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Production of therapeutic oral vaccines from transgenic plants –a promising way in treatment of diseases

Abstract

Discovery of vaccines is the most remarkable invention made in the field of science. These have shown to be successful when produced in animals but come with a lot of limitations. A striking invention was seen when the same vaccines could be produced in plants overcoming the disadvantages of animal vaccines. In this review, there is a detailed description underlying the need of producing vaccines in plants, why use plants as a host to produce vaccines, advantages of producing plant vaccines, existing technologies used in the production of plant vaccines and the strategies that can be employed in increasing its production. The review also focuses on how the small size of the plant genome can be utilized in order to bring about several genetic modifications making them resistant to different platforms in order to integrate the gene of interest. The review also shows lists of recombinant, oral vaccines produced through different technologies produced in plants.

Keywords: *agrobacterium tumefaciens*, oral administration, pollen biodiversity, recombinant vaccines, agrobacterium mediated transfer, plastid transformation, magnifection

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Introduction

Vaccines are regarded as one of the most vital public health discoveries of the 20th century. Downstream aspects in defining the strengths, weakness and well as the potential applications of vaccines developed in animals and plants have been studied extensively in the recent times. Research is now being carried out in developing such vaccines in plants by employing various strategies such as production of transgenic plants which can express a protein that can be further exploited in large scale as vaccines. These vaccines are modified in such a way as to produce resistance to various diseases such as malaria, dengue and so on.[1] The aim of this review will be to move beyond the proof of concept and propose the different advantages and limitations pertaining to vaccine development in plants. The regulatory procedures in developing such vaccines are substantially different from the vaccines developed in animals. Development of transgenic plants in order to produce vaccines can be brought about by the stable integration of a gene into the plant genome and this can now transform plants such as potato, tobacco (regarded as higher order in the plant family) into suitable bioreactors for the actual production of subunit vaccines which can be administered orally as well as parental.[2] The advantages of developing vaccines in plants are the absence of contamination risk, inexpensive, large scale production and most importantly the option of producing edible vaccines. The threat of bio-terrorism can also be eliminated in producing plant vaccines. There are two main ideas on which the development of plant vaccines depend on which is the production of transgenic plants and stable infection of the heterologous gene into the plant genome using precise viral vectors. During the initial period, not all plants were compatible to such vectors due to the inability of carrying large size transgenes. However, advancements are made in this technology by relying on agrobacterium which acts a systemic factor in delivering viral vectors containing the plant genes. There is an extensive list of plant types which can be used for the expression of vaccine antigens such as Nicotiana spp., Arabidopsis thaliana, spinach, carrots, tomatoes etc, in their hairy root cultures and then be amplified in a bioreactor. The romantic notion of 'cheap, edible vaccines' was not discussed previously but has now taken a milestone and producing and resulted in the production of several vaccines based on the above characters. Agrobacterium tumerfaciens is one of the potent gram negative soil bacterium which can be infected in the intracellular spaces of the plants. The characteristic feature which makes the bacteria virulent is the presence of a tumour inducing plasmid called Ti (200Kb) which contains the T-DNA as well as the genes necessary to transfect it to the plant cells. The transfection of this into the plant cells immobilises the plasmid into the nuclei of the cells and hence resulting in the expression of the transgene.[3] This process allows industrial production which occurs without any genetic modification of plants, which is much faster and most importantly biologically safe. Vaccines developed in plants are now advancing themselves towards the Phase 2 and 3 of the clinical trials. Systems through which the production of such vaccines can be brought about can be divided into 3 categories which are: nuclear transgenic technology, chloroplast induced plant protein technology and plant virus technology. These proteins which are now produced by the plants and be applied either topically or can be taken as oral pills or powders. The vaccines produced in the normal fashion have shown a 14 fold increase in the cost of vaccines over the past decade and hence alternate strategies have been investigated in order to produce such vaccines among which production of such proteins in plants have been the prominent one. The expression level of such proteins varies depending on the developmental stages of the leaves as well as the time of harvest. Some of the challenges that are posed in such cases would be the removal of agrobacteria after the infiltration of the gene of interest into the plant using vacuum.[4] Since 1989, there have been many proteins which have proved to be an ideal factor in the development of vaccines or therapeutics using infiltration of whole plants or certain important parts of the plants with Agrobacterium tumefaciens.[5] This bacterium can harbour various launch vectors and with the help of spp such as Nicotiana excelsiana, which is usually selected as the most promising host due to the easy infiltration and high level of reporter protein production act as a whole perfect strategy in producing

