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Biotechnological Techniques to Engineer Disease Free Plants

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ABSTRACT

Plants are prone to various infections and diseases caused by viruses and microbes which adversely affects plants productivity, yield and global food security. Since ages disease free plants were produced using conventional breeding methods, but these approaches are mainly labor extensive and expensive. Thus, various advance technologies have been developed which helps to fight against disease causing pathogens. Techniques like plant tissue culture, use of plantibodies and genetic engineering has helped to gain or to modulate the desired traits in crop varieties. Improvements in Tissue culture techniques like Micropropagation, Meristem Culture and Somaclonal Variation have led to the production of disease and virus free plants. Genetic engineering is also a promising technique as it involves modification and engineering of genes having essential role in providing plant immunity against pathogens. Agrobacterium mediated transformation is associated with plant defense mechanism to produce genetically modified plants. Also nowadays plantibodies is a transgenic approach of introducing antibody specific against a target on pathogen as a transgene in the plant and has helped in making plants pathogens' resistant endogenously. Subcellular expression of which will attack the specific antigen on invading pathogen. All these methods help to obtain disease free plants which will be safe for human consumptions and other applications.

Keywords: *Agrobacterium*, Disease, Pathogen, Plantibodies, Resistance, Transgenics

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1. INTRODUCTION

The world's eighty per cent of population relies on plant derived components for their health. Plants are considered to be safe and are used to treat and prevent specific ailments and diseases in humans [1]. Most of the plants escaped the infection by activating its innate immunity components [2] while some sometimes the plants are exposed to a wide-range of pests and pathogens like bacteria, fungi, oomycetes, viruses, nematodes, and insects. The diseases caused by these organisms in plants represent an important and persistent threat to food supplies worldwide and can affect the plant heath and yield and reducing its productivity These pathogens, are responsible for the major losses in crops that amounts to hundreds of billions of dollars every year, with an average of twenty six percent of worldwide crop production lost each year due to pre-harvest pests and pathogens [3].

Increased human populations, loss of agricultural land because of climate change, erosion and water scarcity requires the reduction of production losses those caused by pathogens [4]. Plant diseases can affect the crops despite the best efforts of skilled farmers who are supported by the plant breeders and the global agrochemical industry. Thus, one of the great challenges for food security in the twenty first century is to improve yield by production of disease-resistant crops.

Since ages several traditional and conventional methods which includes chemical-based pesticides, insecticides, and various breeding practices to develop pathogen resistance. The modern agriculture food production heavily relies on chemical control of pathogens and, breeding resistant plant cultivars. Despite their effectiveness, the control of pathogens chemically is expensive, ineffective, cumbersome and have detrimental environmental consequences, building up in the soil and appearing in water leaching from fields creating risks to the wider environment. Conventional breeding approach has drawbacks as it is usually a lengthy and labor-intensive process for the growing and examining large populations of crops over multiple generations. lack of genetic variability which leads to its failure and has scarcity of resistant germ plasm in nature [5-9]. Thus, its necessary to save them from pathogens by providing strong immunity.

Nowadays one of the primary focus of crop improvement programs is to generate resistance in plants towards these biotic agents. The dependence of food production on chemical control has to be reduced and alternatives to classical and chemical crop protection methods are required for controlling pathogens in the field and development of long-lasting and broad-spectrum disease resistant crops. Thus, its necessary to increase our understanding of the plant immune system in order to develop transgenic crops with enhance resistance to microbial infections and increased yields [10,11] which can be done by altering the genetic composition of plants. Plant biotechnology has become a source of agricultural innovation providing new solutions to age old problems and has helped in the contribution of new plant biotechnological tools to advanced crop breeding [12]. Plant biotechnology will facilitate the farming of crops with multiple

durable resistance to pests and diseases, particularly in the absence of pesticides. Hence, crops should be engineered to meet the demands and needs of consumers.Various methods to enhance plant immunity includes combination of novel molecular tools, screening technologies, development of plant transformation and regeneration technologies, and understanding the molecular mechanism of host pathogen interactions have provided alternative methods to control pathogens through genetic engineering of crop plants [13] (Table 1). Several biotechnological approaches and in vitro techniques are useful for the production of disease-free plants(Table 1), rapid multiplication of rare and endangered plant species and economically important crops which have been resistance to many pathogens, and also biosafe to the environment and consumers [14-16]. Some of these have been discussed in this paper.

