CRISPR/Cas9-based Antibody Production in Plants

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Abstract:

Nowadays demand of antibody production is increased to cure different diseases including diabetes, hepatitis and cancer. For that different types of systems are used for the expression of antibody production. But these were not improved the antibody production. Plant cells have several benefits in comparison with other eukaryotic cells if it is considered as eukaryotic expression system. As compared to the human cell or other microorganisms, the plant cell is safe and decrease the contamination of antibody production. In addition, plants perform proper post-translational modification as a eukaryotic expression system. But recently, transient expression system is used due to the safe and improve the quality and quantity of antibody production. In transient expression system agroinfiltration method are mostly used. The main issue in antibody production is purification. Because in downstream process

antibody is degraded due to the physical and chemical stresses. These issues can be solved with the help of CRISPR/Cas9. Plant antibody can be tagged with the help of CRISPR/Cas9. This review encompasses the applications of CRISPR technology for producing plant-based antibodies.

Keywords: Antigen, Antibody, Plant-based, ZFNs, TALENs, CRISPR

I. Introduction

CRISPR was 1st time identified in 1987 accidentally by Japanese group of scientists in *Escherichia coli*. CRISPR contains short repeats of nucleotides. These repeats are simple. They don't know about these repeats and their importance. They give the name of these repeated sequences was short regularly spaced repeats (SRSS) (Ishino, Krupovic, & Forterre, 2018). In 2002, the CAS gene was discovered by a team of Dutch microbiologist which is associated with CRISPR (Jansen, Embden et al., 2002). Repeated set of these sequences are also observed in archaea and bacteria. It took approximately 20 years to identify that the spacer originates from invaded DNAs. The sequences of the spacer matched with bacterial DNA and acts as a reminiscence of diseases (Bolotin, Quinquis, Sorokin, & Ehrlich, 2005). In CRISPR system comprise in three components, the crRNA, trans-activating (tracrRNA) and CRISPR-associated protein 9(cas9). These joins and form a complex structure, then Cas9 recognize the target region and chop down the DNA. The crRNAs (short CRISPR-derived RNAs), are essential for the identification and cutting of foreign DNA at target site. (Mojica, García-Martínez, & Soria, 2005). CRISPR/Cas provides immunity to bacteria and was identified in a yogurt making experiment in *Streptococcus thermophilus* bacteria (Barrangou et al., 2007). With the passage of time different CRISPR/Cas systems have been identified, these systems have same mechanism but different characteristics such as, RNA read the sequence of DNA in bacterium CRISPR library and then these CRISPR RNA search the viral gene. Next the Cas protein recognize and cut the viral DNA (Jinek, 2012). CRISPR/Cas9 system was engineered and used to edit mammalian cells in 2013 (Mali and Yang, 2013). In barley seed dormancy gene was knock out with the help of CRISPR/Cas9 (Lawrenson et al., 2015). In maize the stress causing gene was disrupted. In result the hybrid maize increased the yield next in 5 to 10 years (Shi et al., 2017). In 2018-19, the germ lines were developed with the help of CRISPR/Cas9. In this ccr5 gene was targeted whose made protein for HIV (Cohen & Corey, 2017).

II. What is antibody?

Antibody is also named as immunoglobulin. It is a defensive protein that is formed by immune system in the presence or in reply to any foreign particle which is known as antigen (Rhoades and Pflanzner, 2003). Because pathogens contain special protein called antigens. When pathogens enter the body of the host, these proteins stimulate the immune system response in host i.e., synthesis of antibodies. Antibodies bind to pathogens and destroy them. More than, memory cells are produced which remain in the host body. These pathogens contain bacteria, virus, incompatible blood cells and pollens are the examples of antigens which acts as an immune response (Maverakis et al., 2015).

III. Structure of antibodies

Antibody is a molecule that is Y-shaped. It comprises two short chains of polypeptides, named as light and heavy chains. Both these chains are parallel to each other.

