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REVIEW

Expression of Single Chain Variable Fragment (scFv) Molecules in Plants: A Comprehensive Update

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Abstract
Single chain variable fragments (scFvs) are generated by joining together the variable heavy and light chain of a monoclonal antibody (mAb) via a peptide linker. They offer some advantages over the parental mAb such as low molecular weight, heterologous production, multimeric form, and multivalency. The scFvs were produced against more than 50 antigens till date using 10 different plant species as the expression system. There were considerable improvements in the expression and purification strategies of scFv in the last 24 years. With the growing demand of scFv in therapeutic and diagnostic fields, its biosynthesis needs to be increased. The easiness in development, maintenance, and multiplication of transgenic plants make them an attractive expression platform for scFv production. The review intends to provide comprehensive information about the use of plant expression system to produce scFv. The developments, advantages, pitfalls, and possible prospects of improvement for the exploitation of plants in the industrial level are discussed.

Keywords Recombinant protein · Targeted expression · Glycoengineering · scFv

Introduction

Antibodies are used extensively as medicines, diagnostic molecules, environment clean-up agents, biosensors, and as a bait in the purification process in various commercial industries [1–3]. Due to the large size, complex nature, and extensive post-translational modifications, monoclonal antibodies (mAbs) with therapeutic applications are produced mostly using the mammalian expression systems. The cost factor involved in maintaining the manufacturing unit according to the GMP (Good Manufacturing Practise) regulations makes the commercial production of mAb an exorbitant process. A single chain variable fragment (scFv) is the smallest fragment of an antibody with the same antigen-binding specificity [4, 5]. Multimeric scFv comprising of more than one pair of heavy and light chains can be generated by changing the length of peptide linker. Multimerization can also be made by using specific peptides which have the natural ability to induce it [6, 7]. Multivalent scFv showing affinity to more than one protein at the same time can be generated through genetic engineering [8–11]. The multivalent scFv binds to multiple antigens at the same time and can improve the diagnostic accuracy in any antibody-dependent procedure.

The concept and practice of using plants as recombinant protein expression platforms have made significant progress in the last two decades. There are many antibodies expressed successfully in plants [12–15]. The differences in the post-translational modifications between mammalian cells and the plant cells were a serious concern in using plants for the recombinant antibody expression. Genetic manipulations leading to the mammalian-like post-translational modifications in the model plant systems resulted in the biosynthesis of antibodies equivalent to mammalian products [16–18]. Even though biologically active mAbs can be expressed successfully in plants, a more feasible approach would be the expression of antibody fragments. The model plants like *Nicotiana* and *Arabidopsis* are studied thoroughly to identify the most appropriate promoter, suitable integration site in the host genome, influence of signal/tag in expression, viability of subcellular targeting/secretion of the recombinant protein, organ-specific expression, expression as transient or stable protein, and the extraction and purification strategies for different target proteins. This review presents a

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comprehensive report on the scFv's and scFv-Fc's expressed so far in plant systems.

Immunoglobulin (Ig) and Single Chain Variable Fragment (scFv)

The conventional antibody consists of two heavy chains and two light chains connected with disulfide bonds. The antibody structure can be divided into a constant Fc domain (crystallizable fragment domain) and the Fab fragment (antibody binding fragment) contains the Fv domains (variable fragment domains) at the end of both the arms (Fig. 1a). In humans, the antibody synthesized is usually glycosylated in the Fc region, which stabilizes the antibody and is necessary for the antibody-dependent immune responses. Enzymatic cleavage of antibody at the N-terminal side of the inter-heavy chain disulfide bridges results in the formation of Fc and Fab fragments [19, 20]. There are two variable regions in a Fab fragment interact with the antigen and each of these units represent the smallest functional antigen-binding domain.

The scFv can be generated by amplifying the variable regions of the Fab fragment from the mAb and by linking it together with a flexible peptide linker (usually (GGGGS)3) [4, 5, 21] (Fig. 1b). Advances in molecular techniques further improved the prospect of engineering scFv to improve its specificity, avidity, affinity, and half-life. Multimerization of the variable domains using, the linker [22], the tetramerization domain of a native protein like p53 [7], the leucine zippers [23], or the C-terminal fragment of C4-binding protein [6] improved the affinity of the scFv to a great extent. The immunogenicity generated by the Fc portion of the antibody is absent in the conventional scFv molecule. The scFv expressing together with the Fc region of IgG (scFv-Fc) is found to be beneficiary with its "effector" functions in many reports [24, 25] (Fig. 1g). Antibody fragments of therapeutic and diagnostic importance have been expressed in mammalian systems [26, 27] plants [28–30] and in prokaryotes [29, 31, 32]. An extensive review of the biotechnological applications of antibody fragments has been given earlier [33, 34]. Plant expressed antibodies are also used in studying the basic metabolism of plants in terms of disease resistance against a pathogen, protein–protein interactions or the specific role of an endogenous protein in a metabolic process by selectively modulating its activity [35].

Advantages of Antibody Fragments and Biopharming

Expression of mAbs in heterologous production systems is precarious as the biological activity of the resultant molecule is dependent on several post-translational modifications.

