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Research review paper

Plants as bioreactors: Recent developments and emerging opportunities

Arun K. Sharma *, Manoj K. Sharma

Department of Plant Molecular Biology, University of Delhi South Campus, New Delhi 110021, India

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ABSTRACT

In recent years, the use of plants as bioreactors has emerged as an exciting area of research and significant advances have created new opportunities. The driving forces behind the rapid growth of plant bioreactors include low production cost, product safety and easy scale up. As the yield and concentration of a product is crucial for commercial viability, several strategies have been developed to boost up protein expression in transgenic plants. Augmenting tissue-specific transcription, elevating transcript stability, tissue-specific targeting, translation optimization and sub-cellular accumulation are some of the strategies employed. Various kinds of products that are currently being produced in plants include vaccine antigens, medical diagnostics proteins, industrial and pharmaceutical proteins, nutritional supplements like minerals, vitamins, carbohydrates and biopolymers. A large number of plant-derived recombinant proteins have reached advanced clinical trials. A few of these products have already been introduced in the market.

Contents

Abbreviations: αAmy3, Rice alpha amylase; *Act1*, *Actin 1* gene; hr-ALS, Herbicide resistance acetolactate synthase; aps, Amplification promoting sequence; BY2, Bright Yellow 2; CVB, Center for Veterinary Biologics; ER, Endoplasmic reticulum; ESP, Endosperm specificity palindrome; GalT, β(1,4)-Galactosyltranferase; hGC, Human glucocerebrosidase; hGM-CSF, Human granulocyte-macrophage colony stimulating factor; HIS-RTB, Hexahistidine tagged ricin B; HLF, Human lactoferrin; HR, Homologous recombination; hr-ALS, Herbicide resistance acetolactate synthase; hST, Human somatotropin; IgG, Immunoglobulin G; *mas*, *Mannopine synthase* gene; *ocs*, *Octopine synthase* gene; ORF, Open reading frame; PHA, Polyhydroxyalkanoate; PHB, Polyhydroxybutyrate; PI, Plasma iron; Prrn, 16S ribosomal RNA promoter; RAST, Radio-allergo-sorbent test; RB-MAR, RB7 matrix attachment region; REB, Rice endosperm bZIP; scFv, Single chain Fv fragments; TA, Tail anchored; TAP, Tandem Affinity Purification; TSP, Total soluble protein; *Ubi*, *Ubiquitin* gene.

* Corresponding author. Tel.: +91 11 24110544; fax: +91 11 24119430.
E-mail address: arun@genomeindia.org (A.K. Sharma).

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1. Introduction

Plants have been serving as an extremely valuable source for treatments and therapies, since our existence. Thousands of plant species are used for medicines and most of the world population uses them to cure acute and chronic health problems. After a shift from wild collection to cultivation, advances in plant biotechnology were aimed at direct improvement and modification of specialized constituents of plants like carbohydrates, proteins, oils, fats and vitamins, over a long time period. However, the use of transgenic plants as "BIOREACTORS" is relatively new bioscience and is gaining momentum. Fig. 1 illustrates various areas of development where plant bioreactors have made significant advancement in recent times. It involves the genetic modification of the host plant through the insertion and expression of new genes. The products that are currently being produced in plants include bioactive peptides, vaccine antigens, antibodies, diagnostic proteins, nutritional supplements, enzymes and biodegradable plastics. The economic driver that is burgeoning this industry includes the ability to synthesize animal proteins, cost benefits, safety issues (lack of pathogen contamination) and easy scale up (Fischer et al., 2004; Lal et al., 2007; Rybicki, 2009; Sharma et al., 2004; Spok, 2007; Stoger et al., 2002; Twyman et al., 2003). Apart from many advantages, there are some problems associated with plants for their use as bioreactors which include differences in glycosylation patterns in plants and humans, inefficient expression and environmental contamination. The primary objective of using plants as bioreactors is to produce the desired proteins/products of therapeutic or industrial importance using plant biotechnology. The constitutional steps involved in the whole process of production of recombinant proteins from plants include: (i) choice of the host species and optimization of coding sequence of the target gene in relation to the host, (ii) selection of expression cassette and creation of the expression vector, (iii) integration of the gene construct into the plant genome and regeneration of plants expressing the desired protein, (iv) identification and stabilization of the plant line for commercial production, and (v) purification and characterization of the recombinant protein. These aspects along with outcome of concerted research efforts in this area as well as limitations in product development and commercialization form the subject matter of this review.

2. Selection of host plant

For the efficient production of recombinant product, selection of the host species is very important. Though earlier, the model system tobacco, which is easy to transform and manipulate, was the system of choice for production of most of the plant-derived recombinant proteins, today a large number of plant species are being used for this purpose including tomato, banana, rice, maize, wheat, carrot, soybean, pea, potato, lettuce and alfalfa. A wide variety of products can be produced in plants, but each one has its own requirement for its production. Therefore, no single species can be ideal for the production of all these products. The choice of the host species is associated with the type of protein in question i.e. the form of the recombinant protein which is to be finally used. The life cycle of the host, biomass yield, containment and scale-up costs are other deciding factors. The performance of any host depends upon many biological as

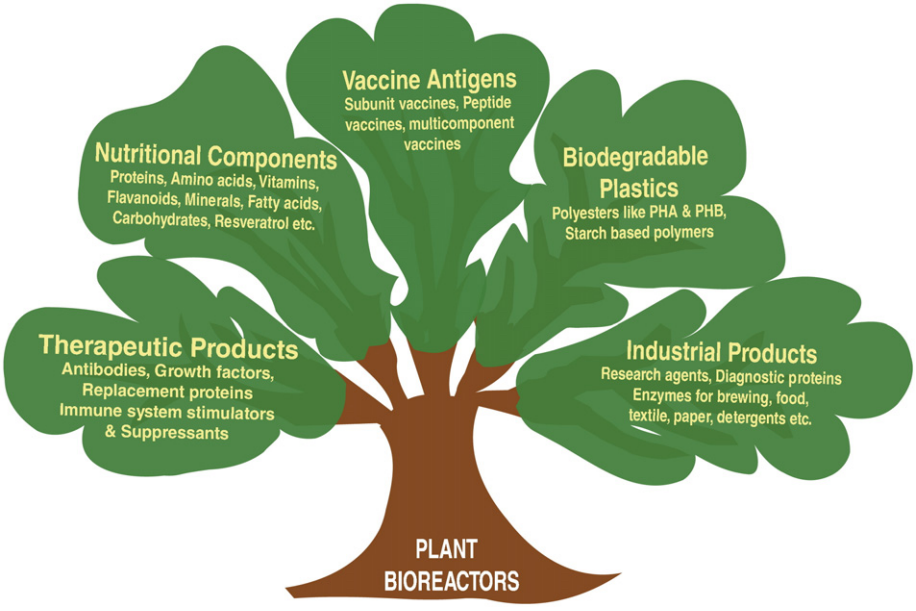

Fig. 1. Tree depicting biotechnological advances using plants as bioreactors.

well as geographical factors and needs to be evaluated on case by case basis. Therefore, success largely depends upon the understanding of species- or tissue-specific factors that affect the recombinant product accumulation and its quality. With the technological developments, a large number of plant species have attracted the attention of scientific community. Generally domesticated species are preferred over the wild species for molecular farming as they are adapted to wide range of environmental conditions because of their commercialization. But wild species could be more advantageous as they will not be mistaken for the food crops and would address the issue of food crop mix-up with transgenic material. The self-pollinating species are advantageous for the containment of the transgenic crops as compared to cross-pollinating crops as chances of spread of transgene through pollen would be minimized. The issue can also be addressed by using plants which can be grown in containment e.g. tomato which can be grown in green houses. Further, the use of plant cell cultures addresses the issue of containment where dedifferentiated cells such as in calli or cell suspensions are used and can be grown on industrial scale using fermenters (Shih and Doran, 2009). For the proteins which need to be purified, the downstream processing may affect the overall production cost to a large extent. Therefore, the host from which the extraction and purification of the recombinant protein is easy and economic, is chosen.