At the end, these chains form the arms of the Y-shaped structure. The structure of Y shape called antigen binding site. The specific antibody determination specific antigen and bind them. One type of antibody binds with specific antigen and more than one types of antibodies recognize different types of antigens and bind them (Figure 1 a, b and c). This type region is called variable region. The Y shape longer region is formed by heavy chains. This is called constant region (An, Zhang, Mueller, Shameem, & Chen, 2014).

Figure 1: (a) Antigen (b) Antibody (c) Antigen-Antibody Interactions: Antibodies formed by B cells are capable of detecting antigens found on or generated by pathogens. This identification happens when the antigen binding sites of the antibody meet and attach to specific antigens whose structure matches the antibody in a lock

and key manner. Due to this selective binding, only a small subset of antigens is detected by each antibody, making them extremely sensitive.

IV. Production of antibodies

Antibody are produced by B lymphocyte. The stem cells in bone marrow develop the B cells. When a foreign particle attacks the host the B cells become activated and develop into cells called plasma cells. Antibodies as the response of specific antigens are produced by plasma cells. The antibody binds with antigen and destroy the foreign particle.

When an unknown antigen attack on the host body, it can take some time (2 weeks) because plasma cell generates the enough antibodies that binds with unknown antigens. If the infection is under control, then the small sample of the antibodies are circulating in the body. Next time when same antigen attacks the host body, these antibodies response will be more active and quicker.

V. Classes

Antibodies has five different classes which play different roles in the human immune response. These classes are following below.

IgG, IgM, IgA, IgD, and IgE

IgE: These found in saliva and mucus membrane. These antibodies are involved in allergic response.

IgA: These mostly located in body fluids and gastrointestinal tracts.

IgM: They play important role in primary immune response.

IgD: Their function is unknown. In the surface of the mature B cells IgD are present.

IgG: They are found mainly in blood and tissue fluids (Rhoades and Pflanzner, 2003).

VI. Types of antibodies

Antibodies are the globular proteins which produced by B cells. Specific antibody attaches with specific antigen and protect the host body. The molecules on the antigens which attach the antibody is called epitopes. The part of antibody that binds to epitope is known as paratope.

Antibodies have two types. 1: Monoclonal antibodies 2: Polyclonal antibodies These antibodies are mostly used in therapeutic and research application. Both antibodies interact with the same antigen. But they have some modification. Monoclonal antibodies are formed by the same plasma B cell clone that is bound to a particular antigen. Contrary, various clones of plasma B cells develop polyclonal antibodies that bind to different epitopes of the same antigen. (Pier, Lyczak, & Wetzler, 2004).

VII. Antibodies production in plants and their importance

Plants are a good source for large-scale antibodies production. Like other eukaryotic cells, i.e. human or microorganisms, they are economically affordable, inexpensive and they cannot be easily contaminate (Stoger, Sack, Fischer, & Christou, 2002); Ma et al., 2003; Ko et al., 2005). In market, other eukaryotic systems are also available but *E. coli* and yeast are used for recombinant proteins. The current preferred systems includes Chinese Hamster Ovary (CHO) (Wurm, 2004) and baculovirus-infected insecticide cells (Berger, Fitzgerald, & Richmond, 2004), but plant antibodies have increased in number and types. Plants play key role in recombinant protein which is made

by mammalian cells, because in mammalian cell the downstream processes create problems. Other than these in plants made antibody production is easily available and sustainable other than mammalian cells (Table 1).

Table 1: Analysis comparison of antibodies expression in mammalian and plant cell

Expression systems	Mammalian cells	Plant cell
Production cost	High	Lowest
Maintaining cost	Expensive	Cheapest
Protein yield	Medium to high	Highest
Gene restriction size	Limited	Not limited
Therapeutic risk	Yes	Unknown
Glycosylation	Correct	Plant specific
Safety	Medium	High
Time required	High	Medium

Plants and mammalian consist of specific glycosylation pattern. Glycosylation is the important region of antibodies, which acts resistance to protein degradation. Glycosylation consists of four polypeptides joined by disulphide bridges. Antibodies require an adequate host expression. Plants and mammalian cells have different glycosylation pattern which are not identical to each other. But plants provide a platform for eukaryotic to express glycoprotein. The antibodies produced by plants are cheap and free from infection by mammalian pathogens for therapeutic purposes. Antibodies production in plants has been in various forms such as, in dicot and monocot plants (Table 2). These are generated by transient or stable transformations via cell cultures and various plant organs.