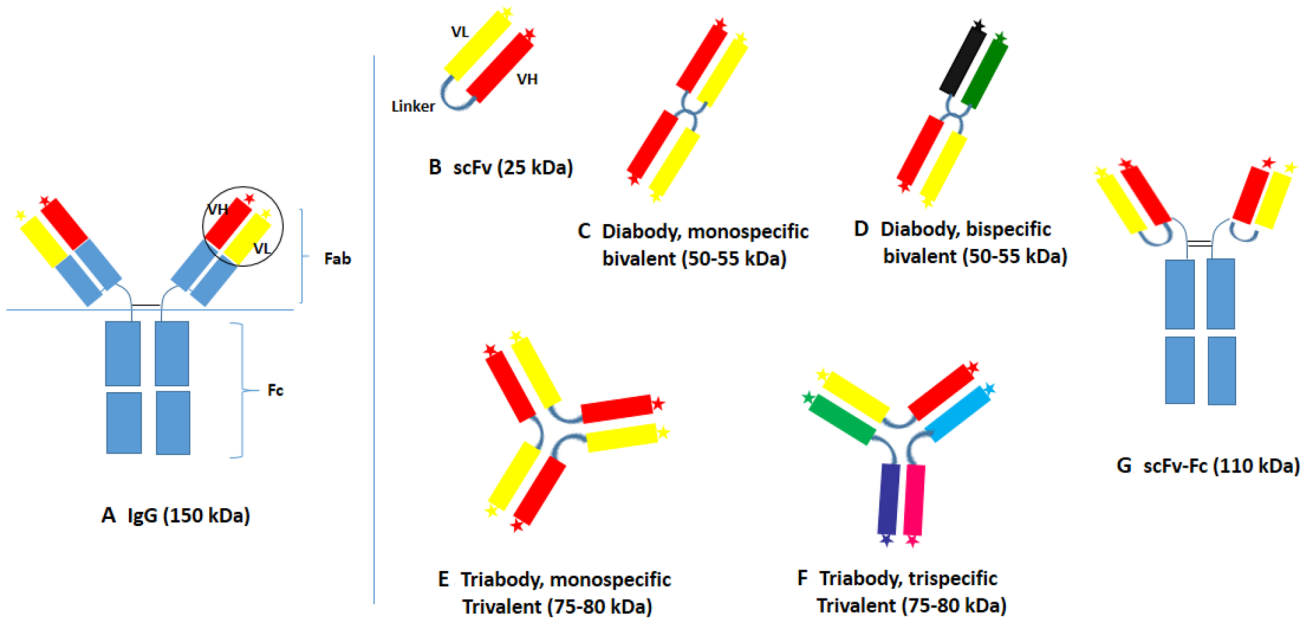

Fig. 1 scFv antibody formats expressed in plants. Immunoglobulin antibody (**a**) showing the variable regions (heavy and light chains in circle). scFv (**b**) represent the variable heavy and light chains connected together with a peptide linker. ScFv can be engineered to generate multivalent, multi-domain structures. dimeric monospecific (**c**) and bispecific (**d**) forms of scFv and multimeric scFv (**e**), molecule generated by the shortening of linker peptide. Multivalent (**f**) scFv with the paratope specificity for more than one antigens, generated by arranging the VH and VL of different antibodies in a specific order. In scFv-Fc (**g**), the scFv is bound to the Fc region of the antibody

Biosynthesis of conventional antibody molecules (150 kDa), through the mammalian expression system and transgenic animals is highly expensive and time-consuming [27, 36]. The scFvs are smaller in size (~30 kDa) with less post-translational modifications. They show a similar specificity and affinity of the parental antibody against the antigen. Due to the smaller size, scFvs show a rapid blood clearance (a useful property in the radiotherapy and other diagnostic applications) and better tissue penetration (which has greater impact when they are used as therapeutics) than the full length mAbs [37–42]. Due to the rapid blood clearance, the in vivo availability of scFv is low compared to the mAbs, which is considered as the major drawback of scFv. In order to increase the bioavailability of scFv in the bloodstream, the diabodies (~55 k Da), triabodies (~85 kDa) and tetrabodies (~120 kDa) were developed by manipulating the length of the linker peptide in between the VH and VL domains [22, 43] or by using the methods which facilitate the multimerization of scFv molecules [7, 23, 44] (Fig. 1). Tandem dimerization of the scFv generated from two different antibody sources can also create a bispecific antibody by the variation in the arrangement of the VH and VL sequences [8, 9, 45] and was proved to be an effective methods to detect specific cell types with fluorescence-tagged scFv dimers [46] (Fig. 1d). Further to that, the multivalency generated by combining the coding sequences of more than one scFv together with the multimerization is used to link different moieties (one may be targeting a cell surface receptor on a cancer cell and other bound to an anti-cancer drug) which facilitate the targeted delivery of the therapeutics to its site of action [47, 48] (Fig. 1f).

Considering the limitations of conventional expression systems like mammalian cells and prokaryotes, it is easy to manipulate the plants to cope with the mounting needs for a suitable recombinant protein production platform (Fig. 2). Contamination with an animal pathogen, one of the major concerns when using the mammalian expression system can be refuted if the plant expression systems are used. Farming of transgenic plants will open up the possibility of expanding the production of the target gene product with very little investment compared to the conventional methods. Targeted expression to seeds and the oil bodies serve a different function. When the protein need to be transported from its place of production or stored for longer, it can be expressed in seeds and for an easy purification, the proteins can be targeted to oil bodies [30, 49–51]. It is reported in the recent past that the plant-based expression systems are efficient to produce sufficient quantity of the recombinant antibodies transiently within a short time span especially during the epidemics like the recent Ebola virus outbreak in Africa [52, 53]. When using stably transformed plants like duckweeds with rapid multiplication rate as the biomanufacturing platform, the target protein can be expressed within a short period and the production can be increased as per the need [54]. The difference in post-translational modification patterns compared to that of mammalian cells has been taken care of in some of the model plants through genome engineering. Successful expression of antibodies, biologically and functionally similar to mammalian products is reported in few plants [16, 55–57]. Recently, Donini and Marusic reviewed extensively the plant-based antibody production platforms including bryophytes and algal species [58]. A comprehensive list of scFvs expressed in plants has been given in Table 1.