Besides tobacco, many other leafy crops used as bioreactors include lettuce, alfalfa and clover. The leading advantages of alfalfa are that it is a perennial plant, fixes its own nitrogen and the glycoproteins synthesized in leaves tend to have homogeneous glycan structures (Fischer et al., 2004). One major disadvantage associated with the leafy crops is their limited shelf life and immediate need of processing. The harvested material does not have any flexibility for extraction times. In contrast, proteins expressed in cereal grains are protected from proteolytic digestion and may remain stable for longer periods at room temperature without significant loss of the activity. Several cereals including rice, wheat, barley and maize have been investigated (Ramessar et al., 2008a). Moreover, the first plant-derived commercialized product was produced in maize (Hood et al., 1997; Witcher et al., 1998). Cereal plants have been adopted as a production platform by the plant biotechnology enterprises like Ventria Bioscience (http://www.ventria.com/).

On the other hand, oil crops offer unique production advantages of inexpensive downstream processing to obtain the desired proteins if they are targeted to the oil bodies. Oleosin, a plant protein, is present on the oil-body surface. The hydrophobic central core part of the oleosin remains inserted into the oil body while the amphipathic and less conserved N- and C-terminals reside on the surface (Huang, 1992). The protein in question can be targeted to oil bodies as an oleosin fusion which can be later removed by centrifugation-based methods that separates oleosin-fused protein from most of the other contaminants (Parmenter et al., 1995). An example of oil crop being utilized for this purpose is the oleosin-fusion platform that has been developed by SemBioSys Genetics Inc. (http://www.sembiosys.com/), where the recombinant proteins are targeted to oil bodies of safflower seeds. According to disclosed information by the company, it is possible to recover more than 1 kg of purified protein from 1 ton of seeds. When proteins in question are recombinant vaccine antigens, food crops like tomato, potato, carrot or banana are preferred as these are palatable and can be eaten raw or with little processing. Further, it would be advantageous to use locally grown plants for the development of plant vaccines as it would save transportation costs.

The use of moss as a bioreactor is one of the major innovations in the manufacturing of biopharmaceuticals, which is cost effective and at the same time, avoids risks associated with environmental release of transgene. Therefore, it has been adopted as the production platform by Greenovation® (http://www.greenovation.com/). Their moss bioreactor is based on the fermentation of the moss protonema. Moss allows humanization of glycosylation patterns and also time taken to reach the market is comparable to traditional systems. A transient expression system allows feasibility studies within 10–12 weeks and stable production strain development takes 4–6 months. Cultivation in suspension allows up-scaling of the photobioreactors up to several 1000 L. Heterologous proteins are secreted into the medium. Downstream processing from this low salt medium involves fewer purification steps. Other aquatic plants and green algae (*Chlamydomonas*, *Wolffia*, *Spirodela*, *Chorella* etc.) can also be used for the production of recombinant proteins. They have high vegetative growth rate which shortens the production cycle. Further, these can be grown under containment. Aquatic plants and green algae can be an alternative to open field plants (Boehm et al., 2001; Franklin and Mayfield, 2005; Kim et al., 2002).

3. Optimization of expression level

For the development of plant-based production platform, one needs to optimize the expression level of a recombinant protein. Several factors are involved in controlling expression of a transgene at various levels which includes transcription, translation, post-translational modifications or storage of recombinant protein in the cell.

3.1. Transcription and translation

3.1.1. Promoters

To achieve high level of transcription, the strength and expression profile of the key regulatory element "promoter" which drives the transcription, play an important role. It contains the sequences which are required for RNA polymerase binding to start transcription and regulation of transcription. The understanding of the promoter components and factors associated with them has opened up an array of possibilities to modulate the expression of a gene in question. In general, promoters can be classified into several categories based on their activity.

3.1.1.1. Constitutive promoters. The promoters that induce the expression of genes irrespective of the environment or developmental factors are called as constitutive promoters. They are generally used for the production of recombinant proteins in all the tissues of a plant. The cauliflower mosaic virus *35S* promoter (Odell et al., 1985) has been used extensively for this purpose and high-level expression of recombinant proteins has been achieved (Gutierrez-Ortega et al., 2005). It is more effective in dicots than monocots probably because of the differences in quality/quantity of regulatory factors. CaMV*35S* promoter has been used to produce several antigenic proteins in plants including CTB, LTB, HBsAg, protective antigen, rabies virus glycoprotein G, SARS virus glycoprotein S (Aziz et al., 2005; Huang et al., 2001; Jani et al., 2002; Kang et al., 2004c; Li et al., 2006b; McGarvey et al., 1995; Satyavathi et al., 2003) and other products of therapeutic or industrial importance like monoclonal antibodies, spider silk, SMAP-29 peptide, streptavidin, avidin and adiponectin (Berberich et al., 2005; Drake et al., 2003; Hull et al., 2005a; Morassutti et al., 2002; Murray et al., 2002; Scheller et al., 2001; Zeitlin et al., 1998).

Ubiquitin promoter is another most commonly used constitutive promoter. It has been isolated from various plants including maize (Christensen et al., 1992), *Arabidopsis* (Callis et al., 1990; Norris et al., 1993), potato (Garbarino and Belknap, 1994), sunflower (Binet et al., 1991), tobacco (Genschik et al., 1994) and rice (Wang and Oard, 2003). Stoger and colleagues transformed rice with the gene encoding scFvT84.66 under the control of maize ubiquitin promoter or enhanced CaMV*35S* promoter. The expression levels of recombinant antibody were found to be comparable in the leaf tissue using either promoter. Using ubiquitin promoter, expression levels in the leaf tissue and seeds were comparable, but recombinant protein was not detected in seeds of transgenic plants when CaMV*35S* promoter was used (Stoger et al., 2000). Several molecules including CTB, LTB,

HBsAg, human interferon, avidin or aprotonin have been produced in plants using ubiquitin promoter (Chen et al., 2004; Hood et al., 1997; Kang et al., 2006b; Kang et al., 2006c; Kumar et al., 2005; Zhong et al., 1999). Recently, Hernandez-Garcia et al. (2009) have reported the characterization of a polyubiquitin promoter from soybean and showed that it is a strong constitutive promoter. Other constitutive promoters used for production of bio-molecules in transgenic plants include *manopine synthase* gene promoter (Arakawa et al., 2001; Kim et al., 2004a; Kim et al., 2004c; Kim et al., 2004d), tobacco cryptic constitutive promoter (Menassa et al., 2004), Mac promoter which is a hybrid of mannopine synthase promoter and cauliflower mosaic virus *35S* promoter enhancer region (Dai et al., 2000; Ziegelhoffer et al., 1999) and rice actin promoter (Huang et al., 2006). Various other constitutive promoters that are characterized and can be used in plants include banana actin promoter (Hermann et al., 2001), C1 promoter of cotton leaf curl Multan virus (Xie et al., 2003), cassava vein mosaic virus promoter (Verdaguer et al., 1996) and nopaline synthase promoter (Stefanov et al., 1991).