2.Plant Tissue culture

One of the biotechnological tools such as Plant tissue culture techniques are used for application-based purposes which helps in plant breeding and crop improvement, conservation of germ plasm, plant genome transformation, and production of plant-derived metabolites of important commercial value which are of great importance in pharmaceutical industries. [14,15,31]. The tissue culture technology has been widely used for the propagation and improvement of important agricultural crops as well as endangered native species. It can be extensively applied to increase crop production and providing plants needed to meet the ever-increasing world demand [32,33]. Plant tissue culture has important role and made significant contributions in agricultural development and productivity. They constitute an indispensable tool in modern agriculture [34]. It helps in the production and propagation of genetically homogeneous plants through micropropagation methodology [35]. Another technique like Meristem culture and somaclonal variation are also utilized for production of disease resistant plants.

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Table 1: List of some of the biotechnological techniques used to generate disease free or disease tolerant plants

S.No.	Name of the technique	Used against	Plant species	Reference
1.	Genetic engineering through MicroRNA technique	Plant viral pathogens	*Arabidopsis*, Tomato	[17.18]
2.	Genetic engineering through CRISPR-Cas technique	Tomato Yellow Leaf Curl Virus	*Nicotiana benthamiana* and Tomato (*Solanum lycopersicum*)	[19.20]
		Cucumber Yellowing Virus, Zucchini Yellow Virus and Papaya Ring Spot Virus	Cucumber	[21]
		Turnip Mosaic Virus	*Arabidopsis*	[22]
3.	Transgenic	Bacterial pathogen like *Ralstonia solanacearum* and *Xanthomonas perforans*	Tomato	[23]
4.	Micropropagation followed by SSR(Simple Sequence Repeat) and SCAR(Sequence Characterised Amplified Region)analysis of micropropagated plants	Casava Mosaic Virus (CMD)	Casava cultivars	[24]
5	Somaclonal Variation	*Bipolaris oryzae*	Rice	[25]
6	RNAi technology	*Sclerotinia sclerotium*	Transgenic tobacco	[26]
7	Transcriptomics	*Xanthomonas oryzae*	Rice	[27]
8	Thermotherapy coupled with Meristem culture	Bean Yellow Mosaic virus	*Gladiolus* sp.	[28]
9	Agrobacterium mediated introduction of rice chitinase gene	Fungal Pathogen *Cercospora arachidicola*	Peanut	[29]
10	Embryo Culture, Somaclonal Variation, Genetic transformation	Alternaria Blight Tolerance	Oilseed Brassicas	[30]

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2.1Micropropagation

At present micropropagation is the widest use of plant tissue culture technology in crops where sexual reproduction is problematic or impractical. Micropropagation is an invitro means of vegetative propagation of economical important plants which are difficult to propagate through conventional methods such as seeds and cuttings. Micropropagation has become a commercial method and provides marked advantages over conventional propagation practices by facilitating the production of large numbers of homogenous plants year-round, the generation of disease-free propagules and a substantial increase in multiplication rates [14]. Micropropagation is presently used as an advanced technique for the production of identical plants for agriculture and forestry [36]. Micropropagation can be explained in four stages. First stage is initiation of cultures, second stage is multiplication, third stage is shoot elongation and rooting and Fourth stage is transplantation and acclimatization. [37]. Nowadays the technique is used routinely to generate a large number of high-quality clonal agricultural plants, including ornamental and vegetable species, plantation crops, fruits and vegetable species. The main advantages of micropropagation are short time span to mass produce plants from a single plantlet which is of aseptic nature, faster growth rate due to nutrient media manipulation, it is approachable throughout the year, virus and microorganism free plantlets, plants can be genetically manipulated for the desirable traits. This technique is independent of seasonal variation because it is grown is controlled conditions and one can easily obtain disease free plantlet. [38]. Some of the plants where micropropagation technique has been applied are**:** *Stevia rebaudiana* Bert [39],*Commiphora wightii* [40],*Agave salmiana* [41],*Morus indica* [42],*Agathosma betulina* [43],*Punica granatum* 'Bhagwa' [44],*Rheum webbianum* [45],*Hippeastrum goianum* [46],*Ribes grossularia* [47],*Blumea lacera* (Burm. f.) DC [48].