Transformation Methodologies, Target Plants and Expression of the Transgene

Among the methods employed for transformation of plant cells for scFv expression, *Agrobacterium*-mediated gene transfer tops the list with more than 95% of representation in the reports. Transformation along with the genome editing techniques to modify the genome of the host plant to facilitate the protein expression in a tailor-made fashion has been the subject for a number of recent reviews [59–62]. In general, *Agrobacterium tumefaciens* transfer the T-DNA region from its Ti plasmid into the genome of the host plant, with the help of its *vir* gene products. Replacing the tumor-inducing genes with the gene(s) of interest (within the right and left borders), and co-cultivating the *Agrobacterium* containing the modified Ti plasmid with in vitro raised plant cells or wounded tissue leads to the transfer of cloned DNA into the plant cells followed by integration into the genome [62–64]. This method is cheap, easy to handle and produces transgenic lines carrying only one or a few copies of the transferred gene. While most of the transformation experiments using the model plants to express scFv followed the *Agrobacterium*-mediated gene transfer, there were reports on the use of plant virus [65] and polyethylene glycol [66, 67] as the method of transformation (Table 1).

The model plants, *Nicotiana* (both *tabacum* and *benthamiana* sps), and *Arabidopsis thaliana* were the most favored expression systems for scFv (Fig. 3). Well-characterized in vitro propagation, transformation, and maintenance methods of these plants are the factors behind their selection. The protein was expressed in whole plant tissue [66, 68, 69], leaves [70–75], flower [76], seeds [77–81], hairy root culture [82] or tobacco BY-2 (tobacco cultivar Bright Yellow) cells [8, 83, 84] (Fig. 4). Even though there is a dearth of information about the level of expression either due to the lack of reports or due to the usage of measures which are difficult to compare (like TSP, µg/L, mg/kg and mg/Hector), the recent reports with standard measurements show that the model plants express proteins in good quantity [85]. In tobacco plants, exploitation of the

Table 1 Transgenic plants expressing scFv/scFv varients

S. nos	Model plant	Antigen	Antibody format	Transformation method	Expressed in	Protein yield	References
1	*Arabidopsis thaliana*	Hepatitis A (HA78) and HIV (2G12)	scFv-Fc	Agrobacterium	Seeds, leaves	0.8–9.4 mg/g dry wt	[112]
2	*A. thaliana*	Maltose Binding Protein (MBP 10), Hepatitis A (HA 78, HA 16) and Hantaan virus nucleocapsid protein (EHF 34)	scFv-Fc	Agrobacterium	Seed	19–28 µg/mg seed	[49]
3	*A. thaliana*	Fungal mycotoxin Zearalenone	scFv	Agrobacterium	Leaves	–	[70]
4	*A. thaliana*	Human creatine kinase-MM (CK-MM)	scFv	Agrobacterium	Leaves	0.01% TSP	[29]
5	*A. thaliana*	human epidermal growth factor receptor 2 (HER2)	scFv-Fc	Agrobacterium	Seeds	1.1% TSP	[30]
6	*A. thaliana*	Herbicide Chloropropharm	scFv	Agrobacterium	Whole plant	–	[66]
7	*A. thaliana*	Gibberlin	scFv	Agrobacterium	Whole plant	–	[69]
8	*A. thaliana*	B-lymphocyte antigen CD20	scFv-Fc	Agrobacterium	Seeds	6.12% TSP	[81]
9	*A. thaliana*	Tumor necrosis factor (TNF)-α	scFv-Fc	Agrobacterium	Seeds	0.27–0.46 mg/g seed	[79]
10	*Chrysanthemum*	PVX	scFv	Agrobacterium	Leaves	–	[152]
11	*Hordeum vulgare*	β-Lactoglobulin (BLG)	scFv	Agrobacterium	Seeds	55 mg/kg grain	[80]
12	*Kalanchoe pinnata*	PVX	scFv	Agrobacterium	Leaves	–	[153]
13	*Nicotiana benthamiana*	Mouse B cell lymphoma, 38C13	scFv	Virus	Leaves	–	[65]
14	*N. benthamiana*	tomato spotted wilt tospovirus (TSWV)	scFv	Agrobacterium	Leaves	–	[32]
15	*N. benthamiana*	Glycoprotein G1 of Tomato spotted wilt virus	scFv	Agrobacterium	Whole plant	–	[68]
16	*N. benthamiana*	Cucumber mosaic virus (CMV)	scFv	Agrobacterium	Leaves	–	[154]
17	*N. benthamiana*	HER2	scFv	Agrobacterium	Leaves	–	[155]
18	*N. benthamiana*	Tomato yellow leaf curl virus (TYLCV)	scFv	Agrobacterium	Leaves	–	[156]
19	*N. benthamiana*	Beet necrotic yellow vein virus (BNYVV)	scFv	Agrobacterium	Leaves	–	[157]
20	*N. benthamiana*	RNA-dependent RNA polymerase (RdRp)	scFv	Agrobacterium	Leaves	–	[158]
21	*N. benthamiana*	Anti-rabis	scFv	Agrobacterium	Leaves	–	[132]
22	*N. benthamiana*	CD20	scFv-Fc	Agrobacterium	Leaves	28 mg/kg	[127]
23	*N. benthamiana*	CD20	scFv-Fc	Agrobacterium	Hairy root	16 mg/L	[18]

Table 1 (continued)