Table 2: Recombinant proteins produced from plant cells and plant tissue culture.

Host	Recombinant protein	Protein Yield	Reference
<i>Nicotiana tabacum</i>	Human serum albumin	0.25 mg/l	(Drake et al., 2003)
<i>Nicotiana tabacum</i> cv. BY-2	Human erythropoietin	1 pg/dry weight	(Matsumoto, Ikura et al., 1995)
<i>Oryza sativa</i>	Human α 1 antitrypsin	200 mg/l	(Huang et al., 2001)
<i>Nicotiana tabacum</i> cv. BY-2	Human interferon α 2b	28 mg/l	(Xu, Tan, Goodrum, & Kieliszewski, 2007)
<i>Oryza sativa</i> cv. Donjin	Human growth hormone	57 mg/l	(Kim, Baek et al., 2008)

<i>Oryza sativa</i>	Cytotoxic T cells surface antigen	76.5 mg/l	(Park, Lee et al. 2010)
<i>Oryza sativa</i> cv. Taipie	Human lysosome	3-4% TSP	(Huang et al., 2002)
<i>Nicotiana tabacum</i> cv. NT-1	Human alkaline phosphatase	27 mg/l	(Becerra-Arteaga, Mason, & Shuler, 2006)
<i>Hordeum vulgare</i>	Human collagen $\alpha 1$ chain	2-9 mg/l	(Weathers, Towler, & Xu, 2010)

Besides this, transgenic antibodies, were tested in several organelles for instance plasma membrane, cytosol, chloroplast, vacuole, ER, ER-derived protein and protein storage vacuoles were tested in several parts (De Jaeger, De Wilde, Eeckhout, Fiers, & Depicker, 2000); Vine et al., 2001; Jobling et al. 2003; Petrucci et al., 2006; (De Muynck, Navarre, Nizet, Stadlmann, & Boutry, 2009; Wilde et al., 1998).

In the laboratory, the yield was reported in comparison to CHO cells and it was higher in industrial processes (Bendandi et al., 2010). In 1989, the 1st work on tobacco plant were done in the field of PMAbs, in this field the single gamma or kappa immunoglobulin chains was expressed, which created a complete functional antibody as a result of sexually crossed to yield progeny. Hiatt's work has shown that the plant cell machine is capable of synthesizing, folding and secreting IgG full-size functional antigen binding antibodies and his work has also been accompanied by many groups (De Neve et al., 1993); Düring et al., 1990; Hein et al., 1991).

Human IgG was produced in the plant cells in large number as the same date. IgA is now the prevalent isotype in humans that has caught the attention of plant biotechnologists in their passive immunotherapy strategies. Chimeric IgG/A antibodies show their proper folding and assembly in *Nicotiana tabacum* and *Oryza sativa*. IgG is also produced in corn in its both monomeric and secretive form (Wycoff, 2005). On the other hand, the full-size antibodies, plants produced successfully number of antibody fragments and fusion proteins (Conrad & Floss, 2010).

Single-chain variable fragments (scFv) which consist on production of 7 in 145 Plants of two VH and VL variable regions of an antibody that is artificially attached to a stable polypeptide. They are produced in various species of plants for instance tobacco, tomato, pea; plant organs can also be used including leaves, fruits, seed and can also be produced in plant cell cultures (Table 3) (Fiedler et al., 1997; (Stoger et al., 2002). With the help of tobacco Ma and coworkers assembled performed recombinant platform and recognized its Secretory IgA-G from streptococcal antigen (SA) I/II cell surface adhesion molecule of *Streptococcus mutans* and *S. sobrinus*, which considered as passive mucosal immunotherapy (Ma et al. 1994). Antibody production levels as compared to ER-retained protein are extremely variable, scFvs are well expressed and level ranging are 6.8% total soluble protein (Fiedler et al., 1997).