3.1.1.2. Tissue-specific promoters. These promoters control gene expression in tissue or developmental stage-specific manner. The transgene driven by such promoter will only be expressed in specific tissues leaving all other tissues unaffected. They are very helpful to concentrate the transgenic product in certain organs like seeds or fruits to limit any possible negative effect on plant growth and improve the harvesting efficiency. Several such promoters have been used for targeting the expression of foreign bio-molecule to specific organs of plants. The potato tuber-specific patatin promoter (Jefferson et al., 1990) has been used to target several molecules including CTB, LTB, HBsAg, dextran, mutan or alternansucrase to potato tubers (He et al., 2007; Joung et al., 2004; Kok-Jacon et al., 2005a; Kok-Jacon et al., 2005b, 2007; Lauterslager et al., 2001; Richter et al., 2000; Shulga et al., 2004). Fruits are preferred targets for the expression of vaccine antigens which can be used with little processing. A fruit-specific *E8* promoter was identified from tomato (Deikman et al., 1992) and used for the expression of various antigens in fruits (Jiang et al., 2007; Ramirez et al., 2007; Sandhu et al., 2000). Several promoters have been characterized which restrict the foreign bio-molecule expression in the seeds only (Lau and Sun, 2009). These includes arcelin promoter (Osborn et al., 1988), maize globulin-1 promoter (Belanger and Kriz, 1991), maize zein promoter (Marks et al., 1985; Russell and Fromm, 1997), 7S globulin promoter (Fogher, 2000), rice glutelin promoter (Wu et al., 1998) and soybean P-conglycinin a'-subunit promoter (Chen et al., 1986). Seed-specific promoters have been used to produce LTB, pro-insulin, laccase, human T-cell epitopes, VP2 protein of bursal disease virus, human placental acid beta-glucosidase, provitamin A, lectoferin and phytase (Aluru et al., 2008; Bailey et al., 2004; Brinch-Pedersen et al., 2006; Chikwamba et al., 2002; Farinas et al., 2005; Hood et al., 2003; Li et al., 1997; Reggi et al., 2005; Takagi et al., 2005). Arcelin promoter has been found to be capable of transcribing a heterologous DNA at high levels in plants. The arcelin 5-1 promoter of common bean was used to express ScFv which accumulated up to 36% of TSP (De Jaeger et al., 2002). Ventria Bioscience, a biotech company, is using seed-specific expression of foreign molecules as the expression technology and has produced lectoferin and lysozyme in the seeds of maize and rice (http://www.ventriabio.com/products/). A seed storage protein oleosin can be used as "carrier" for recombinant protein expression in seeds (Parmenter et al., 1995). Since these proteins are immiscible, it helps in the purification of recombinant protein fused with them. The core technology of SemBioSys Genetics Inc. (a biotech company) also utilizes plant seed oil-bodies and has been involved in the production of insulin, apolipoprotein AI, animal vaccines and nutritional oils (http://www.sembiosys.com/).

Chloroplast-specific promoters have also been utilized for targeting the foreign protein expression into chloroplasts. The 16S ribosomal RNA promoter (*Prrn*) or *psbA* gene promoters are commonly used for chloroplast transformation. Several molecules including CTB, LTB, protective antigen or insulin have been produced in chloroplasts using either *Prrn* or *psbA* promoter (Daniell et al., 2001a; Kang et al., 2004a; Koya et al., 2005; Ruhlman et al., 2007; Staub et al., 2000). Plant biopharmaceutical-based venture "Chlorogen Inc." has developed a proprietary chloroplast transformation technology (CTT™) for the production of recombinant molecules in tobacco chloroplasts and has been involved in the production of several bio-molecules like cholera vaccine, interferon, insulin and polymers. Dow AgroSciences LLC has secured exclusive rights to Chloroplast Transformation Technology (CTT™) from Chlorogen, Inc. (http://www.dowagro.com/newsroom/corporatenews/2007/20070910a.htm?filepat).

A leaf-specific promoter, *rbcS* gene promoter, has also been used to produce smallpox subunit vaccine, E1 endoglucanase or xylanase in the plant leaves (Dai et al., 2000; Golovkin et al., 2007; Hyunjong et al., 2006). Recently, Wakasa et al. (2009) have described a callus-specific system for the production of foreign molecules in transgenic plants.

3.1.1.3. Inducible promoters. The transcriptional activity of the chemically regulated promoters is modulated by chemical compounds like alcohol, tetracycline, steroids etc., whereas the physically regulated promoters are those whose activity depends upon environmental factors such as desiccation, salt stress, temperature, light etc. The constitutive expression of foreign bio-molecule may interfere with plant growth and development and in the more severe cases; it may block the plant regeneration. To certain extent the lethality problem can be overcome by using tissue-specific promoters but chemical-inducible systems offer high inducibility and specificity for regulated gene expression. These are quiescent in the absence of inducers. Use of chemical-inducible promoters in combination with the chemical responsive transcription factor can further restrict the target transgene expression to specific organs, tissues or even cell types (Zuo and Chua, 2000). For the inducible gene control system to be highly efficient, inducer should have high specificity to the promoter, fast response upon induction, rapid switching off upon withdrawal, non-toxic nature to plant, easy applicability and should not be present in the plant. A sucrose starvation-inducible promoter of rice *alpha amylase* (*αAmy3*) gene has been used to express human interferon gamma (Chen et al., 2004) and human α1-antitrypsin (Terashima et al., 1999). Inducible promoter has also been used to produce human granulocyte-macrophage colony stimulating factor (hGM-CSF) in rice cell suspension culture and it was found that the yield of hGM-CSF was significantly higher as compared to that produced by transgenic tobacco expression system using a constitutive promoter (Shin et al., 2003). Rice *αAmy3* gene promoter has also been used to derive the expression of human growth hormone in rice cell suspension culture (Kim et al., 2008). Another inducible system has been described which uses hydroxy-3-methylglutaryl CoA reductase 2 promoter, which is inducible by mechanical stress (Cramer and Weissenborn, 1997; Cramer et al., 1996). CropTech Corp. has used this mechanical stress inducible system. The activation of transgene expression takes place when the tobacco leaves are harvested and sheared during their processing leading to induction stimulus (Ma et al., 2003). Chemically inducible systems including tetracycline-, ecdysone-, estrogen-, glucocorticoid- or ethanol-based inducible systems, which have been used in plants, are reviewed by Padidam (2003). Recently Corrado and Karali (2009) have discussed about various inducible systems and their relevance to plant biotechnology. In general, these systems contain two transcription units. The product of first transcription unit is a transcription factor that responds to a chemical. The second transcription unit contains an element on which the activated transcription factor acts. The transcription factor that responds to a chemical may be regulated using a constitutive promoter or a cell/tissue/developmental stage-specific promoter, providing additional control on expression of transgene. Recently, a system for bacteriophage T7 RNA polymerase-directed, tissue-specific and inducible overexpression of transgene in plants has been described (Nguyen et al., 2004).

3.1.1.4. Artificial promoters. A set of minimal elements that include TATA box, necessary for recruiting the RNA polymerase II, a transcription start site and the CCAAT consensus sequence, are required for an active eukaryotic promoter. In addition to these minimum requirements, promoters have a diverse range of elements, which either up-regulate or down-regulate the activity of the promoters (Novina and Roy, 1996). Artificial promoters have been constructed in the past by combinatorial engineering of *cis* elements which include enhancers, activators or repressors, upstream to core promoter. It has been found that the promoter strength depends upon the motif copy number and the spacing between them (Gurr and Rushton, 2005; Rushton et al., 2002). In order to increase the strength of CaMV*35S* promoter, multiple copies of its enhancer element have been used (Guerineau et al., 1992). The CaMV*35S* promoter with its duplicated enhancer sequence has been used to optimize the expression level of several recombinant biomolecules in plants and enhanced expression levels have been achieved (Ashraf et al., 2005; Dong et al., 2005; Hull et al., 2005a; Mishra et al., 2006; Ruggiero et al., 2000; Salmon et al., 1998; Sharma et al., 2008a,b). A hybrid promoter, *mac*, was constructed by incorporating part of *mas* promoter and enhancer region of CaMV*35S* promoter. It enhanced the GUS expression by 3 to 5 times when compared with the GUS expression under the control of CaMV*35S* promoter with double enhancer in the leaves and 10 to 15 times in hypocotyls as well as roots (Comai et al., 1990). This promoter was used to express a thermostable cellulase E2 or E3 from *Thermonospora fusca* in alfalfa, potato and tobacco (Ziegelhoffer et al., 1999). Galvin and associates tested many combinations of regulatory sequences from octopine synthase and mannopine synthase gene promoters, and a hybrid promoter "*(Aocs)3AmasPmas* or superpromoter" was constructed by combining a triple repeat of *ocs* activator sequence, *mas* activator element and *mas* promoter (Ni et al., 1995). Super promoter has been used for the expression of SARS Coronavirus spike protein in tomato as well as tobacco (Pogrebnyak et al., 2005). Velten et al. (1984) isolated a dual plant promoter fragment that was found to initiate the expression of the gene coding for neomycin phosphotransferase in either orientation. Bidirectional promoters have also been synthesized which are capable to transcribe the gene upstream as well as downstream to it. The construction of bidirectional promoter was reported for the first time in 1995 (Baron et al., 1995). In plants, the strategy to engineer bidirectional promoters was used successfully to engineer *gus* reporter gene and *npt* II gene under the control of recombinant bidirectional promoter of *Arabidopsis* (Xie et al., 2001). A tetracycline-inducible bidirectional promoter system has been described that produces simultaneous and rapid co-induction of two separate reporters (Sammarco and Grabczyk, 2005). Zhang et al. (2008) modified the CaMV*35S* promoter to make it bi-directional and used to express reporter proteins in tobacco. Chaturvedi et al. (2006) constructed an artificial bidirectional promoter that comprises multiple *cis* regulatory DNA sequence elements, arranged to give a transcription initiation module. It activated transcription simultaneously in both directions at comparable levels. Recently, Lv et al. (2009) have constructed bidirectional promoters by fusing CaMV*35S* minimal promoter to 5′ end of either grp1.8 or 4Cl1 promtoer. The regulatory complexities in *cis* and *trans*-regulatory mechanisms and technological advances for the development of synthetic promoters have been reviewed by Venter (2007).