S. nos	Model plant	Antigen	Antibody format	Transformation method	Expressed in	Protein yield	References
24	*N. benthamiana*	Metalloproteinase BaP1	scFv	Agrobacterium	Suspension culture	71.75 mg/L	[84]
25	*N. benthamiana*	Anti-CD20 2B8 (glyco-engineered)	scFv-Fc	Agrobacterium	Leaves	20–35 mg/kg	[75]
26	*N. benthamiana*	Omp D	scFv scFv-Fc	Agrobacterium	Leaves	45–82 µg/g	[93]
27	*N. benthamiana*	West Nile Virus (WNV)	scFv-C_H	Agrobacterium	Leaves	–	[56]
28	*N. benthamiana*	WNV	scFv-C_H (tetravalent)	Agrobacterium	Leaves	0.8 mg/g	[159]
29	*N. clevelandii*	Porcine coronavirus transmissible gastroenteritis virus (TGEV)	scFv	Agrobacterium	Leaves	2% TSP	[145]
30	*N. tabacum*	Oxazolone (Ox), kresoxim-methyl (Kres)	scFv	Agrobacterium	Seeds	0.5% TSP	[77]
31	*N. tabacum*	Fungal cutinase	scFv-SK, scFv-CK	Poly Ethylene Glycol	Leaves		[67]
32	*N. tabacum*	Abscisic acid (ABA)	scFv	Agrobacterium	Leaves	0.05–4.8% TSP	[160]
33	*N. tabacum*	Paraquate, Atrazine	scFv	Agrobacterium	Leaves	0.014% TSP	[161]
34	*N. tabacum*	Human RBC	scFv	Agrobacterium	Leaves	2.9 to 3.28% TSP	[72]
35	*N. tabacum*	Cutinase of *Botrytis cinerea*	scFv-SK scFv-CK	Agrobacterium PEG	Leaves	0.01–1%	[162]
36	*N. tabacum*	Hapten oxazolone	scFv	Agrobacterium	Leaves	0.67% TSP	[163]
37	*N. tabacum*	Phytochrome	scFv	Agrobacterium	Leaves	–	[164]
38	*N. tabacum*	Hepatitis B surface antigen	scFv	Agrobacterium	Leaves	0.031–0.22% TSP	[111]
39	*N. tabacum*	Tomato spotted wilt virus (TSWV) movement protein (NSM)	scFv	Agrobacterium	Leaves	5.9–8% TSP	[73]
40	*N. tabacum*	Tobacco mosaic virus (TMV)	scFv	Agrobacterium	Leaves	–	[103]
41	*N. tabacum*	TMV	scFv	Agrobacterium	Leaves	8.5 µg/g tissue	[165]
42	*N. tabacum*	Human tumor-associated antigen tenascin-C	scFv	Agrobacterium	Whole plant	–	[68]
43	*N. tabacum*	Picloram	scFv	Agrobacterium	Whole plant	–	[166]
44	*N. tabacum*	*Salmonella enterica* LPS	scFv	Agrobacterium	Leaves	41.7 µg/g tissue	[167]
45	*N. tabacum*	Botulinum neurotoxin A (BoNT/A)	scFv	Agrobacterium	Whole plant	2 kg/Hector	[168]
46	*N. tabacum*	Human epidermal growth factor receptor 1 (HER1) and HER 2	scFv (bispecific)	Agrobacterium	Leaves	–	[9]
47	*N. tabacum*	Double stranded RNA	scFv	Agrobacterium	Leaves	–	[169]

Table 1 (continued)

S. nos	Model plant	Antigen	Antibody format	Transformation method	Expressed in	Protein yield	References
48	*N. tabacum*	PVX	scFv	Agrobacterium	Leaves	–	[170]
49	*N. tabacum*	Tomato spotted wilt virus	scFv	Agrobacterium	Leaves	–	[171]
50	*N. tabacum*	T84.66 (From mouse human chimeric IgG1)	scFv	Agrobacterium	Leaves	15 µg/ml	[102]
51	*N. tabacum*	T84.66 (ScFv diabody)	scFv	Agrobacterium	Leaves	–	[44]
52	*N. tabacum*	*A. thaliana* CDK	scFv	Agrobacterium	Leaves	1.2–1.8%TSP	[90]
53	*N. tabacum*	Stolbur phytoplasma (mollicute)	scFv	Agrobacterium	Leaves		[115]
54	*N. tabacum*	TMV (virion, coat protein)	scFv (bispecific)	Agrobacterium	Suspension culture	0.0064–1.65 TSP	[8]
55	*N. tabacum* (*BY2 cells*)	Auxin Binding Protein (ABP1)	scFv	Agrobacterium	Cells	–	[83]
56	*N. tabacum*, *Pisum sativum*	Eimeria (oocysts, sporocysts and sporozoites)	scFv	Agrobacterium	Leaves, seeds	1.6–1.9 mg/g dry seed	[78]
57	*N. tabacum*	Human chronic gonadotropin HCG (β-subunit)	scFv	Agrobacterium	Leaves	20–40 mg/kg fresh leaf	[71]
58	*N. benthamiana*	β-1, 3-Glucan	scFv-Fc, VH + hHC, VL + hLC	Agrobacterium	Leaves	40–60 mg/kg plant tissue	[92]
59	*Oryza* sps	*E. coli*, K-99	scFv	Agrobacterium	Leaves	–	[172]
60	*Petunia hybrida*	Petunia dihydroflavonol 4-reductase (DFR)	scFv	Agrobacterium	Leaves, flower	0.3–1% TSP	[76]
61	*Pisum sativum*	Abscisic acid	scFv	Agrobacterium	Seeds	–	[106]
62	*Solanum* sps	Potato virus X (PVX)	scFv	Agrobacterium	Leaves	–	[74]
63	*Solanum tuberosum*	Fungal cutinase	scFv-CK, scFv-SK	Agrobacterium	Leaves	0.02–0.1% TSP	[173]