2.2 Meristem Culture

A method in which apical meristem is used to produce disease free plants is meristem culture. Apical meristem is a dome of tissue located at the extreme tip of a shoot. The apical meristem along with the young leaf primordia constitutes the shoot apex. Anatomically apical meristem is divided into two parts. One is pro-meristems and other one is peripheral meristems. Peripheral meristem consists of protoderm, procambium and ground meristem. There is a lack of vascular tissue formation which is the main reason for disease free propagation. Apical meristems cells are genetically stable. When used as an explant source these plants have highest potential of generating plants which have similar genotypic and phenotypic composition. [49]. The dark-green "island" areas of the growing point (meristem) are either free of virus or to contain virus only low concentrations. Meristem or shoot tip is been isolated from a stem by a V-shaped cut. The small portion of the apical meristem is cut off from the infected plant body as the apical meristem is virus free and placed in agar medium and a healthy virus free plant is obtained from the infected plant [50]. Meristem culture has made important contributions to the crop improvement program. Meristem culture has been applied to Dahlia, carnation and white potato by

several researchers and has been succeeded in the elimination of virus in those plant (50). Meristem culture has been done in various plant species like: *Medicago truncatula* [51],Strawberry *(Frangaria* x *ananassa)* [52],*Allium tuncelianum* [53],*Solanum tuberosum*.L [54],*Gentiana Kurroo* Royle [55],*Manihot esculenta* [56],*Fragaria chiloensis* (l.) Duch [57],Black pepper [58],Commercial Fig cultivars 'Sabz' and 'Jaami-e-Kan'[59], *Hosta capitate* [60].

2.3 Somaclonal variation

In vitro regenerated plants can show some modifications (somaclonal variation) as a result of the mutagenic effect of the culture or the chimeric nature of the cultured tissue. Somaclonal variations (soma means vegetative and clone means identical copy) are genetic and epigenetic changes observed in plants which are regenerated from cultured somatic cells [37]. Somaclonal variations is a technique under plant tissue culture in which plant itself generate variations via genetic or epigenetic changes [5] which are very similar to the divergence caused by physical and chemical mutagens [61]. Variants having genetic changes are also termed as mutations as they are heritable and genetically stable [62] while epigenetic variants are restricted to somatic cells making them non heritable, temporary and reversible [63]. Heritable variants are the results of point mutations, methylation of DNA sequences, changes in chromosome number and structure, recombination and transposition in nuclear, chloroplast or mitochondrial genome [64, 65] leading to the stable changes, which can be sexually transmitted to the offspring's [66]. Thus, it this much popular in comparison to others. It is easy to perform, simple and genetic variations based somaclonal variants are of greater importance for obtaining disease resistance crops. Thus, providing a tool in which natural tendency of plant is used for crop improvement. There are two broad methods in context of somaclonal variations for obtaining plants with desired traits [67]. The traditional and bulky method involves in field screening of large population of in vitro raised plants. While another technique is more specific and convenient in which callus is in-vitro raised on selection media which contains culture filtrate of pathogens or fungal toxins. In this case selection media containing phythopathotoxins, components from pathogen cell wall acts as the pressure agent [68] and result in the generation of disease resistant callus from which resistant plants are obtained. In these selection systems can either be stepwise/gradual/long term culture in which culture are exposed in step-wise manner to increasing levels of selecting agent [62, 69] or shock treatment/short term culture in which cultures are straight away subjected to a shock of high concentration of selection agent, only those which could tolerate that will survive [70]. Then after every passage of three to four weeks on stress media culture are monitored on the basis of various growth parameters. The cultures showing best performance are selected as putative resistant variety. After selection cultures are further screened for the stability of variation by sub culturing them on media devoid of pressure agent and then again culturing on selection media. Those which survived are the disease resistant. There are various advantages of this technique which make relatively inexpensive, no knowledge of the gene responsible for trait is required as it is

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due to the random changes during in-vitro cultures, it can be performed for vegetatively propagating, species having Somaclonal variation has been a valuable tool in plant breeding; wherein variation in tissue culture regenerated plants from somatic cells can be used to develop crops with desirable traits. Characteristics for which somaclonal mutants can be improved during in vitro culture includes resistance to disease, herbicides and tolerance to environmental or chemical stress, as well as for increased production of secondary metabolites. Selection is done by employing a stress-causing agent in tissue culture containing dividing cells [71]. Somaclonal variant based selection has been proved to be the efficient and successful method examples of those are tabulated in Table 2.