3.1.1.5. Trans-acting factors. *Trans*-acting factors are also important to optimize the transgene expression level in plants. The strategy has been used successfully to enhance the expression level of reporter transgene (Yang et al., 2001) where a transcription factor (REB; rice endosperm bZIP) has been engineered in rice along with human lysozyme gene driven by rice globulin promoter where the presence of REB enhanced the expression level of lysozyme. These *trans*-acting factors either directly bind to the promoter driving the expression of transgene or interact with other factors recruiting them to promoter. Transcription factors active in prokaryotic environment can be engineered to enhance the expression level of the transgene targeted to plastids of the plant cell. T7 RNA polymerase expressed from the nuclear genome of the plant cell has been found to enhance the expression level of transgene transcribed by T7 RNA polymerase, targeted to plastids (Magee and Kavanagh, 2002; McBride et al., 1994). For the expression of transgene in plants, a *trans*-activation system has been described, recently, that utilizes the viral vector to provide the inducer. This virus-mediated transactivation system was tested for the expression of intracellular domain of the diabetes associated autoimmune antigen IA-2ic and anti-tetanus antibody 9F12, where it displayed tight control on transcription (Hull et al., 2005b).

3.1.2. Position and context of the initiation codon

The translation initiation in majority of eukaryotic cellular mRNAs depends on the 5′-cap and involves ribosomal scanning of the 5′ untranslated region for initiation codon. Recent studies have identified an increasing number of upstream open reading frames (ORFs) that have key translational regulatory properties. The first ATG codon in optimal context has been shown to be the exclusive site of initiation even when the second initiation codon was positioned just a few bases downstream. This was true even when the second initiation was in the same favorable context (Kozak, 1995). The sequences surrounding the initiation codon also play an important role in translation initiation and AACAAUGGC, UAAACAAUGGCU and GCCAUGGCG have been identified as good context for plant genes (Lutcke et al., 1987). The positions −3 and +4 are highly conserved in plant mRNAs. A purine at −3 position and a GC at +4, +5 are the most conserved bases in plant genes (Joshi et al., 1997). In most of the high expressing plant genes, alanine follows N-terminal methionine. The sequence GCT TCC TCC (consensus from high expressing plant genes) was used after the initiation codon in a reporter gene. It augmented the expression of reporter gene in an incremental fashion with the insertion of each successive codon in tobacco and also increased the stability of the reporter protein by 2-folds. With the construct having Met-Ala-Ser-Ser-GUS, 30- to 40-fold increase in GUS activity was reported (Sawant et al., 2001). Plant-preferred translation initiation context has been used for the expression of *ctx*B gene as a C-terminal fusion protein with native plant protein "ubiquitinin" fragment in tobacco and a significant enhancement in the expression levels of transgene has been achieved (Mishra et al., 2006). Plant-preferred translation initiation context nucleotide sequence, where ACC or ACA precedes ATG, has also been used to optimize transgene expression in plants (Sharma et al., 2008a,b; Stomp et al., 2004).

3.1.3. Control of gene expression by 5′ untranslated region

The 5′ UTR is very important for translation initiation and plays a critical role in determining the translational efficiency. Regulation of translation initiation by majority of eukaryotic cellular 5′ UTRs or leader sequences is well understood and several factors involved in this process have been characterized. The leader sequences are capped and serve well to enhance the translation of foreign genes (Kozak, 1990; Tyc et al., 1984). Use of 5′ UTR of rice polyubiquitin gene *RUBI3* along with its promoter was reported to enhance the expression of GUS at mRNA level as well as translational level suggesting that 5′ UTR plays an important role in gene expression (Lu et al., 2008; Samadder et al., 2008). The untranslated leader sequences of alfalfa mosaic virus mRNA 4 or tobacco etch virus have been found to enhance the transgene expression by several folds due to enhanced translational efficiency of transcripts (Datla et al., 1993; Gallie et al., 1995). These have been used for the optimization of expression of several foreign molecules in plants (Haq et al., 1995; Huang et al., 2001; Huang et al., 2005b; Kong et al., 2001; Mor et al., 2001; Sharma et al., 2008a,b; Wang et al., 2008). Untranslated leader sequence from tobacco mosaic virus has also been used for the same purpose (Hood et al., 1997; Kang et al., 2004c; Wang et al., 2001).

3.1.4. Control of gene expression by 3′ untranslated region

The 3′ untranslated region plays an important role in gene expression as it contains message for transcript polyadenylation that directly affects mRNA stability (Chan and Yu, 1998). Heterologous 3′ untranslated regions from plants or plant viruses, have been used in the past to stabilize the recombinant transcript (Hood et al., 1997; Huang et al., 2005a; Ko et al., 2003; Staub et al., 2000). Some A/U rich sequence elements which destabilize the mRNA have been identified in the 3′ untranslated regions. These elements have been shown to cause rapid degradation of mRNA (De Rocher et al., 1998; Green, 1993; Newman et al., 1993; Ohme-Takagi et al., 1993). A sequence element "AAUAAA" has been characterized in the coding region of *cry3Ca1* gene which caused premature polyadenylation and resulted in poor expression of transgene in transgenic plants (Haffani et al., 2000). Therefore, such sequence elements and consecutive stretches of AT nucleotides should be avoided for heterologous transgene expression. The modified polyadenylation sequence element has been used to optimize the recombinant protein expression in plants and significantly higher accumulation of mRNAs has been reported (Kang et al., 2004c; Mishra et al., 2006; Richter et al., 2000).

3.1.5. Effect of sequence within the coding region

Any secondary structure present in the coding region slows down the progression of the 40S ribosome-initiation complex and, therefore, provides more time for the recognition of the preceding AUG codon. This enhancement critically depends on the position of secondary structures in the coding region. The introduction of hairpins near the beginning of the coding sequence at different positions shows different levels of enhancement. The maximum enhancement was reported when there was 14 nucleotide gap between AUG and the base of the stem-loop structure (Kozak, 1990) and can be considered for transgene engineering in plants. Because of extensive third-position degeneracy of the genetic code, species-specific codon bias has evolved during the course of evolution and therefore, optimization of coding sequence according to the host plant is another important parameter to enhance the recombinant protein expression (Daniell et al., 2009; Kang et al., 2004c; Koziel et al., 1996). It has been reported that the presence of rare codons may form secondary structure and slow down or pause ribosome movement through that region (Wolin and Walter, 1988). Since a strong correlation exists between frequency of codon usage and the presence of their cognate tRNAs, a cluster of rare codons may block the translation completely as all the tRNAs are trapped in the protein complexes (Chen and Inouye, 1990; Thanaraj and Argos, 1996; Varenne and Lazdunski, 1986). By removing such undesired signals, one can dramatically increase expression of a transgene in plants (Kang et al., 2004c; Mason et al., 1998). The *ctx*B gene was synthesized by optimizing the codon usage according to tobacco plant where rare codons were replaced with codons frequently used in tobacco and GC content was increased from 33% to 45%. The modified *ctx*B gene was expressed at significantly higher levels (~15-folds) as compared to native gene expressed under CaMV*35S* promoter (Kang et al., 2006b; Kang et al., 2004c). Codon optimized genes have also been synthesized for other bio-molecules for plant expression (Kang et al., 2006a; Kang et al., 2004b; Mason et al., 1998; Oszvald et al., 2007; Stomp et al., 2004; Streatfield et al., 2001). Sequences which are involved in post-transcriptional processing and directly influence the expression level of transgene, have been identified in the coding regions. When the gene encoding immunoglobulin A was expressed in tobacco, it was found that most of the recombinant hybrid immunoglobulin A/G was secreted into the apoplast but a portion was delivered to vacuole where it was degraded because of lytic activity of leaf vacuoles. Based on deletion analysis of the transgene, a motif was identified that is recognized as a vacuolar sorting signal in plant cells (Frigerio et al., 2000). In plants, vacuolar sorting signals can be present either on N-terminal, C-terminal or internal regions of propeptides. A few such sequence elements have been identified but there is no consensus sequence that represents the vacuolar sorting signal (Paris and Neuhaus, 2002; Vitale and Hinz, 2005). Therefore, in order to avoid vacuolar delivery, identification of such signals followed by their deletion or mutation, is required.