Table 3: Therapeutic antibodies from transgenic plants

Plant	Antibodies	Treatment	References
Soybean	IgG against HSV-2	HSV Treatment	So et al. 2013
Tobacco	Hybrid IgA-G	Anthrax	Hull et al. 2005
Rice	ScFv against CEA	Tumor marker	Torres et al. 1995
Tobacco	IgG against HIV	For HIV	Rosenberg et al. 2013
Cereal	ScFv against CEA	Clinical test	Stoger et al. 2000
Tobacco	IgG against Ebola virus	For Ebola virus	Castilho et al. 2011
Tobacco	IgG against RSV	For RSV	Zeitlin et al. 2013

In 1993, in camels and llamas a light chain class of antibodies was identified (Hamers-Casterman et al., 1993). The V_HH or nanobody is a variable domain that functions like a typical antibody and can also bind an antigen on its own. The molecular weight of V_HH is 14–15 kDa and their structure extremely resembles to the human family III VH domain (Serge Muyldermans, 2001). Nanobody can be formed with the help or by using prokaryotic or eukaryotic host organism. And with the help of eukaryotic organism enhance their biophysical and pharmacological characteristic, the drug development, diagnose the infection, and can be cure to treat cancer like diseases (S Muyldermans et al., 2009; Teh & Kavanagh, 2010; Van Bockstaele, Holz, & Revets, 2009) verified nanobody production system as the potential of plants by producing anti-hen egg white lysozyme at an exceptional high level in *Nicotiana* leaves. They got 0.1–0.22 mg g⁻¹ fresh weight yield in post-purification which compared to the (Giritch et al., 2006; Huang et al., 2010) when making full-size IgG. (Ko et al., 2005) formed a neutralizing anti-rabies virus IgG in tobacco. The accumulation of terminal sialic residues for the achievement of complete human pattern. The first approach is the manufacturing of the full mammalian *N*-acetylneuraminic acid (Neu5Ac) biosynthesis pathway into *Arabidopsis thaliana* in this direction (Castilho et al., 2010). Similar method was used in sialylation of the recombinant monoclonal antibody 2G12, which is reported in *Nicotiana benthamiana* which consisted the full neutralizing activity (Table 4) (Castilho et al., 2011).

Table 4: Antibodies recombinantly produced in plant cell/culture.

Recombinant protein	Host	Reference
Human anti-rabies virus mAb	Tobacco	Girard et al. 2006
ScFv antibody	Tobacco	Firek et al. 1993
LO/BM2, a therapeutic IgG	Tobacco	De Muynck et al. 2009
MAK33, IgG	Potato	De Wildo et al. 2002
CO17-1A, IgG2a	Tobacco	Ko et al. 2005
cPIPP, IgG1	Tobacco	Sriraman et al. 2004
H10, IgG1λ	Tobacco	Villani et al. 2009

Other than these to increase the high production levels, many technical problems are faced to deal it with, such as the subcellular location for the efficiency of targeting, how antibody degradation happens and the process to solve this issue, and which step are required to be taken for enhancing the patterns of glycosylation and for the optimization of downstream processing (Table 5).

Table 5: Advantages and disadvantages of mammalian cell and plant cell expression

	Advantages	Disadvantages
Mammalian cell	<ul style="list-style-type: none"> • Post-translation modification of protein • Easy collection and attractive yield 	<ul style="list-style-type: none"> • Viral safety • Risk of contamination • Growth is difficult
Plant cell	<ul style="list-style-type: none"> • Low production cost • Easy production • Increased viral safety 	<ul style="list-style-type: none"> • Glycosylation/posttranslational modification • Culture parameter being uncontrolled

A. Tools for antibody production in plants

The demand for monoclonal antibodies has increasing as a therapeutics and diagnostics. To overcome this problem alternatives ways are used such as expression systems are developed. For that various expression systems have been used such as transient or stable expression. But there are some problems in expression system, localization of subcellular and proteolytic degradation. Because antibodies contain two polypeptides, the addition of a single DNA build attaches the both chains (heavy and light) at a time. Two different approaches have been used for that: 1 - The plants which are independent and containing one transgene of individuals by cross pollination and plants shows both chains (heavy and light) 2- The DNA constructs is also called co-transformation. They contain two different vectors in many cases resulted, in the addition or deletion of transgenes in the one or same locus and, occasionally, complex insertion patterns involving transgene repeats (De Block & Debrouwer, 1991; De Neve et al., 1993; Ramessar et al., 2008).