Col-0 plant-specific glycosylation knockout mutant TKO, *scFv* single chain variable fragment, *VH* variable heavy chain, *VL* variable light chain, *hHC* human heavy chain, *hLC* human light chain, *scFv-SK* scFv with an Endoplasmic reticulum targeting signal, *scFv-CK* scFv directed to cytoplasm, *CK-MM* creatine kinase with two subunits of the muscle type, *RBC* red blood cells, *LPS* lipopolysaccharide, *CDK* cyclin dependent kinase, *CD 20* B-Lymphocyte antigen CD20 (Cluster of Differentiation), *BaP1 Bothrops asper* proteinase 1, *biscFv* bispecific scFv, *OmpD* outer membrane protein D, C_H heavy chain constant domain

hairy root formation for protein expression and rhizosecretion opens the possibilities of reducing the downstream processing cost of recombinant protein production. Developing specialized cell types like BY-2 or identifying the plants which have high multiplication potential can positively influence scFv production using plants. Another reason for using the model plants is the availability of genome engineered plants to support post-translational modification compatible with the mammalian expressed proteins [75]. Use of non-conventional plant groups such as algae and bryophytes, which were shown to be good protein expression platforms [58] can be an option for scFv expression.

In plants, the heterologous expression of a gene can be transient or stable. In a stably transformed plant, the transgene is integrated into the genome and will pass on to the successive generations. The resultant product of the transgene continues to present throughout the plant body or in specific tissues (targeted expression). In transient expression, the transgene expresses the protein without a proper integration in to the genome and the transgene will not be transferred to the next generation. Vectors specifically designed for the transient expression of various target genes have been established [8, 86–88]. Agro-infiltration method was used commonly to establish transient transgenics. While most of the reports on scFv expression in plants deal with

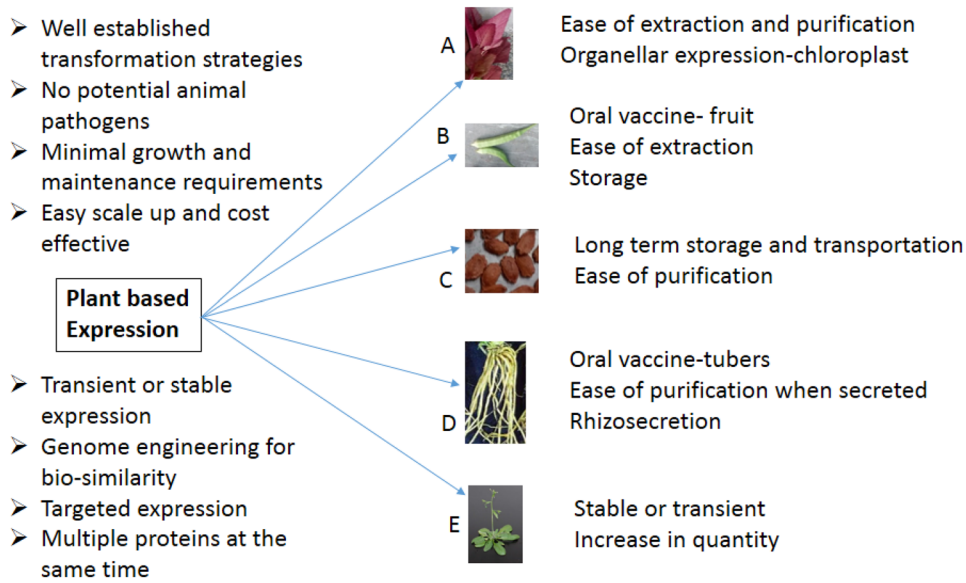

Fig. 2 Advantages of plant expression systems. Protein expression in leaves (**a**), fruit (**b**), seed (**c**), roots (**d**) and whole plant (**e**)

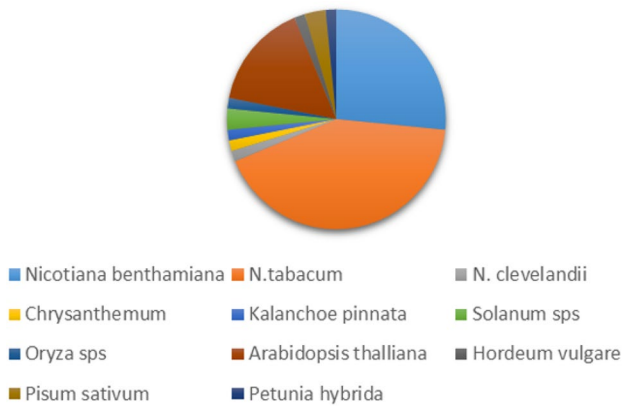

Fig. 3 Plants used for scFv expression

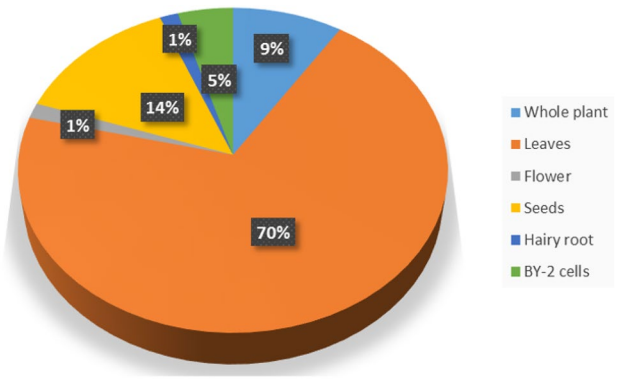

Fig. 4 Tissue specific expression of scFv in plants

the generation of stable transgenics [29, 76, 89] there are few successful reports on the expression of biologically active scFv using the transient expression systems [65, 71, 90–92]. It was reported that compared to scFv-Fc format, the scFv format express better (a twofold increase) in *N. benthamiana* [93]. *Nicotiana benthamiana* hairy root culture was used successfully to express a glyco-engineered scFv antibody (CD20 2B8-Fc) based on the mAb Rituximab. Addition of the plant growth regulator 2,4-D was found to be enhancing the secretion of recombinant antibodies about 28-fold to the culture vessels. The purified CD20 2B8-Fc effectively bound to the CD20 antigen on the surface of human Daudi cells [18]. Transient expression is favored when the protein is needed within a short span of time in a comparatively large quantity [53, 93, 94] and for the expression of proteins which have an adverse effect on the host cells when over-expressed [95].