such proteins. The most commonly induced viral vectors in the agrobacterium are pBID4-GFP (Tobacco mosaic virus-based) which now in the aid of several chemicals such as acetosyringone in filters the vector into the spp of N.excelsiana. Several plant derived vaccines in the above approach are used as specific idiotypes for the treatment of non-Hodgkins lymphoma and influenza. Agrobacterium induced plant vaccines based on Tobacco mosaic virus has been successful in the lab scale and now produce vaccines in the larger scale to act against pathogens such as human papilloma virus, Yersinia pestis, Bacillus anthracis, and smallpox virusin N. benthamiana leaves. This method also promises the idea of producing multiple proteins in the same plant system. This can be seen in the example where tumour specific recombinant proteins are produced in the glycosylated form against epidermal growth factor receptor as well as production of monoclonal antibody specific for anthrax antigen.[6] Plants as hosts to produce vaccines Delivery of vaccines to the body made in plants is known to have several advantages such as low cost as well as their abilities in eliciting the required cell-mediated and humoral responses without evoking much pain and discomfort than the parenteral delivery. Oral vaccines are now the rigid walls which act as a source of excellent defence mechanisms against various diseases as well as protect the cell walls from the acidic environment of the stomach, enabling the target antigen to reach the gut associated lymphoid tissue (GALT) and thereby providing immunity. The immunogenicity of the plant made vaccines is increased by techniques such as Chloroplast transformation, addiction of adjuvants and targeting proteins (Table 1, Table 2 & Table 3).[7]

Table 1 List of oral vaccines produced in plants using various virus causing diseases

Oral vaccines developed in plants		
Pathogen	Antigen	Plant
HepB virus	SurfaceAg	Potato
Capsid Pro	VP6	Potato
HIV-1	p24-Nef	Tobacco
HPV	HPV16-L1	Tobacco
Rabies virus	G protein	Tomato

Table 2 Statistics of recombinant vaccines produced in plants from the year 1992-2002.[4]

Year	Vaccine antigen	Species
1992	Hepatitis virus B surface antigen	Tobacco
1995	Malaria parasite antigen	Virus particle*
1995	Rabies virus glycoprotein	Tomato
1995	Escherichia coli heat-labile enterotoxin	Tobacco
1996	Human rhinovirus 14 (HRV-14) and human immunodeficiency virus type (HIV-1) epitopes	Virus particle*
1996	Norwalk virus capsid protein	Tobacco
1997	Diabetes-associated autoantigen	Tobacco
1997	Hepatitis B surface proteins	Potato
1997	Mink Enteritis Virus epitope	Virus particle
1997	Rabies and HIV epitopes	Virus particle
1998	Foot and mouth disease virus VP1 structural protein	Arabidopsis
1998	Escherichia coli heat-labile enterotoxin	Potato
1998	Escherichia coli heat-labile enterotoxin	Potato
1998	Rabies virus	Virus particle*
1998	Cholera toxin B subunit	Potato
1998	Human insulin-Cholera toxin B subunit fusion protein	Potato
1999	Foot and mouth disease virus VP1 structural protein	Alfalfa
1999	Hepatitis B virus surface antigen	Yellow lupin, lettuce
1999	Human cytomegalovirus glycoprotein B	Tobacco
1999	Dental caries (S. mutans)	Tobacco
1999	Diabetes-associated autoantigen	Tobacco
2002	Respiratory syncytial virus	Tomato