3. Genetic Engineering

Recombinant DNA technology also called transgene technology or genetic engineering, is the most powerful and revolutionary of the new genetics developed in the last half of the twentieth century. Genetic engineering techniques are used only when all other techniques have been exhausted, especially when the trait to be introduced is not present in the long reproductive cycle or sterile species [66].

germplasm of the crop or it is taking a very long time to introduce and/or improve such trait in the crop by conventional breeding methods [82]. Genetic engineering is a DNA recombination technique that has made possible gene transfer between dissimilar genera or species [83, 84]. Genetic engineering is an exceptional way of breeding as compared to conventional breeding [85]. This approach has been demonstrated to provide enormous options for the selection of the resistance genes from different sources to introduce them into plants to provide resistance against different biotic stresses [86]. It introduces specific traits into plants i.e., traits like resistance to fungal, bacterial and viral diseases, herbicide and drought tolerance plants have been developed.

Genetic engineering allows for introduction of R-genes from unrelated plant species, which often remain functional in the new host plant [87]. The R-gene *Rxo1* from maize was

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Table 2: List of in vitro raised plants having somaclonal variations resulted in disease resistance

S.No.	Common name	Scientific name	Resistance to	Pressure agent	Reference
1	Apple	*Malus pumila Mill*	*Dematophora necatrix*	Fungal culture filtrate	[72]
2	Canola	*Brassica napus*	*Sclerotinia sclerotiorum*	in- vivo inoculation with mycellial disc	[73]
3	Carnation	*Dianthus caryoplyllus L.*	*Fusarium oxysporum*	Culture filtrate	[74]
4	Chrysanthemum	*Dendranthema grandiflorum*	*Septoria obesa*	Culture filtrate	[75]
5	Garlic	*Allium sativum L.*	*Sclerotium cepivorum*	Culture filtrate	[76]
6	Sugar beet	*Beta vulgaris*	*Fusarium oxysporum*	Culture filtrate	[77]
7	Turmeric	*Curcuma longa L.*	*Fusarium oxysporum*	Culture filtrate	[78]
8	Banana	*Musa acuminata L.*	*Fusarium wilt*	Culture filtrate	(79)
9	Sugarcane	*Saccharum officinarum*	*Colletotrichum falcatum*	Inoculated with red rot suspension culture	[80]
10	Potato	*Solanum tuberosum*	*Pectobacterium atrosepticum*	Inoculated in two different media one with calcium another without with needle dipped in suspension culture	[81]

successfully intro- duced into rice and conferred resistance against bacterial streak disease caused by *Xanthomonas oryzae*pv. Oryzicola [88]. Another example is the R-gene *RCT1* from *Medicago truncatula* that was expressed in alfalfa and conferred resistance to *Colletotrichum trifolii*, and RPI-BLB2 from wild potato

Solanum bulbocastanum conferring resistance to *Phytophtohora infestans* in cultivated potato [89].

Development of a genetically engineered crop is done through five steps. The first step is to extract DNA from the organism known to have the trait of interest. Gene cloning is a second step, where gene of interest is isolated from the entire extracted DNA, followed by mass-production of the cloned gene in a host cell. After cloning, the gene of interest is designed and packaged so that it can be controlled and properly expressed nside the host plant. The modified gene is then mass-produced in a host cell for making thousands of copies. After the gene package is ready, it is introduced into the cells of the plant being modified through a process called transformation. Of these the most common used to introduce the gene package into plant cells is *Agrobacterium*-mediated transformation [82]. Almost all plant taxa (including ferns) have been shown to be amenable to *Agrobacterium*-mediated transformation [90], although in some species only a few genotypes can be transformed efficiently.

3.1 *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens is a gram-negative bacterium of the family Rhizobiaceae. It is natural plant pathogenic soil bacterium known as 'nature's own genetic engineer' which has the natural ability to genetically engineer plants. The genes encoded in Ti plasmid region are called T-DNA. This causes tumorous growth called "crown gall" disease in plants. It causes crown gall disease in a wide range of plant species. This bacterium is modified in lab and it transfers gene of interest into plants without causing symptoms of disease [91, 92]. The T- DNA constitutes various genes including virulence (vir) genes which regulates the process of plant infection and T -DNA integration into the host chromosome. It also contains tumor inducing genes and genes that expresses for the opines, specific compounds acting as carbon source and making bacterium self-sufficient to make its own food within the plant. This has been proved to be one of the useful genetic transformation technologies for the production of genetically modified plants [93].