In general, sequences of introns or intron splice sites should be avoided from the transgenes but in some cases, the presence of intron sequences has been found to contribute positively to gene expression levels (Callis et al., 1987; Fiume et al., 2004; Huang and Gorman, 1990; Rethmeier et al., 1997). The position of intron in the gene determines its effect on expression enhancement as introns placed at all the locations do not contribute equally. The stimulatory effect of the intron has been found to decline with increasing distance from the promoter and may lose their stimulatory capability completely when placed in 3′ UTR (Bourdon et al., 2001; Mascarenhas et al., 1990; Rose, 2002; Snowden et al., 1996). Using intron-I from *shrunken-I* gene of maize at 5′ end of reporter gene, over 100-fold increase in reporter protein activity has been reported (Maas et al., 1991). Recently, it has been reported that introns in 5′ UTR are not randomly distributed and are more likely to be located closer to ATG codon. Using 5′ UTR from *EF1αA3* gene from *Arabidopsis*, reporter gene expression has been found to increase by 10-folds. The deletion analysis of introns showed that the expression enhancement is controlled by at least three elements distributed in the 5′ region of 5′ UTR intron (Chung et al., 2006). Introns placed near promoter have been found to enhance mRNA accumulation as well as its translation. But in a few cases the enhancement of mRNA accumulation because of the presence of 5′ UTR could not account for an increase in the reporter enzyme activity suggesting that introns enhance transcription and translation by two distinct mechanisms (Bourdon et al., 2001; Matsumoto et al., 1998; Rose, 2004).

3.1.6. Uniformity of transgene expression

Generally, transgene expression varies in the transgenic plants generated using the same construct, even in the same environment. It may be because of position effect, transgene copy number or silencing (Bhat and Srinivasan, 2002; Butaye et al., 2004; Fischer et al., 2008). There is a possibility to engineer desirable elements in the expression cassette to obtain the uniform expression levels. Nuclear matrix attachment regions (MAR), also called global regulatory sequences, that are thought to influence gene expression, can be used to enhance the transcriptional activity of the transgene. They are supposed to place the surrounding loci in the regions which are suitable for recruitment of transcription factors to promoters (Spiker and Thompson, 1996; Streatfield, 2007). Further, these AT-rich elements have been shown to reduce position effect by forming chromatin loops and therefore, increase transgene expression (Allen et al., 1993; Allen et al., 1996; Laemmli et al., 1992; Liu and Tabe, 1998; Mlynarova et al., 1995). Also these have been found to maintain expression level in subsequent generations (Vain et al., 1999). Based upon the sequence elements characterized in naturally occurring MARs, artificial MARs have been synthesized and tested for their effect on transgene expression in plants with respect to their orientation, plant promoter and different species (Nowak et al., 2001; Van der Geest et al., 2004). For the production of polyhydroxyalkanoates, the *bkt*B, *phb*B, *phb*C genes from *Ralstonia eutropha* and *tdc*B gene from *Escherichia coli*, flanked by RB7MAR (RB7 matrix attachment region) of tobacco, have been transferred to oil palm (Yunus et al., 2008). Similarly, gene coding for phytase enzyme flanked by RB7MAR has been transferred to tobacco and *Medicago truncatula*, and significantly higher expression levels have been achieved (Abranches et al., 2005). Targeting of transgene into plastids also eliminates the position effect because transgene is inserted in the functional region of the chloroplast through homologous recombination. Moreover, gene silencing has not been observed in plastids (Daniell et al., 2001b). Several foreign

proteins have been expressed in plastids and have been discussed later in this review.

3.2. Post-translational modifications of recombinant proteins

Once the protein is synthesized, it undergoes several modifications before final delivery to its target. These modifications include glycosylation, phosphorylation, methylation, ADP-ribosylation, oxidation, acylation, proteolytic cleavage involving the polypeptide backbone and non-enzymatic modifications like deamidation, glycation, racemization and spontaneous changes in protein confirmation (Gomord and Faye, 2004). Many heterologous systems involving bacteria, yeast, insect cells, mammalian cells or plants have been used for production of recombinant proteins. Each system has its own advantages and disadvantages. Glycosylation is important for various polypeptides to gain biological activity (Rothman et al., 1989; Sethuraman and Stadheim, 2006). Prokaryotes do not glycosylate the proteins and hence they cannot be used to produce recombinant proteins that require glycan residues for their activity. Glycosylation occurs in endoplasmic reticulum (ER) and Golgi apparatus. The glycosylation machinery of ER is conserved in most of the species and adds similar glycans that belong to oligomannose category (Chen et al., 2005; Sturm et al., 1987). Yeast, insects, mammals and plants attach high-mannose glycans to the same Asn residue in the ER, but they differ in trimming and further modification of the glycans in the Golgi apparatus. Plants have the capacity to add complex N-linked glycans with a core substituted by two N-acetylglucosamine residues which is similar to the glycosylation pattern observed in mammals (Wilson, 2002). However, plants do not add galactose and terminal sialic acids but add plant-specific α-(1,3)-fucose and β(1,6)-xylose residues, which are not desirable (Bardor et al., 2003; Cabanes-Macheteau et al., 1999). Various strategies have been developed to humanize the glycan patterns generated by transgenic plants and have been discussed later.

3.3. Sorting and targeting protein for higher accumulation

Most of the proteins are synthesized in cytosol however some proteins are made in mitochondria or plastids. Secretory proteins have N-terminal transit signal peptide and/or trans-membrane domains. The proteins are either delivered to ER, Golgi complex, hydrolytic compartments of the cell or are secreted out of the cell. Secretory proteins often undergo post-translational modifications like formation of disulfide bonds, N-glycosylation or proteolytic maturation steps. Endoplasmic reticulum, protein storage vacuoles and oil bodies are the desired targets for recombinant protein accumulation in transgenic plants.