Table 3 List of plant vaccines produced due to plastid transformation

Pathogen	Antigen	Plant species(chloroplast)	Immunological response
Bacillus anthracis	83kD antigen 83kD antigen	Tobacco	subcutaneous immunisation in mice produced IgG
Vibrio cholerae	Cholera toxin B	Tobacco	
Clostridium tetani	TetC	Tobacco	oral vaccines inducing CD4+ cells and IFN-γ production
E.coli	heat labile enterotoxin B	Tobacco	LT-B protein with GM-1 binding properties

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Cost effectiveness

Novel approach to improve the quality and standard of the vaccines was increased with the production of the plant vaccines. These vaccines can be produced in the cheapest ways and in very high amounts. Plants such as potato, corn are easily accepted by the patients and antigens derived from such plants are usually stable and optimum and don't pose any risk of contamination. These can also be stored for long period of times. Traditional vaccines are prepared as attenuated versions of the pathogens of interest following the inactivation of the disease. In contrary plant derived vaccines do not undergo the risk of an organism being involved and such vaccines have the potential for 0 contaminations.[8] AIDS is a type of syndrome which 95% of the people in the developing countries suffer from. For this reason, it is very essential that the HIV vaccination has to be cost effective. Production of such recombinant proteins in plants offers many such advantages, with lesser manufacture and processing requirements. The amplification of such potential proteins can be increased by simply increasing the planting area for whole plant systems or by further scaling up the process by the principle of bioreactors. The cost of producing such proteins in plants is approximately 100 fold lesser than producing them in animals.[9] Ability to produce multiple proteins Plant species have a unique property of expressing multiple proteins by encoding many genes. This can be explained with an example where the native genes for the daily activity are synthesised systematically by plants through a process called as regulation. Once such multiple transgenes get integrated into the host chromosomes, they then become a part of the genome and inherit this successively to the next generation. The duration of these genes expressing certain proteins can be as long as they are present inserted within the genome. Antibiotic screening can perhaps be used to locate the gene encoding a protein each time. However, multiple protein expression depends on certain factors such as the age, flowering stage etc. The production of multiple antigens in plants gets tricky when the complicated scenario of post translational modifications and senescence is being studied. In this way, the protein obtained has to be modified and then subjected to a bioreactor for its large scale production. Easy administration the vaccine antigens developed in the plants can be eaten readily with the edible part of the plant. Transgenic plant technology is advantageous for the reason being it is safe and needleless and hence can be easily administered.[10] Oral administration is a very straight forward and an easy process to deliver a vaccine to the mucosal lining of the gut. This comes with a challenge of inducing mucosal immunity and conferring protection against pathogens that invade these surfaces. However these plant vaccines have the potential to confirm immunity as soon as they have been administered. Oral dosage of a plant vaccine is stimulated simultaneously with proteolysis in the gastrointestinal tract. For this reason, it is very important to protect the antigen from proteolysis for the oral delivery to be effective. Some of the plant tissue materials can store the antigens for long period of time. These plant recombinant antigens can be preserved in encapsulated forms and hence can be taken in the form of pills. The advantage of the plant material to store the antigens at ambient temperatures prevents the cold storage of vaccines thereby limiting its costs. There is an array of options available in order to produce plant vaccines in the palatable form. The expressed antigen has to be purified before packaging it in the form of a capsule for oral administration.[11]

Biosafety

Entry of genetically modified products into the market on a broader scale also brings with it several ethical and environmental concerns. The most frequently described is its biosafety. Phase 1 and 2 clinical trials have confirmed that plant derived vaccines can be allowed as they proved to confirm resistance to the mucosal lining. However the question arises as to how these proteins expressed in the plants undergo post translational modifications. These vaccines hence have to be administered carefully. Following strategies were employed concerning the biosafety of the vaccines:

I. Establishment of male sterility to overcome the problem of pollen biodiversity
II. Use of tissue specific promoters to inhibit the effects of development of the plant.
III. Following the principles of maternal inheritance using chloroplast thereby limiting pollen-mediated gene flow.