Two essential genetic components are necessary to be present on the bacterial Ti plasmid, one is the T-DNA and the other is Virulence region. T-DNA- contains conserved twenty-five -basepair imperfect repeats known as border sequences at the ends of the T-region. Virulence region should at least compose of seven major loci which encodes for the bacterial proteins which helps in the T-DNA processing and transfer. Plant VirE2- protein 1 (VIP1) has been shown to play a role in this process and recent reports demonstrates that VIP1, one of the transcription factors, is also involved in plant immunity responses. *Agrobacterium* is able to activate and abuse VIP1 for transformation (Fig.1).

Proteins namely VirD2 and VirE helps in the integration of the T -DNA. After the T-complex enters the plant nucleus, the associate virulence proteins and host proteins may need to be remove the T-DNA to allow efficient T-DNA integration. VIP1 belongs to subgroup I of the bZIP family of *A. thaliana* [94]. Involvement of VIP1 in the transformation process was indicated by the observation that plants expressing a VIP1 anti-sense gene showed reduced

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transformation rate, and that over-expression of VIP1 increased the transformation efficiency of plant cells by *Agrobacterium* [95, 96]. VIP1 participates in plant immunity signaling and was phosphorylated by MPK3 (mitogen-activated protein kinase 3). Phosphorylation of VIP1 by MPK3 is required for VIP1translocation into the host-cell nucleus and for activation of defense gene expression [97]. Spatial restriction of defense regulators by the nuclear envelope and stimulus-induced nuclear translocation constitutes an important level of defense-associated gene regulation [98, 99].

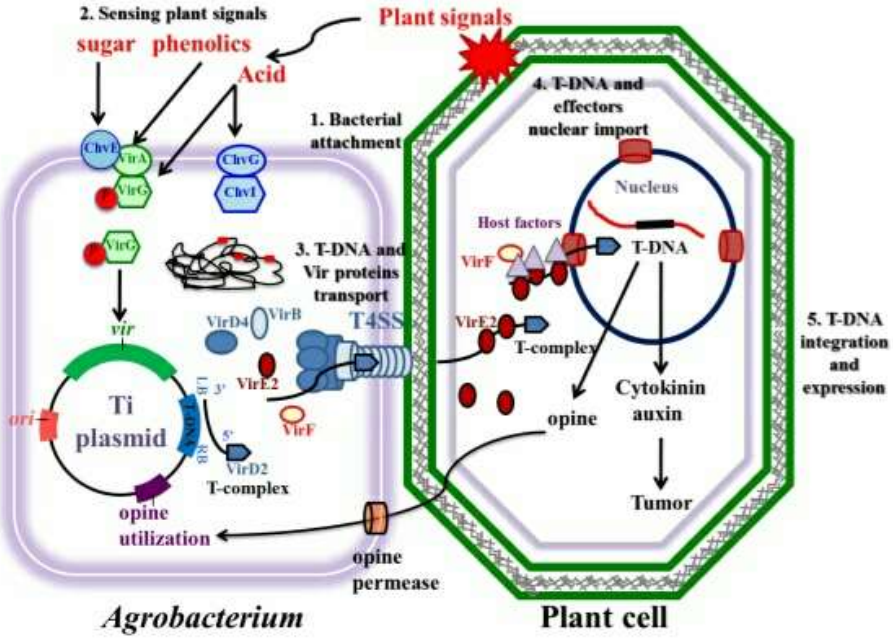

Figure.1 Major steps of the *Agrobacterium tumefaciens*-mediated plant transformation process (100)

Agrobacterium holds ability to transfer some part of its own genetic material into other plant species by a simple process called transformation. Genetic transformation is a widely used technique for molecular breeding to create new characteristics into the existed genomes. Genetic transformation is a method to produce recombinant antibodies, vaccines and antibodies [101]. Plants were used to produce plant based recombinant proteins and this technique is called as molecular farming [102] Using genetic transformation techniques, it is possible to introduce genes for herbicide tolerance, bacterial, fungal or virus resistance in some important agricultural crops. This is the most suitable method of non-sexual gene transfer and there are many

useful crops that are tested and are good candidates for agriculture use. Using recombinant DNA technique many plant and bacterial genes encoding enzymes has been engineered which makes plant crops tolerant to broad spectrum and environmentally safer herbicide.