Promoters, Signal Peptides, and Targeted Expression

The use of different promoters in recombinant protein expression systems, in general, has been reviewed extensively [96–99]. The promoters used in heterologous expression systems vary significantly in their nature, activity, location, and mode of action. Studies revealed that in plants, the signal peptides and promoters are available to express, guide and store the protein in almost all cellular and extracellular targets [99]. Among the different types of promoters, the constitutive [100], and target specific [49, 50, 79, 101] promoters were used widely for scFv expression. The constitutive promoters CaMV35S [44, 78, 102, 103], Ubiquitin [51], and promoters of the seed storage proteins, globulins, glutelins, albumins, prolamins, and few other proteins like Sucrose binding protein [104], Unknown Seed Protein [77, 105, 106], and oleosin [107, 108] are used with or without

the signal peptides for targeted expression and storage of heterologous proteins in transgenic plants. Yamamoto et al. reported the combination of a geminivirus replication sequence along with a double terminator in the transient protein expression vectors resulted in a twofold increase in the protein level compared to the transient expression using single terminator [109].

One of the major disadvantages, when plants are used as an expression platform, is the plant-specific secondary modifications added on to the proteins, which may make them immunogenic and hence useless in many applications. Addition of plant-specific *N*-glycans takes place in the endoplasmic reticulum and in the Golgi apparatus. Preventing the protein from reaching these targets keeps the protein away from any modifications. It can be done mainly using two methods; (1) by using the glycosylation mutants developed in model plants such as *Arabidopsis* and *Nicotiana* to facilitate similar glycosylation as that of mammalian system [55, 89, 110], and (2) engineer the construct by incorporating a signal peptide which either retain the expressed protein in the ER [49] or target the protein to the external medium, apoplastic space or to a tissue. The KDEL signal sequence is used extensively in plant recombinant scFv expression to retain the protein in the ER lumen [49, 111–113]. ER retention in many occasions is found to be beneficiary as the protein expressed show higher stability and concentration in the ER [113]. The 2S2 signal peptide derived from *Arabidopsis* seed storage protein is commonly used in plants to target the scFv to seeds [29, 51, 79, 112, 114]. There are reports on the use of signal peptides like pectate lyase SP [115] and murine signal peptide MSP [51, 102] with the plant expression systems for the secretion of scFv expressed.

While the transient expression is performed mostly with leaves, stable transgenics direct the protein expression to specific tissues. Expression of recombinant protein in seeds is considered as a suitable strategy for scFv expression. The scFv targeted to oil bodies in seeds was purified with less difficulty [108]. The same study reported that in the seeds, the shelf life of scFv was also increased. The seed-specific promoters, *Phaseolus vulgaris* β-phaseolin [30, 49, 50, 79, 101], legumin B4 [77, 116] unknown seed protein [77] and oleosin [108] were used to target scFv to the seeds in model plants. Zakharov and co-workers studied the seed directed expression of reporter genes using four seed-specific promoters in different transgenic plants including tobacco, pea, narbon bean, and linseed. The study proposes to engineer the promoter to impart more specificity for either pollen or seed to refute the ambiguity in non-specific expression [117]. In another study, the scfv targeting the surface epitopes of the sheep gut nematode *Trichostrongylus colubriformis* was fused to a trimeric polyoleosin and expressed in *Arabidopsis* seeds using the seed-specific oleosin promoter. The fusion product was accumulated in the oil bodies to a range of nearly 0.9% of total seed protein, showed a similar expression profile as that of oleosin [108]. Gomes and co-workers used a SUMO (Small Ubiquitin-like Modifier) fusion protein with a scFv against the metalloproteinase BaP1 (*Bothrops asper* P1) increased the protein expression in *N. benthamiana* [84]. The advantages of targeting the protein expression to seeds along with the technical details on expression strategies and the issues to be considered when the product is having a pharmaceutical application have been explained well in one of the pioneering reviews on the seed-specific expression of recombinant proteins [118].

Negative Effects on the Host System, Transgene Stability and Product Inconsistency

The expression of certain recombinant proteins in a heterologous system may cause minor to severe side effects to the host. This topic was studied well in the prokaryotic expression system compared to mammalian or plant expression systems. In prokaryotes, especially with the induced expression of recombinant proteins, the host cells are affected due to; (a) target protein [119, 120], (b) inducer [121], or c) the toxicity of expressed protein [31, 122]. There are few studies at the transcriptome level in plants that tried to relate the effect of transgene expression with the physiological and physical status of the transgenic plants and found that there are no major differences observed in transgenic plants compared to the non-transgenic control under the conditions used [123–125]. De Wilde and co-workers studied the effect of recombinant antibody expression in the transcriptome and proteome of *A. thaliana* seeds collected 13-day post-anthesis. The study revealed that there was an upregulation of genes corresponding to protein folding modification, translocation, vesicle transport, and protein degradation. It was observed that the production of a recombinant antibody in the ER triggers ER stress, causing disturbance of normal cellular homeostasis [95].