Development of plant vaccines is indeed a biotechnological innovation, which can be allowed not only for its technical merits as well as for its social acceptance. Some of the plant derived vaccines have completed all the phases of clinical trials and hence can be regarded as biologically safe product.[12] Since, these vaccines have proved to exhibit high expression levels the whole process can be fully contained in a greenhouse producing approximately 500kg of recombinant protein per year. Most of the transgenic plat vaccines have completed all the phases of the clinical trials and hence have proved to be safe and can therefore be released into the market. Expressing vaccine candidates in fruit and vegetable tissues have also confirmed their resistance and thereby providing immunity. Some of the productions emerge where the whole tissue is edible and hence can be consumed directly without purification. This is another character describing the biosafety of plant vaccines. The achievement of this goal correlated with the GMP standards. The principle of this can be substantiated with the potential of these plants producing multiple proteins. Hence to increase its productivity there is a need for plant bioreactors to scale up the production of such products. Bottlenecks such as low yield and inconsistent quality along with certain ethical issues have been overcome by the vaccines developed capable of undergoing downstream processing which make it even more feasible to be used more often.[13]

Existing technologies used in the production of plant vaccines the production of foreign proteins in transgenic plants has become an alternative source for to conventional production systems such as the microbial bio fermenters etc. These transgenic plants act as the well experienced and suited bioreactor and bring about the production of several therapeutics most important being the vaccines. The products derived from the plants are particularly attractive as they are free from human diseases as well as from few mammalian viral vectors.[14] Agrobacterium mediated transfer of the gene of interest into the plant Agrobacterium spp- "natural genetic engineer" is used in several biotechnological purposes to carefully transfer DNA to the plant cells which are subjected to genetic engineering. This approach is used quite a lot in today's scenario and has been successful in producing transgenic tomato, potato, cotton etc. There is a wide array of species which come under the genus Agrobacterium. For example; A. tumefaciens causes crown gall disease in plants and another spp called Aradiobacter is considered as avirulent. Plants when infected with A. tumefaciens containing the nopaline type Ti plasmid pTiC58 results in the crown gall disease. The disease gets its name due to the presence of small tumours seen in the soil surface during the integration of the bacterial DNA to the plant genome. This causes tumour production and changes in the metabolism of the plant. This procedure is of prime importance because any desired gene of interest resistant to a variety of factors such as Bacillus thuringiensis