The result of cross pollination transformation is better than the co-transformation or different plasmids. But due to the time consumption this method was not properly used (Voss et al., 1995).

VIII. Transient expression system

The stable transformation and transient expression provide good advantages on the antibody production. Transcriptionally active foreign coding sequence can be easily entered in the plant cell. The most widely used 1; agroinfiltration 2; recombinant viral vectors 3; vector consisting on viral clone and agroinfiltration (Figure 2).

Agrobacterium-mediated transformation system has been used for checking the construct or to produce large number of antibodies or proteins. With the help of agroinfiltration mAbs have been shown to produce in leaves or fruits (Orzaez, Mirabel, Wieland, & Granell, 2006; Vaquero et al., 1999) a yields of up to 20 mg kg⁻¹ fresh weight. *Agrobacterium* embryogenic cultures are penetrated, the syringe is used and infiltrated the intercellular space of the mesophyll cells in plant leaf (D'Aoust et al., 2009).

Figure 2: Development of transgenic plant through different methods. Transgene can be stably integrated into plant either in nucleus or leaves/seeds (b) For transient expression, the transgene can be integrated through either RNA virus or agroinfiltration method.

IX. Zinc finger nucleases

The 1st targeted DNA strand was artificially designed with the help of ZFNs (Yang, Ding, Xu, Li, & Xiong, 2017). This is derived from eukaryotic organism which consist of protein, and act as a DNA binding domain of ZFN and the nucleotide chopper domain Folk 1 which is discovered by *Flavobacterium okeanoikoites* ((De Souza, 2012). The ZFPs creates two antiparallel b-sheets inverse of an alpha-helix and these proteins consist on 30 nucleotides (De Souza, 2012). With the help of ZFP a single cutter domain attaches more than one ZFPs, the number of these are 3 to 6. ZFP consist on 18 base pair DNA-binding have 9 base pair, which makes it more precise and robotic in gene editing (Sander et al., 2011). In many prokaryotic and eukaryotic targeted regions, the ZFNs have been magnificently consumed for HDR and (NHEJ) based knockout and knock in (Carroll, 2011). In therapeutic, biopharmaceutical industries the suitable production of protein has been modified from CHO cells by KI of GS and dihydrofolate reductase (DHFR) genes.

ZFNs are not only used for industries but nowadays due to some restrictions such as, all targeted regions can't be modified. And specific regions can be disrupted some other genes or non-targeted regions (Sander et al., 2011).

X. Transcription Activator-Like Effector Nucleases (TALENs)

Transcription activator-like effector nucleases is a moderate new genome-editing tool. In the pathogenic bacteria *Xanthomonas* the TAL proteins are discovered and play the major part of TALENs, (Li et al., 2015). TALENs consist on 33- 34 base pairs having specific recognition and binding efficiency. The TAL identify the single nucleotide, and this nucleotide is specific and not interrupted the other regions or domains which exist their neighbor. The TALENs construct used the knockout or knock-in the gene because this gene editing tool is effective. ZFNs like, TALENs also consist of two protein domains, that cuts the strand and TAL repeats and help domains to attach specific binding site and making a notch (Christian et al., 2010). TALENs have low off-targeted because this construct produces sticky ends on the DNA (Aryan, Anderson, Myles, & Adelman, 2013). By using this construct N-glycan have been modified, and knockout the two core genes (1,3)-fucose and (1,2)-xylose. These genes significantly affect the immunogenic protein such as their activity and stability. With the help of TALENs, these two genes were knockout in *N. benthamiana*. The single gene have 50% reduction and both genes on same alleles have 73% reduction or knockout of genes. TALENs construct on *N. benthamiana* have 100% result in 1st time and completely gene knockout is occurred (Jinek et al., 2012)

TALENs is more efficient in knockout is 30 to 100% nut knock-in range from 1 to 10%. Various animals such as zebrafish, chickens, frogs, rats, and mammalian cells are genetically modified through TALENs (Sander et al., 2011; Aryan et al., 2013; Kondo et al., 2014).