Compared to CHO (Chinese Hamster Ovary) expression system, it is difficult to predict the expression efficiency of proteins in model plant systems. For example, the quantity of protein (mg/kg fresh tissue) expressed in *N. benthamiana* leaves vary from 28 mg/kg (anti-CD20 scFv, [126] to 45–85 mg/kg (anti-OmpD scFv [93]. The 2.5-fold change observed with the same model system for two different target proteins can be due to multiple factors involving the codon usage, mRNA stability, and proteolysis that are reported in plant expression systems [127, 128]. The structure and specific characters of the protein play a crucial role in its stability within the host and proteolysis can be a mechanism adopted by the host to maintain its functional integrity. Various methods like protease inhibitors, supplementing the suspension culture medium with stabilizing

agents, knocking down the protease encoding genes, subcellular targeting of recombinant proteins and the use of fusion proteins are the major strategies to reduce proteolysis [129]. In another report, when two formats of the scFv (scFv and scFv-Fc) against the OmpD protein of *Salmonella typhimurium* expressed in *N. benthamiana*, the scFv version showed a greater expression (85.5 µg/g) than the scFv-Fc (45.9 µg/g). More detailed studies are necessary to prove the structure/function-based target protein cleavage in the expression host.

Unlike the prokaryotic system, expression of the transgene in the successive generations is a matter of concern in higher organisms like animals and plants. The factors controlling the expression of transgenes in plants include its copy number, place of integration, physiological status of the target tissue, nature of the protein, sorting signals, and subcellular localization [79, 130, 131]. There are only a few reports which studied the expression of antibody/antibody fragments in the transgenic progenies for three or four generations. In one of these studies, the protein (anti-TNF alpha scFv) concentrations were 0.270 µg/g, 0.37 µg/g, and 0.46 µg/g for the 5-day, 10-day and 20-day old seeds, respectively in the T2 generation. The concentration of the protein in the T3 generation was more (0.501 ± 0.0785 mg/g), especially in the homozygous genotypes [79]. While the recent strategies are targeted for transient expression, the tissue-based approach may be adopted only to those proteins which need special care.

Inconsistency in biological efficacy is one of the major problems of biosimilar proteins expressed in plants. The Mak-33 scFv encoding gene was expressed in tobacco as cytoplasmic and ER-targeted protein. Measurement of the affinity of the scFv with the antigen indicated an 80% reduction compared to parental Fab [29]. The reducing environment of cytosol considered as non-favorable for the disulfide bond formation in the proteins. While the transient expression of scFv in tobacco protoplast resulted in the absence of a disulfide bond and reduced activity, the scFv expressed in the cytosol of stably transformed tobacco plants was with disulfide bonds and showed excellent biological activity [67]. In one of the recent study published by Phoolcharoen et al. on the transient expression of a scFv against the rabies virus alone or in conjugation with a central nervous system-specific peptide, they showed that mice infected with the virus when treated with the scFv and scFv-peptide, both the molecules could cross the blood–brain barrier and the scFv-peptide specifically bound to the target confirming the bioavailability of the scFv [132, 133].

Glycoengineering for the Mammalian Type of Glycosylation

The allergic reactions to glycoproteins lead to the production of IgE antibodies in human beings. There were many studies conducted in the past to link the presence of IgE antibodies in patients and use that information for the diagnosis or treatment of allergic diseases. Surprisingly, none of these studies indicated a clear correlation between the presence of IgE in blood and its clinical effects [134, 135]. Immunogenicity induced by plant-specific glycans is one of the main objections against plant-based production of biotherapeutics. Contrary to this argument, experimental evidence showed that the plant-specific glycans do not interfere with the immune system and at times it is rather beneficial [56, 136, 137]. The N-glycosylation pattern of plant glycoproteins is different from the animals. Plant-derived recombinant proteins with N-linked glycosylation have a terminal β 1, 2-xylose and core α 1, 3-fucose. β 1, 4-galactose and sialic acid residues are absent in plants [138, 139]. O-linked glycosylation is also different in both plants and animals [140]. Taliglucerase alfa (TGA) expressed in carrot cells is an acid beta-glucosidase used in the treatment of Gaucher disease. Rup and co-workers studied the immunogenic effects of TGA over one year among adult and pediatric patients. The results indicated that 8/63 patients developed anti-plant-glycan antibodies. The detailed study of various disease parameters showed that the treatment was as efficient as of any other enzyme replacement therapies used, and the safety indicators were well within limits [17]. This observation substantiated the results obtained by Landry and co-workers on the use of a plant-based H5N1 vaccine. According to the results, only 14.8% of the patients that receive the vaccine against avian H5N1 influenza developed serum antibodies against plant-specific sugars [94]. Positive effects of humanized glycosylation in plant-derived antibody are reported in the case of ZMapp. ZMapp is a cocktail of three mAbs expressed in glyco-engineered *N. benthamiana* used for the treatment of the Ebola virus [141]. The medicine was approved by the FDA for usage during the 2014 Ebola outbreak in West Africa. Phase II clinical trials in four countries have been completed for ZMapp in 2015 [142]. Other examples are the synthesis of idiotype-specific vaccines for non-Hodgkin's lymphoma [143] and follicular lymphoma [144].

To synthesize mammalian biosimilar proteins glycosylation machinery of the host plant need to be manipulated. Glycosylation mutants developed in *A. thaliana* and other model plants for protein expression had similar glycosylation as that of mammalian cells [16, 18, 75]. Even though there were minor differences in the glycosylation, scFv expressed in *A. thaliana* showed biological effects similar to the parental antibody [56, 112]. In a phase-I clinical trial to assess the safety and immunogenicity of recombinant idiotype-specific scFvs developed against non-Hodgkins lymphoma in *Nicotiana*, McCormick and co-workers observed that the vaccine has induced an idiotype-specific immune response [144]. Monger and co-workers studied the effect of vaccinating piglets against gastroenteritis virus infection with different concentrations of scFv with or without an adjuvant. Both low

