to produce a diverse range of biotherapies in tobacco. However, compared to the highly studied model plant organism *Arabidopsis thaliana*, there is still relatively little known about the basic molecular biology of tobacco. While U.S. biodefense government sectors DARPA, Biomedical Advanced Research and Development Authority (BARDA), Defense Threat Reduction Agency (DTRA) and the National Institute of Allergy and Infectious Diseases (NIAID) have been quite generous in funding applied technologies related to tobacco to combat infectious diseases, basic tobacco research has been rather neglected. Even when tobacco-based biotherapeutics show good efficacy in an animal model, and would be attractive for at least pilot-level funding, some plant biologists have anonymously expressed their frustration to this author that there is a certain stigma to using the words "plant virus" and "tobacco" within a U.S. National Institute of Health (NIH) R21 or R01 proposal call. It is plausible that some grant reviewers may have the impression that these projects are more appropriate under the umbrella of U.S. Environmental Protection Agency (EPA) or U.S. Department of Agriculture (USDA), even if all proposed work is related to the pathogen of interest and has nothing to do with tobacco biology. Furthermore USDA and EPA funding calls generally aren't applicable to tobacco projects with NIH-based applications, therefore a perpetual cycle exists that creates roadblocks in tobacco biotherapeutic research even though the track record of research for using tobacco systems shows this is a proven technology (Table 1).

While one can argue that private industry should now drive internal investment in tobacco basic research, partnerships between academia and industry often leads to innovation breakthroughs. Multiple scientific publications have come through collaborative efforts between Medicago and

Quebec University researchers. KBP has previously had direct ties with academic faculty from the Owensboro Cancer Research Program (OCRP). Fraunhofer actively works with numerous U.S. agencies along with international universities to advance plant-based therapies through the various clinical trial stages. By having a two-armed collaborative approach with academic basic research driving the science and private industry driving the efficacious product, newer *Agrobacterium*-related technologies should continue to advance the technology while lowering the production and overhead costs of the final product.

6. Perceptions Related to Tobacco Biotechnology

Public acceptance for repurposing plants for innovative biotechnology applications is largely complicated by the issue of genetically modified (GM) crops. While tobacco-related technologies are not designed to be edible, there are increasingly vocal opponents to plant biotechnology in general at both the political and societal level [122,123]. Even though detailed risk assessments by multiple nations have shown GM crops do not pose a greater risk for environmental contamination more than traditional breeding, public perception by a vocal minority (media outlets, blogs, *etc.*) sometimes unjustly lump all plant biotechnologies as being unsafe regardless if they are edible [124–126]. While U.S.A. public perceptions of plant biotechnology is considered to be more favorable than European perceptions, hotly contested ballot measures at the state level in the USA for mandatory GM labeling do imply a growing resentment towards GM technologies [123,127]. It should be emphasized that there is no scientific evidence of transient *Agrobacterium* systems accidentally transferring recombinant DNA to seed to create a tobacco GM organism. It should also be noted regarding public perception, the *N. benthamiana* tobacco strain is not favorable for cigarette production and therefore does not promote the use of a cancer-causing product or directly benefit "big tobacco" companies. While there is no quick remedy that can collectively change the opinions of the vocal minority that may be against tobacco-*Agrobacterium* technologies, the growing body of published literature and the recent publicized success of Zmapp™ against Ebola virus is a positive step. Hopefully in the coming years the public opinion for tobacco-expressed biotherapeutics will continue to warm to the notion that tobacco is an eco-friendly alternative for cheaply producing a range of beneficial products as it relates to human health.

7. Conclusions

In the last decade tobacco is increasingly being used as a host species for the production of antigens and antibodies. In some instances, tobacco systems offer a cheaper, faster and safer alternative source for biotherapeutic production versus traditional technologies. Basic research investments in green plant biotherapeutics at the government level are needed so that basic science (academic) and applied applications (biotechnology companies) can collectively work together to advance this emerging technology even further.

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Conflicts of Interest

The author declares no conflict of interest. The inclusion or exclusion of discussed companies, therapeutics, or research in no way reflects a financial endorsement by the authors or their employer.

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