3.3.1. Endoplasmic reticulum

ER of plant cells can tolerate unusually high accumulation of proteins without compromising plant development and reproduction. This property of ER retention has been exploited for recombinant protein accumulation. ER resident proteins have a C-terminal tetrapeptide, usually KDEL or HDEL, which is recognized by a receptor located in the Golgi complex (Gomord et al., 1997; Pelham, 1990). The proteins having C-terminal tetrapeptide bind to receptor in Golgi complex and are retrieved back into ER. Adding ER retention signal, along with signal peptide, results in increased accumulation of foreign protein in ER (Arakawa et al., 1998; Ko et al., 2003), indicating that the compartment has very low hydrolytic activity and is very plastic. Plants also accumulate many proteins which do not have a C-terminal retention signal in the ER e.g. prolamins. They form large aggregates within the ER lumen, the protein bodies (Shewry and Halford, 2002; Vitale and Ceriotti, 2004). Another ER retention signal from γ-zein, a prolamin of maize, has been characterized recently which is more efficient than KDEL signal (Mainieri et al., 2004). When fused with the proteins destined to different locations of the cell, it resulted in high accumulation of foreign protein in ER. In order to compare the activity of γ-zein ER-retention signal to KDEL signal, N-terminal 89 amino acids of γ-zein or C-terminal KDEL retention signal was fused to phaseolin and transgenic tobacco plants were tested for accumulation of phaseolin protein in ER. Using γ-zein signal, the foreign protein accumulated up to 3.5% of total extractable protein, whereas, it was only 0.5% when KDEL sequence was used. This remarkably high accumulation does not compromise the ER functions. As both the signal sequences resulted in accumulation of protein in ER, higher accumulation may be due to zein-mediated protein body formation which does not occur using KDEL signal (Vitle and Pedrazzini, 2005). It can be used for the expression of foreign proteins in plants. Also, as the protein bodies are insoluble (Mainieri et al., 2004), they can be easily purified by centrifugation and it further makes the production of recombinant protein economical. Virgilio et al. (2008) used either N-terminus domain of γ-zein or entire zeolin sequence to target human immunodeficiency virus negative factor (Nef) protein to protein bodies. It was found that N-terminal domain was not sufficient for high accumulation of Nef protein, but fusion with whole zeolin resulted in high accumulation (up to 1%) of Nef protein in protein bodies. Recently, because of the expression of avian infectious bronchitis virus M protein and GUS fusion, an unusual organization of the membranes of endoplasmic reticulum has been observed. The oligomerization of GUS domain of fusion protein was found to be responsible for this organization. It resulted in enhanced accumulation of the recombinant fusion protein and can be used as mechanism for retaining proteins in specific membrane compartments (Noizet et al., 2008).

3.3.2. Oil bodies

Oil bodies are the organelles that originate from the ER and function to store plant seed oils. These are surrounded by a phospholipid monolayer that is in direct contact with the lipid content. The oil-body membrane contains a very abundant protein termed as oleosin (Murphy, 1993; Zweytick et al., 2000). Foreign proteins can be targeted to the seeds of transgenic plants as translational fusions with oleosins. The fusion proteins are correctly targeted to oil bodies and remain tightly associated with them (Parmenter et al., 1995). The oil bodies and proteins associated with them can be easily separated from the majority of other seed cell components by floatation centrifugation, taking advantage of their low density. To facilitate the recovery of pure protein, a specific protease cleavage site can be inserted between oleosin and the desired recombinant protein. Thrombin inhibitor, hirudin, was produced in transgenic seeds of *Brassica napus* using this strategy (Parmenter et al., 1995). The engineered oil bodies with their associated proteins can be used as affinity matrices for the selective, non-covalent binding of desired target molecules. For this, the oil-body proteins may be genetically fused to a ligand having specificity for the desired target molecule (Moloney et al., 1999). The expression of recombinant protein as translational fusion with oleosin protein exposes the recombinant protein to cytosol, but at the same time it protects the foreign protein from cytosolic degradation. Because the fusion protein is not exposed to the environment of ER lumen, it avoids the post-translational modifications.

Recently, Maggio et al. (2007) have explored another strategy to target the recombinant protein to membrane system without passing through ER lumen. They have used the C-terminal hydrophobic region of tail anchored (TA) protein. TA proteins are synthesized on free ribosomes in the cytosol. The C-terminal region targets as well as anchors the protein on the membrane surface. The C-terminal region emerges from ribosome only upon translation termination, it is very unlikely for this hydrophobic region to interact with signal recognition particle, which binds only signal peptide or signal anchors as long as they are part of a nascent polypeptide (Borgese et al., 2003). The TA domain of mammalian cytochrome b5 was used to express recombinant antigens. The results indicate that these molecules are more

stable than the cytosolic counterparts and may provide a good strategy for preventing the rapid degradation of recombinant proteins, thus improving yield (Vitle and Pedrazzini, 2005).

3.3.3. Plastids

Semi-autonomous organelles, plastids are of several kinds e.g. chloroplasts, chromoplasts and leucoplasts, and each have a different function. Each cell contains a large number of plastids, ~100 chloroplasts per cell and each chloroplast contains about 100 genomes. Therefore, their transformation permits the introduction of thousands of copies of transgenes per plant cell. It dramatically enhances the protein production in the cell. Several proteins have been expressed in plastids and an increased foreign protein expression has been reported (Chebolu and Daniell, 2007; Daniell et al., 2001a; De Cosa et al., 2001; Kang et al., 2003; Molina et al., 2005; Ruf et al., 2001; Ruhlman et al., 2007; Staub et al., 2000; Tregoning et al., 2003). The vector used for chloroplast transformation uses two targeting sequences that flank the transgene and insert them through homologous recombination, at a predetermined location in its genome. It eliminates the position effect and results in uniform expression of transgene (Daniell et al., 2001a). Human therapeutic protein, interferon gamma was fused to His-tagged GUS and expressed in tobacco chloroplasts. The INF-γ-GUS fusion protein accumulated up to 6% of total soluble protein (Leelavathi and Reddy, 2003). In the tobacco chloroplasts cry1Ia5 protein accumulated up to 3% of TSP in the leaf tissue. A uniform expression level was observed in T1 and T2 progeny (Reddy et al., 2002). De Cosa et al. (2001) reported exceptionally high accumulation of *Bt* Cry2Aa2 proteins (up to 46% of TSP) when the transgene was engineered in chloroplasts. Recently, plastoglobules, the sub-chloroplastic compartments, have been targeted for recombinant protein accumulation (Vidi et al., 2007). These are low density particles, associated with the thylakoid membranes. Like the oil bodies they are light in weight and contain only a few proteins (Ytterberg et al., 2006), which is advantageous for purification. Chlorogen, a biotechnological company, has adopted the chloroplast transformation as platform technology for the production of foreign proteins in plants. The company has patented genetic sequences or regulatory signals which direct foreign genes to function specifically within the chloroplasts and is developing a wide range of protein molecules including vaccines and other therapeutic proteins of industrial applications. Chlorogen Inc. has provided the exclusive rights of proprietary licensed technology to Dow AgroSciences (http://www.dowagro.com/newsroom/corporatenews/2007/20070910a.htm).

3.3.4. Plant vacuoles

Plant vacuoles not only maintain the cell turgor but also store proteins and secondary metabolites. The protein storage seed vacuoles of most of the plants are an attractive target for recombinant protein accumulation. Signal sequences that are responsible for targeting the protein to protein storing vacuoles have been identified (Brown et al., 2003; Frigerio et al., 1998; Jolliffe et al., 2004; Koide et al., 1997; Matsuoka and Neuhaus, 1999) but no consensus protein sorting signal has been optimized so far. It has been observed that both ER-stored and vacuolar storage proteins are found in rice. Therefore, it is possible that the high synthesis of these proteins has a dominant effect on the localization of foreign proteins expressed in the secretory pathway (Vitle and Pedrazzini, 2005). The effect of high-level production of storage proteins on sub-cellular localization has also been observed in transgenic wheat (Arcalis et al., 2004). In rice seed endosperm, recombinant lysozyme accumulated in ER-derived protein bodies and protein storage vacuoles (Yang et al., 2003). Recombinant human serum albumin and *Asgergillus niger* phytase were delivered to protein storage vacuoles together with storage proteins when expressed in wheat endosperm (Arcalis et al., 2004). In contrast to protein storing vacuoles, vacuoles of vegetative tissues like leaves have higher hydrolytic activity and recombinant protein targeted would degrade. Therefore, mechanisms or the signals required to store recombinant proteins in the vacuoles need further exploration.