XI. CRISPR/Cas9

CRISPR-Cas is a defense mechanism in microorganism such as bacteria. By using this system different pathogens have been controlled by bacteria. From *Streptococcus pyogenes* Cas9 endonuclease has been also discovered and it is the mostly used in this study (Ran et al., 2015). Complex of CRISPR RNA (crRNA) and transactivating (tracrRNA) have been formed by the binding of Cas9 protein. The Cas9 endonuclease cut the targeted DNA at particular sequences by the restrictions of crRNA (Figure 3) (Brinkman, Chen, Amendola, & van Steensel, 2014). With the help of CRISPR-Cas9, Cas9 endonuclease target the specific genomic loci, and cleavage it at specific sites (Doudna and Sternberg., 2017). CRISPR-Cas9 used the repair, disruption, insertion or deletion of specific trait and its applications in different areas such as agricultural biotechnology, biomedical research, and medicine, (Hwang et al., 2013). Now this system is used for genetic manipulations in animals, fish and plant etc.

Figure 3: Artificially designed CRISPR/Cas system. In artificial system, a guided RNA (gRNA: crRNA + tracrRNA) is used to target the DNA sequence along with Cas protein.

In plants the *N. benthamiana* plant is mostly focused. Because *N. benthamiana* plant is model plant for research. This is mostly used in transient expression or stable transformation. By using CRISPR we knock-out, knock-in and modified the gene. This system is also used for the production of antibody in plants.

Pharmaceutical glycoproteins in vivo from *N-glycans* formed in plants can affect proteins behavior (Parekh et al., 1989). For that, there has been trying to modify the N-glycosylation pathway in plant species in a number of varieties with the help of T-DNA insertion (Strasser et al., 2004), RNAi (Cox et al., 2006; Sourrouille et al., 2008; Strasser et al., 2008), chemical mutagenesis (Weterings and Van Eldik, 2013) and genome editing tools or target the specific traits (Li et al., 2015; Hanania et al., 2017; Mercx et al., 2017). Genome editing tool CRISPR/Cas9 was used to suppress two b-1,2-xylosyltransferase and four a-1,3-fucosyltransferase genes in *N. benthamiana*. For that, different multiplexed gene knockout and crossing F-KO and X-KO, and confirmed the knockout of four (XylT), eight (FucT) or 12 (XylT and FucT) target genes and verify the limit of our experiment and the absence of each glycosyltransferase activity in N-glycans of total endogenous proteins and N-glycans of the recombinant antibody 2G12 shown briefly in the plants. To knockout the complete genes in *N. benthamiana*, this the 1st report.

A. Advantages

The CRISPR technology has several advantages of producing plant-based vaccine. The CRISPR-Cas9 system offers simplicity in plasmid design and construction

other than ZFN and TALEN systems. It is easily programmable to change the guide sequences of sgRNA to any DNA sequence of interest. CRISPR is capable of altering high-fidelity chromosomal markers, whereas ZFN/TALEN is vulnerable to CpG methylation. It is multiplexed genome editing gRNA.

B. Limitation

One of the limitations of using CRISPR-Cas9 gene editing system is off-targeted mutation. Because sometimes Cas9 enzyme cut the gene at the wrong site. Through the off-targeted the function of a gene may be altered, and results are unstable. The 2nd limitation is the joining of homologous pair to each other after the breaks. Therefore, NHEJ are mostly used. Limited number of PAM sequences. CRISPR cannot work without PAM sequences.

XII. Conclusion

According to this review of literature, antibodies become the part of life to control the diseases. For that, different methods and techniques are used to overcome the problems. Other than these, different cells are also used such as, mammalian cells, insect's cells, and microorganisms etc. But these are expensive, highly health risk such as allergies reactions. All of these the plants are the best source for antibodies production due to limited time requirement, health issues etc. In plants purification is a main issue. This issue can be solved with the help of CRISPR- based techniques. Tag the protein with the help of CRISPR and sought out this problem.

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