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can be incorporated into the plant genome via the Agrobacterium species which can then be introduced into the plant genome in order to produce several pharmaceutical products such as vaccines. The preliminary step in inducing the transformation of the plant cell is to integrate a tumour inducing plasmid region in the bacterium to the plant genome. The region which gets integrated is called as the T zone containing the Ti plasmid. The plasmid contains the T region flanked by left and right repeats called the TL and TR respectively which is approximately 25bp. The TL and the TR region is covered by several growth factors such as auxin, cytokinin and opine. The left border of the plasmid contains the virulence region encoding genes such as VirD1, D2, C1, C2 etc. Nicking of the border is brought about by VirD1 and D2 protein; VirC1 and C2 play an important role in virulence and hence are required to induce the disease in the plant. There are other virulence proteins such as Vir A and G which act as a 2 system sensory system in detecting plant phenolic compounds that induce tumor formation and wounding. VirA phosphorylates itself as well as phosphorylates and activates VirG. The transfer of the T-DNA is then facilitated into the plant genome with the help of other proteins such as VirE2 and VirF. Lying just next to the virulence region is the Origin of Replication which brings about the actual process of the replication of the different proteins in the plant genome. Either the whole TDNA can be inserted or just the region containing the virulence proteins can be incorporated. However, different scientists have shown different parts of the Ti plasmid which can be transferred to the plants and hence could confer resistance. For example, Ooms et al observed and confirmed that the TDNA borders could also efficiently induce the infection in plants; Ramanathan and Veluthambi have proved that the TL border induces more infection than the TR border. Wenk et al proposed that the entire Ti plasmid along with the DNA sequences is required in order to bring about the infection in Nicotiana plumbaginifolia and Arabidopsis thaliana. Konov et al suggested that the Vir protein D2 did not play a substantial role in the infection. In this way different experiments were performed in different laboratories in order to understand the significance of the transfer.[15] In the development of vaccines, the gene of interest to be inserted into the host is first targeted into the Ti plasmid. This gene could be encoding a protein and this in the form of the TDNA or other parts of the plasmid can now be infiltrated to the leaves of the plant. Ideally the duration for full expression in the case of Agrobacterium mediated is 3-4 days. However, this method is not suitable for large scale production and hence is regarded as the First Generation Production Systems. Organelle transformation of the plant such as the plastid biotechnology has become an attractive biotechnological tool in producing different biopharmaceuticals particularly vaccines. The high copy number of the plastids enables transformations of the plant very effectively. Another factor lies in the pattern of inheritance which the plastids follow called as the maternal inheritance. These and many more characteristics of the plastids define them as the ideal "green bio factories" which provides a platform for immense vaccine production. Some of the reasons why chloroplasts can be used to produce vaccines include high production level, low production cost and eradication of animal prone diseases. There are two main methods involved in the transformation of the plastids: polyethylene glycol mediated transformation and gene gun mediated plastid transformation. In the first method, protoplasts are isolated and are transformed using PEG. The gene encoding the protein required for vaccine production is now gently incorporated into the plastid for transformation. There are about 100 plastids each enclosing 100 copies of plastid DNA which brings about the actual transformation. A gene gun or biolistic particle delivery system is a device used for injecting plant cells. The payload is an elemental device made up of a heavy metal coated with the plasmid DNA. The device is not restricted only to nucleus but can transform any organelle such as the plastid. The gun is a Crosman air pistol filled with tungsten particles. The target of a gene gun in the plants is usually callus growing on a medium in a petri dish. The gold particles from the gun are now dispersed into the medium and causes disruption of the callus causing the plant cells to encapsulate gold particle which now is integrated into the plastid to bring about transformation.[16]

Magnification

Recently developed new technique in plant biotechnology overcomes the limitations of using agrobacterium mediated transfection. This process is used to simultaneously facilitate transient gene amplification encoding a protein alongside brining about high level of expression in all the mature leaves of the plant in which the gene of interest was targeted. This technology combines the advantages of three biological systems such as vector efficiency, efficient DNA delivery by agrobacterium and speed and high expression levels of the protein integrated. This process brings out the highest level of protein expression compared to the other technologies which therefore do not require large scale up by using a fermenter. At the manufacturing stage, this system offers many advantages such as rapid production cycles, high product yield etc. Through this method many vaccines have been produced such as vaccines to treat Non Hodgkins Lymphoma and several other diseases.[17]

Conclusion

Pilot studies have explained and confirmed that the expression of antigens in plants is successful and this can be confirmed with the number of vaccines that have been produced through this approach. These oral vaccines produced in the plants have passed all the phases of the clinical trials and are hence termed now as bio safe and can be used for the treatment of different diseases. In the case of plant made HIV vaccines, it is evident that these have to be taken in the form of booster doses in different schedules. Looking at the progress made in the field of plant vaccines there may come a day when heat labile plant vaccines will be in use which would certainly keep HIV at bay. Use of viral vectors in order to produce vaccines in plants has also shown significant increase. Peptide and subunit vaccines are primarily produced in this discipline. This area has not only shown the expression of viral proteins but has also been successful in stimulating the required mucosal immunity in humans considering these vaccines. The need of large scale fermenters are also minimized when vaccines are developed with the help of technologies such as plastid transformation technology and the latest one called as the Magnifection. In order to further improve the statistics of plant vaccine production the following strategies can be employed as better and improved ideas.

Future perspectives

Future perspectives in the area of plant based vaccines include integrating multi-disciplinary strategies like designing formulations which involve incorporating antigen expression such as oral adjuvant and focussing on conducting pre-clinical trials and using the results for further phases of analysis. Improving downstream processing of the proteins produced in the plants for increasing quality is another area to bring about a platform for the production of vaccines.[17]

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Conflict of interest

The authors declare that there is no conflict of interest.

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