3.4. Copy number and site of integration of transgene

Theoretically, increase in transgene copy number in the transgenic plants should result in increase in recombinant protein expression level but multiple copies do not always result in higher expression levels. Integration of multiple copies of transgenes often results in transgene silencing and the transgenes are prone to rearrangements (Hobbs et al., 1993; Linn et al., 1990; Svitashev et al., 2002). Therefore, for more predictable transgene expression, single-copy insertions are preferred as these are less likely to be targeted by silencing mechanisms (Jones et al., 2005; Kohli et al., 2003). Alternatively, independent single-copy transgenic lines having high transgene expression levels can be crossed and transgenic plants with increased copy number as well as expression level can be generated reducing the silencing because of multiple copies (Streatfield, 2007). A *cis*-acting regulatory element, amplification promoting sequence (aps) was identified in the intergenic spacer region of tobacco ribosomal DNA, which was found to be responsible for increase in copy number of rDNA by DNA amplification (Borisjuk et al., 2000; Borisjuk et al., 1988; Hemleben and Zentgraf, 1994). Such elements have also been described in animals (McArthur and Stanners, 1991; Pasion et al., 1987; Wegner et al., 1989). The *aps* element contains an 11 bp A + T rich sequence, which is located within the origin of DNA replication. This *aps* sequence was used with herbicide resistance *acetolactate synthase* (*hr-ALS*) gene in tobacco and analysis showed that *aps* increased the copy number as well as transcription of the adjacent heterologous genes and enhanced herbicide resistance was also observed (Borisjuk et al., 2000). More recently, the gene encoding soybean protease inhibitor, Bowman–Birk inhibitor, was transferred to tomato plants with or without *aps* element. The *aps* element caused a 3-fold increase in mRNA as well as protein (Yakoby et al., 2006). A tobacco-based GAT™ protein expression system has been developed by Phytomedics Inc. for large scale and efficient recombinant protein production (http://www.phytomedics.com/). GAT™ is a gene amplification technology that uses *aps* sequence elements to increase the copy number of the transgene in the plant genome.

The site of integration of a transgene directly influences its expression level. A transgene may be integrated in a highly transcribed region or nontranscribed/least transcribed region. In the first case transgene would have high rate of transcription and so the high expression level. Further, the relative tolerance of a particular region for invasion by foreign DNA also directly affects transgene expression. In the plant genomes, heterochromatin regions, intercalary heterochromatin and repetitive DNA stretches have been shown to be associated with transgene silencing (Matzke and Matzke, 1998). In higher eukaryotes, the integration of DNA occurs mainly by illegitimate recombination at non-specific sites in the genome (De Buck et al., 1999; Hohn and Puchta, 2003). Homologous recombinations (HR) can be used to integrate the transgene at predetermined site in the highly transcribed regions but the procedure has not been optimized yet for homologous recombination-mediated transgene integration in plants. To increase the HR frequency in plants, several approaches have been followed to modulate the plant hosts by transferring HR related genes (Gherbi et al., 2001; Kumar and Fladung, 2001; Shalev et al., 1999; Terada et al., 2002). Chromosome breaks have been found to enhance frequency of homologous recombinations and have been reviewed recently (Puchta, 2005). Zinc finger nuclease containing a DNA recognition domain has been used to create site-specific chromosomal breaks, thereby, enhancing the frequency of localized homologous recombinations in plants. The outcome of the described system is very promising where they have shown 10^4-folds enhancement over frequency of unassisted homologous recombinations (Wright et al., 2005).

3.5. Strategies to achieve uniform transgene expression

Variation in transgene expression in the transgenic plants is another major issue. Large variations in transgene expression with undesired and unpredictable pattern have been observed among the transgenic plants generated under similar conditions (Bhat and Srinivasan, 2002; Hobbs et al., 1993; Miki, 2002; Vain et al., 2002). Various strategies have been explored to stabilize the transgene expression in plants, which include transfer of specific elements along with transgene and identification of low copy number transgenics, preferably single-copy insertions. Last two decades have seen the identification of several genetic elements in the 5′ or 3′ regulatory regions, coding sequence or the surrounding environment which directly influence the transgene expression (Butaye et al., 2005). Recently, De Paepe et al. (2009) has described a strategy to generate high frequency of single-copy T-DNA transformants. They used CRE-expressing *Arabidopsis* plants and found that 70% of the transformants in the CRE background was single copy and expression was stable as well as uniform. An alternative strategy is the creation of plant artificial chromosomes (Somerville and Somerville, 1999), which can be used as independent platforms to develop a stabilized environment for the uniform transgene expression. Artificial chromosome-mediated gene delivery does not interfere with host genes and eliminates the possible entry of transgene into the inactive region of the genome. The technology would make it possible to introduce the whole biochemical pathways into the plants conferring new properties to the plant. Taking one step ahead in this direction the identification and cloning of functional plant centromeres in *Arabidopsis* have been described that will help in the construction of stably inherited plant artificial chromosomes (Copenhaver et al., 1998; Copenhaver and Preuss, 2000). Recently, construction of minichromosomes has been reported in maize where telomere-mediated chromosomal truncation method was used. Small chromosomes created by this technology can carry site-specific recombination or other sites that allows further additions (Wright et al., 2005; Yu et al., 2007). Ananiev et al. (2009) reported the presence of minichromosomes in the callus cultures for one year. Further, these were detected in the root tips of regenerated plants, indicating normal replication and transmission through mitosis during organogenesis. Agrisoma Biosciences Inc., a private biotechnology company is also involved in the development of plant artificial chromosomes (http://www.agrisoma.com/home_frames.htm).

3.6. Downstream purification strategies

Along with high level of transgene expression to provide good yields in plant-based production system, efficient recovery of the recombinant proteins must also be optimized. Plants contain several undesired molecules (proteins, oils, phenolic compounds etc.) that must be removed during purification of the recombinant protein. The processing of plant tissue for the recovery of recombinant proteins generally includes fractionation of plant tissue, extraction of recombinant protein in aqueous medium and purification. Several strategies have been developed to improve the downstream processing of plant-produced recombinant proteins. Secretory systems are advantageous for the recovery of proteins as the plant cells need not to be disrupted (Schillberg et al., 1999; Drake et al., 2009). It avoids the release of the phenolic compounds and other contaminants from the plant cells but then the stability of the recombinant protein is an issue which needs to be taken care of. Another approach is to target the proteins into the protein bodies, oil-bodies or plastoglobules. Protein bodies are insoluble and oil-bodies or plastoglobules are light in weight and can be separated by simple floatation centrifugation (Parmenter et al., 1995; Torrent et al., 2009; Vidi et al., 2007). The use of affinity tags with the desired protein is a powerful approach for recombinant protein recovery, but the tag needs to be removed from the final product. Several affinity tags have been described for recombinant protein purification (Terpe, 2003). N-terminal hexahistidine tagged ricin B (HIS-RTB), the lactin subunit of ricin, was expressed in tobacco. The recombinant HIS-RTB was purified from tobacco leaves using lactose affinity column, and was found to be functional (Reed et al., 2005). His-tagged amarantin was expressed in tobacco and the presence of His tag was used for single-step purification of recombinant protein using immobilized metal-ion affinity chromatography (Valdez-Ortiz et al., 2005). The effects of three affinity tags, i.e. eight amino acid tag StrepII, His_6 and 181-amino acid Tandem Affinity Purification (TAP) tag were studied for the purification of recombinant membrane anchored protein kinase. The protein purified using His_6 tag was of low purity whereas the recombinant proteins having TAP or StrepII tag were purified to homogeneity. While StrepII-tag purification achieved high yield and purity which was comparable to that obtained with TAP-tag, it was considerably easier and faster (Witte et al., 2004). In addition to aid in the purification of recombinant protein from plants (Conley et al., 2009; Joensuu et al., 2009), elastin-like polypeptide tag has also been found to enhance expression of the protein target (Conley et al., 2009). The possibilities of using histamine, tryptamine, phenylamine or tryamine as affinity tags for affinity purification of recombinant proteins have also been evaluated (Platis and Labrou, 2008). The protein splicing elements (inteins) that can catalyse self-cleavage (Liu, 2000) have also been utilized in protein purification purposes. The SMAP 29, a mammalian antimicrobial peptide (Bagella et al., 1995), was expressed with intein fusion tag in tobacco plants. The recombinant protein was targeted to apoplast, using β-conglycinin transit peptide. The plant-expressed recombinant SMAP-29-intein tag fusion protein was purified using chitin column. Although the system needs to be optimized according to the chemical features of the target product, SMAP purification demonstrates that the self-cleaving intein tags can be used as purification tags with the recombinant proteins (Morassutti et al., 2002). The nucleoprotein of *Tomato spotted wilt virus* can be used as affinity tag for easy purification from plant cells (Lacorte et al., 2007). The nucleoprotein is highly stable in plant cells and can be easily purified using ultracentrifugation (De Avila et al., 1990).