For this bacterial gene is engineered in such a way that its enzyme is insensitive to herbicide and then transfer it to plant. The plant can also be engineered so that they express genes that detoxify herbicide. Genes obtained from *Bacillus thuringienesis* has been engineered and transfer to plants that act as insecticides [103].

3.2 Plantibodies

With the rapid growing technology several techniques have been introduced that involves genetic modification and gene editing methodology. One such promising technique is the transgenic modification of plants which proved to be solution for several plant related problems.

By method of gene transfer and special techniques in genetic engineering scientists have been able to genetically alter a number of common crops creating new varieties with selected characteristics that are better suited to farmer's needs. These new varieties are known as transgenic varieties and they have the features that can improve production of crops. [104]. Many genetic engineered crops have been developed and commercialized with improved production efficiency, increased market focus, and enhanced environmental conservation. Transgenic plants of over fifty species that contain genes from other plant species, bacteria, viruses, and animals, are currently available.

Although plants do not naturally make antibodies, plantibodies have been shown to function in the same way as normal antibodies.Agricultural crops such as tobacco, tomato, potato, soya bean, alfalfa, rice, and wheat are commonly used for the production of plantibodies [105]. Now a day's antibody production in plants has acquired significance as an emerging system for the production of many recombinant proteins that can be used for therapeutic purposes [106]. There are many sources of transgenic plants like Tobacco, Alfa alfa and soya bean are another leafy crop used to produce recombinant antibodies. Cereals, seeds and tubers are better sources of plantibodies when we are mainly targeted for long term storage [105].

Plantibodies were first demonstrated by Hiatt and colleagues and Duering and colleagues [107, 108]. Around 1990s, plants were first considered as a potential host for producing antibodies and the word "plantibody" was coined [109]. These researchers demonstrated that plants could express and assemble functionally active antibodies thus opening a new era in plant biology research. Since then, the technology of expressing antibodies in plants has advanced rapidly with a view to their utilization for therapeutic, diagnostic and agricultural purposes [110, 111]. Plants have been utilized for the expression of antibodies specific for pathogenic viruses, nematodes, fungi etc. Plant produced antibodies has become one of the predominant strategies for the protection of crop plants against pathogens [13].

"Plantibodies" terminology describes the products of plants that have been

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genetically modified to express antibodies and antibody fragments in plants. These antibodies can be recombinantly designed to target different pathogens. Plantibodies is an attractive approach to increase plant immunity by genetically transforming plants to produce antibodies and endogenously become resistant to pathogens. In this technique different plant cell compartments are transformed with functional antibodies and single chain variable fragments (scFv fragments). This process primarily involves generation of recombinantly produced antibodies by cloning and expression of antibody binding or variable domains (Fv) in vectors like bacteria, mammalian cells, yeast, and plant cells. Screening of the scFv library can be done using different techniques like ELISA and Phage display to find the specific scFv that bind efficiently to the antigen. The recombinantly produced antibodies can then be integrated into the plant genome via projectile bombardment or *Agrobacterium tumefaciens* mediated transformation (Fig. 2).