and high doses of vaccine with or without an adjuvant did not make any significant adverse effects. They also reported that the difference in glycosylation did not impart immunogenicity [145]. Underglycosylation of the native proteins along with aberrant glycosylation of scFv was noticed when an anti-MBP 10 scFv was expressed in the glycosylation mutant (alg 3-2) of *A. thaliana* [89]. In another study, anti-HA 78 and anti-2G12 (Loose et al. 2011) and MBP-10, HA 78, HA16 and EHF 34 [49] were also had the altered glycosylation pattern on the scFv. Aberrant deposition of ER-specific chaperons was reported when the protein was targeted to the ER [49]. The anti-CD20 scFv-Fc expressed in *N. benthamiana* glyco-engineered for xylose/fructose N-glycosylation were devoid of plant-specific N-glycosylation. The biological activity studies with purified scFv-Fc exhibited an improved binding affinity compared to the non-engineered form of scFv and rituximab. Cytotoxicity induced through the antibody-dependent cell-mediated pathway and the complementary dependent pathway was strongly enhanced [18, 126]. The data indicate that the major factor which prevents the use of plant system is the cost associated with the downstream purification of the recombinant protein rather than the post-translational changes associated with plant expression. Developing an expression system that may secrete the protein to the medium makes the purification process easy, and will resolve this issue.

Making the Plant System More Industrial Friendly

Validation of the cost factor had been done with several recombinant proteins including mAbs to evaluate the feasibility to develop the process for commercial purposes. With the ever-increasing demand for antibody fragments in the commercial market, there will be a continuous search for more economically viable platforms for its production. In this regard, it is worthwhile to look into the plant expression system to establish a cost-effective production platform and search for the points of concern to rectify it. While the seed-based propagation of plants is time-consuming, transient expression of proteins in plants can be attained in a few days. In *N benthamiana*, the transient expression system generated the recombinant antibody against the influenza virus in a time-bound manner. The agro-infiltration method took less than a week including the time needed for downstream processing to get the purified antibody [94]. It indicates that once the transformation and purification methods are established with a production platform, the recombinant protein can be made at will whenever it is needed. This technology will have great significance during conditions like the sudden surge of seasonal epidemics such as H1N1 or Ebola.

The argument on the cost factor was the focus of many research papers published recently [85, 146–148]. Unlike the uniformity that exists among the microbial or mammalian expression systems in terms of its growth requirements, amount of protein expression, and the strategies adopted for purification, plant expression systems exhibit significant differences among themselves. Plants offer an option to select the site of expression (leaves, whole plant, seeds) type of system (hairy root, plant, suspension culture) and the purification methods. Therefore the studies failed to derive any conclusions in general as the expression systems they have looked in to were different in many aspects. Schillberg and co-workers observed that plant systems have a relatively low yield, inconsistent productivity, and inefficient large-scale downstream processing. In the tobacco chloroplast, GFP was expressed at a concentration of 4 mg/g FW and Cry2Aa2 at 5 ng/g FW. Tobacco BY2 cell-based expression produced GFP at around 270 µg/ml. The study estimated that the production cost of 1 g of human M12 antibody in *Nicotiana* was around 1137 EUROs. The results also have shown that 84% of the total process cost was on the downstream processes and the cultivation account for only 16% [85]. When the same antibody purified from BY-2 cells, 77% of the total process cost was on the downstream processing. They also observed that the production rate of BY-2 cells was 20-fold lower than the whole tobacco plants. Another study estimated that the production cost of an antibody using the plant expression platform is about 100 EURO/g [149]. According to some previous reports, in CHO cells, the production cost is around 180 EURO/g for an antibody [148] and sometimes it can be as low as 22 EURO/g [150]. According to Lim et al., the production cost for 1 g of a typical glycosylated protein using different expression systems such as *Pichia pastoris*, mammalian cells, *Escherichia coli*, and *Pseudomonas fluorescens* is around 148, 206, 588, and 129 USD, respectively [148]. Sugar profiles attached to proteins can influence the activity. Due to the less complex machinery than the mammalian cells, plants are more amenable for specific glycan modification.

Tuse and co-workers used SuperPro Designer modeling software to predict the techno-economic advantage of the plant expression system using human butyrylcholinesterase and cellulose, two enzymes with diverse origin and application. The study proposed significant cost advantages with the plant expression compared to the conventional production systems for the above-said candidate molecules [146]. Buyel reviewed the additional streams which can be incorporated with the plant production platforms to increase its commercial potential. Other than the target proteins, the biomass can further be exploited to improve the revenue. The side stream of plant-based processes can double the revenue depending on the additional products developed in an integrated approach [147]. Plants are again endowed with additional

features like rhizosecretion, which has not yet been exploited to its full potential [151]. The secret is in the selection of correct expression platform which is fast-growing, easy to manipulate genetically, needs less maintenance cost and have the provision to reduce downstream processing costs.

Conclusions and Future prospectus

As the mAb expression is more complex, it may be possible to look for antibody fragments, which offer similar activity as that of the mAb. Heterologous expression of antibody fragments in plants can be an effective and low-cost strategy for its large-scale pharmaceutical production. The major concerns in this system like the development of stable transgenics, expression of the protein, purification of the protein, structural and functional similarity with the parental antibody, and scaling up of the production platform been studied in detail and measures to tackle issues related to each of these topics were developed. The added advantages like the absence of any animal pathogens, targeted expression in organelles/storage organs, long-term storage of expressed protein in the target tissue, feasibility for oral consumption, facilitated purification as in the case of oil body coupled expression, and development of genome-edited expression platforms make plant-made recombinant proteins more lucrative. With the high demand for therapeutic antibodies in human and veterinary therapeutics, the magnitude of production needs to be enhanced. Combining the advantages of the plant expression system along with the favorable features of antibody fragments over mAbs may pave the path to the rapid biosynthesis of antibody-based diagnosis/therapeutics, which will increase the availability of the product and may lead to making the usage affordable.

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Compliance with Ethical Standards

Conflict of interest The author declares no conflict of interest.

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