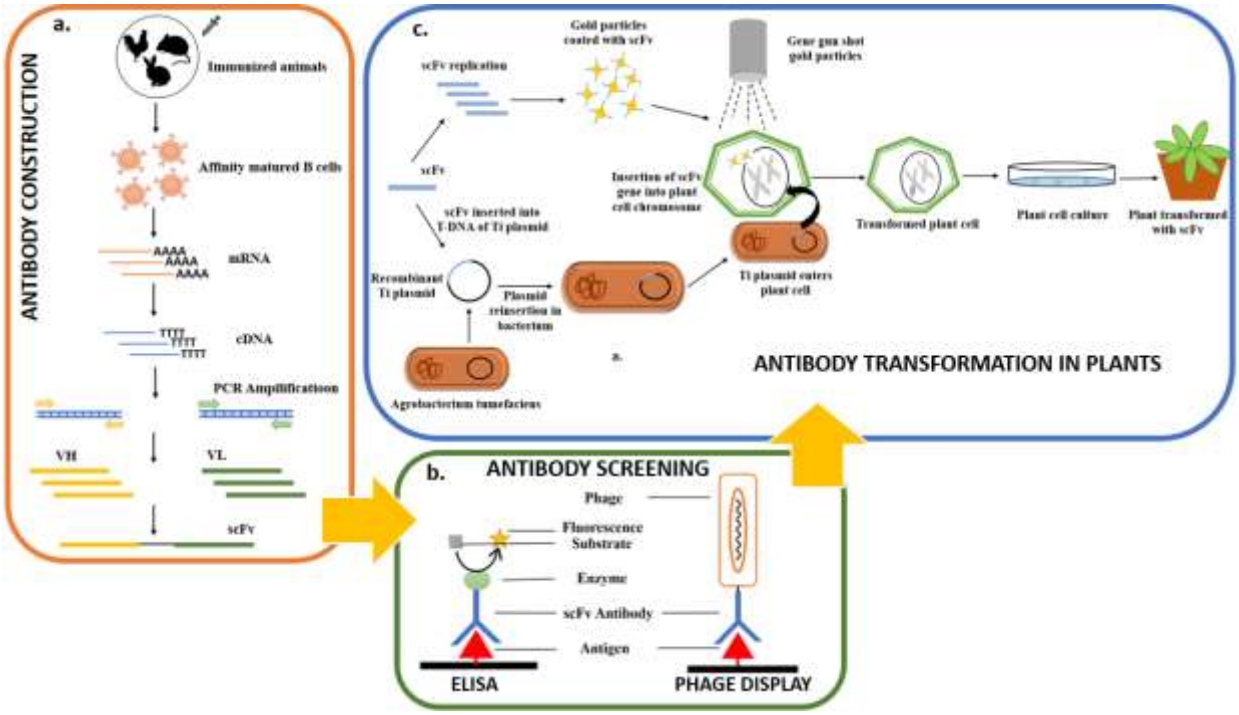

Figure.2 Method for transgenic modification of plant to produce plantibodies. a. Recombinant production of antibody fragments (scFv) from the antibody repertoire obtained from the animals immunized with antigen of interest. b. scFv library can be screened on the basis of efficient antibody-antigen interaction using techniques like ELISA and Phage Display. c. Production of transgenic plant by incorporation of best binder scFv gene in plant genome via gene gun method or *Agrobacterium tumefaciens* mediated gene transfer.

There are several additional advantages that make it a potential strategy for generating pathogen resistant transgenic lines. The transgenically produced antibodies expressed in the plant seeds can be stored stably at room temperature for a long time. Also, expression of antibody fusion in plants makes it highly specific against pathogen preventing its attack on other microorganisms present in the surrounding environment. Firstly, Resistance against fungal infection has been done. *Fusarium* is the fungal pathogen globally known for causing devastating diseases in many cereal plants. *Fusarium* sp. causes severe diseases like *Gibberella* stalk rot and ear rot in maize and *Fusarium* head blight and seedling blight in wheat. Scientists world-wide have suggested several strategies that can be a potential way to develop resistance in plants against this fungal pathogen [112]. A study expressed fusion proteins in plants that can specifically target *Fusarium verticilloides*[113]. The fusion protein comprises of the recombinant scFv and an antifungal protein (AP) Alkaline phosphatase. The scFv segment recognises the *F. verticilloides* soluble cell wall bound proteins (SCWPs) and AP provides the specificity to the target. The antibody specific to the antigen generated by an immunized chicken antibody library was identified using phage display technology. Further protein analyses validated the antibody specificity and affinity towards the surface antigen of *F. verticilloides* conidiospores and mycelia. Another interesting report [114] identifies the membrane bound protein glyoxal oxidase (GLX) in *F. graminearum* as specific antigen for an antibody CWP2 isolated from *Fusarium* resistant animal immune system. The function of GLX is Hydrogen peroxide (H_2O_2) synthesis, required for regulation of mycotoxin production in fungus.Thus, CWP2 transgenic expression in plants improves its heath by developing fungal resistance.

Secondly resistance against viral infection is also needed as viruses are the deadliest pathogens, responsible for severe plant disease. Small genome size and simple infection cycle allows rapid mutations in viruses. Therefore, the technology also has to evolve along with the viruses. The major viral antigen targets are protease domains, coat proteins and proteins involved in viral replication against which specific antibodies can be prepared. The activity of anti-viral antibodies towards antigen is dependent on several factors including the quantity (stability and yield), cellular localisation, and binding efficiency [115]. DNA methylation explored as an important factor contributing to transgene silencing in transgenic *Brassica rapa* (Chinese cabbage) [116]. The construct of scFv, bar and GFP genes with upstream 35s promoter was inserted in *B. rapa* leads to development of transgenic plant resistant against Turnip mosaic virus (TuMV). The antiviral scFv cDNA fragment was developed by the reverse transcription of the anti-ssDNA monoclonal antibodies isolated from a pre-immunised mouse. Interestingly, the resistant T1 transgenic plants produced TuMV susceptible T2 transgenic plants. Further expression study reveals DNA methylation of the promoter region confer transgene silencing and thus, decreases resistance towards viral pathogen.

Recently, a transgenic *Nicotiana benthamiana* produced with single chain variable

fragment (scFv) via Agrobacterium transformation [117]. The transgenic plant developed resistance against Beet necrotic yellow vein virus (BNYVV) which is known for causing Rhizomania, the most damaging sugar beet roots disease. The recombinant scFv developed was specific towards a major coat protein (CP21) of BNYVV. From the studies the plant clones were observed to highly express the recombinant scFv in cytosol with lowest viral susceptibility as compared to the clones with mitochondrial or apoplast localised scFv. Camelid nanoantibodies also known as nanobody (Nb) described as more promising tool in therapeutics and medicine compared to the scFv or whole immunoglobulin [118]. Because of their unique structural features like small molecular size, presence of only heavy chain fragments no light chains, greater specificity, high solubility and stability. Nbs library constructed by immunizing camel (*Camelus dromedaries*) with *Grapevine fanleaf virus* (GFLV), known for causing fanleaf degenerative disease [119]. Transgenic expression of Nb specific to GLFV in grapevine rootstock and *Nicotiana benthamiana* helps to develop resistance against the viral pathogen.

Next was to provide the plants resistance against bacterial infection. Trangenic production of bacteria resistant plants involving several genetic strategies including attacking bacterial natural defense system, expression of lytic peptides, regulating bacterial pathogenicity and inducing antibacterial proteins. Studies have shown that induction of recombinant antibodies is a potential approach to develop bacterial resistance in plants. Bacterial pathogen, *Candidatus Liberibacter asiaticus* (CaLas) globally known for causing Huanglongbing (HLB) or citrus greening disease in various citrus plants [120]. scFv antibodies developed specific to the surface proteins of CaLas [121]. Phage display technology has been used for screening and isolation of antibodies with high specificity and affinity for the pathogen surface antigens. The study proposed the application of these antibodies in diagnostic assays and development of CaLas resistant citrus plants.

Production of antibodies in plants has numerous applications not only to the pharmaceutical industry but also to the plant breeders [122]. The production of antibody fragments by plants is not only cheaper but also more efficient. Since plants do not produce antibodies naturally the purification process is much simpler and plants are capable of producing unlimited amounts of protein [122]. Therapeutic applications of plantibody are the treatment of infectious disease, inflammation, autoimmune disease or cancer. Using plants for the production of recombinant proteins has advantages compared with other expression systems such as animal systems, bacterial systems, yeast systems [123]. Plantibodies work in a similar fashion to mammalian anti-bodies; however, compared to conventional methods using mammalian cells, the use of plants for antibody production offers several unique advantages, one of these is the cost of antibodies produced by plants is substantially less than that from their animal counterparts [124].

Despite the technological advances in developing disease resistance strategies, the evaluation of these transgenic plants for resistance under field conditions has been reported in only a few studies, and the

commercialization potential for bacterial and fungal resistance remains to be seen. Adaptation of these technologies will only progress once the costs associated with growing, developing and registering the transgenic technologies are balanced by the gains observed by the producers and ultimately with the consumers of the plants [125].

Conclusion

Hence, one of the ultimate objectives of crop engineering program is to develop stable disease resistance plants. Biotechnological techniques have been shown to be a robust method to develop resistance in plants against various pathogens. The main goal of these techniques is to protect the plant against the infection caused by pathogen. All the techniques enlisted in above review have been successful in generating biotic resistance variants. The tissue culture-based techniques and genetic engineering methods involving *Agrobacterium* mediated transformation have been proved to be most efficient method till date. Nowadays various other methods such as miRNA-based approach, CRISPR cas9 technology etc. are widely used for these purposes. Further in-depth study is required to figure out advanced technologies that helps to overcome rapid mutations in pathogen. The need of today is to discover a novel method with maximum efficiency to generate stable resistant crop variant to fulfill the increasing demands of growing population.

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