4. Humanizing the protein production in plants

Plant specific glycan residues might be responsible for immunological reaction, which could be a limitation to the use of plants for production of pharmaceutical proteins (Bardor et al., 2003; Chargelegue et al., 2000; Faye et al., 1993). Several approaches have been developed and rapid progress has been made towards humanizing plant glycosylation patterns. The first approach is based on the fact that ER of plants and human add similar type of glycan structures (Faye et al., 2005; Pagny et al., 2000). Retaining the recombinant glycoprotein in endoplasmic reticulum avoids further modification of the glycoprotein in the Golgi apparatus, where plant-specific oligosaccharides are added (Lerouge et al., 1998). The SEKDEL (Ser-Glu-Lys-Asp-Glu-Leu) or KDEL (Lys-Asp-Glu-Leu) or HDEL (His-Asp-Glu-Leu) peptides are often used to retain the protein in endoplasmic reticulum (Fujiyama et al., 2009; Gomord et al., 1997; Haq et al., 1995; Ko et al., 2003; Wandelt et al., 1992). The second option is to modify the enzymatic machinery of the Golgi apparatus. It can be done either by knocking out genes related to β(1,2)-xylosylation and α(1,3)-fucosylation (Strasser et al., 2004; Von Schaewen et al., 1993) and/or by adding new glycosyltransferase to modify the processing of glycan structures (Frey et al., 2009; Palacpac et al., 1999). Strasser et al. (2004) generated knockout *Arabidopsis* plants with the complete deficiency of β(1,2)-xylosyltransferase (XylT) and α(1,3)-fucosyltranferase. These plants were able to produce N-glycans with two β-N-acetyglucosamine residues but lacked β(1,2)-linked xylose and α(1,3) linked fucose. Recently MDX-060 mAb was produced in *Limna minor*, where glycosylation was optimized by co-expression of an RNAi transcript designed to silence endogenous α(1,3)-fucosyltransferase and β(1,2)-xylosyltransferase activities. The mAb with optimized glycosylation had a single major N-glycan species without detectable plant-specific N-glycans. Plant-

produced mAb showed better antibody-dependent cell-mediated cytotoxicity and was indistinguishable from the mAb produced in Chinese hamster ovary cells (Cox et al., 2006). RNAi technology has also been used to down-regulate the endogenous *XylT* and *FucT* genes in *Nicotiana benthamiana* which was used for expression of anti-HIV monoclonal antibody 2G12 (Strasser et al., 2008). The β(1,4)-galactosyltransferase is an important enzyme, that can convert typical plant N-glycans into mammalian-like n-glycans. It competes for the same acceptor substrate as XylT and FucT. The β(1,4)-galactosyltransferase was overexpressed in plants and resulted in partial elimination of α(1,2)-xylose and β(1,3)-fucose (Bakker et al., 2001).

Plants do not add terminal galactose which contributes to the immunological functions and the correct folding of antibodies (Wright and Morrison, 1998). In order to add terminal galactose to plant processed antibody proteins, human β(1,4)-galactosyltranferase (*GalT*) gene was transferred into *Nicotiana tabacum* L. *cv.* Bright Yellow (BY2) cells lacking *GalT* gene. The galactosylated N-glycans accounted for 47.3% of the total sugar chains in transgenic plants (Palacpac et al., 1999). In another approach, the genes encoding N-terminal domain of *Arabidopsis thaliana* xylosyltransferase and the catalytic domain of human β(1,4)-galactosyltransferase I were fused and the chimeric gene was expressed in tobacco. Along with high-level galactosylation of N-glycan, it led to a steep decline in plant-specific Xyl and Fuc residues (Bakker et al., 2006).

Plant glycoproteins also lack terminal sialic acid (Lerouge et al., 1998), an important component in human glycoproteins, and may make these proteins short-lived and ineffective (Kelm and Schauer, 1997). Endogenous sialylated glycol-conjugates have been found in the plants which means that sialylation pathway exists in plants (Shah et al., 2003). However, it has been found that Neu5Ac, the major sialic acid present in humans is not synthesized in plants in detectable amounts (Paccalet et al., 2007; Seveno et al., 2004; Zeleny et al., 2006). Many components of sialic acid synthesis pathway have been successfully engineered in plants producing biologically active recombinant proteins. These include mammalian α-2,6-sialtransferase, a glycosyltransferase of the mammalian *trans*-Golgi cisternae expressed in *Arabidopsis* plants (Wee et al., 1998), human CMP-N-acetylneuraminic acid synthetase and CMP-sialic acid transporter in tobacco suspension-cultured cells (Misaki et al., 2006), genes encoding Neu5Ac lyase from *E. coli* and Neu5Ac synthase (neuB2) from *Campylobacter jejuni* in BY2 cells as well as alfalfa (Paccalet et al., 2007). Finally, the engineering of gene coding for epimerase along with other components in the same system would complete the sialation pathway in plants (Saint-Jore-Dupas et al., 2007).

5. Bio-molecules produced in plants

5.1. Vaccine antigens

A vaccine is an antigenic preparation used to establish immunity against a disease and the main aim of the vaccination is to eradicate infectious diseases. In the beginning, the technology of expressing antigenic determinants in plants was tapped for production of edible vaccines and local production of engineered edible plants for consumption was proposed (Artnzen, 1997; Prakash, 1996). Later, with the technology developments, role of health care workers was discussed in order to define doses and "Edible Vaccines" are now more popular as "Plant Vaccines". Although the amount of protein required for oral delivery will vary among various antigens, oral delivery needs larger doses of the antigens in general (Rice et al., 2005; Streatfield and Howard, 2003). For defining vaccine doses, powdered formulations can be produced from seeds or freeze-dried fruits and leaves using standard inexpensive food industry milling and processing techniques. Large scale batches of powdered plant materials can be tested to ensure consistency of vaccine dose and adequate quality control. This material may also be supplemented with mucosal adjuvant, vitamins and/or other vaccines to make it more effective. If vaccines are supplied in powdered formulations, it may also improve acceptability of vaccines in countries which are against growing genetically modified crops. The idea of plant vaccine is coming closer to reality. In recent years, a large number of antigens have been produced in plants and shown to activate the immune response against the antigen in the animal models. Dow AgroSciences has already received the first ever regulatory approval for a plant-made vaccine from the USDA Center for Veterinary Biologics (CVB) in January 2006. It entered the regulatory process in the US for approval of a plant-made vaccine against Newcastle Disease (http://www.dowagro.com/uk/media/General/20061017.htm).

Several antigenic determinants belonging to various pathogens causing variety of diseases including bacterial and viral diarrhea, anthrax, rabies, cancer, SARS, measles, HIV, diphtheria, pertusis, tetanus, tuberculosis, respiratory syndrome, Alzheimer's disease, malaria, foot and mouth disease of cattle, gastroenteritis, hemorrhagic disease, bursal disease, goat plague, rinderpest virus, cytomegalovirus infections, parvoviral infections of dogs, avian influenza and bovine pneumonia have been produced in plants (Khandelwal et al., 2003; Sharma et al., 2004; Streatfield and Howard, 2003; Tiwari et al., 2009; Youm et al., 2008). These antigens have been characterized either by western blots or ELISA, using antibodies raised against them. Many plant-produced subunit vaccine candidates have been used to immunize (orally or parenterally) model animals as well as humans and different levels of immune responses have been recorded (Arakawa et al., 1997; Chen et al., 2006a; Dong et al., 2005; Gil et al., 2006; Haq et al., 1995; Jani et al., 2004; Lauterslager et al., 2001; Nochi et al., 2007; Spitsin et al., 2009; Yu and Langridge, 2001; Zhang et al., 2006). For better protection efficacy, production of multicomponent vaccines is an attractive alternative. Several antigens can be targeted to mucosal system as chimeric products and the use of adjuvants like cholera toxin B subunit or heat-labile enterotoxin B subunit as one of the components, make the antigen delivery more efficient. The oral administration of CTB linked to antigens has been shown to trigger acceptable peripheral and mucosal immune responses that were otherwise unattainable when the antigen was given alone (Dertzbaugh and Elson, 1993). Several antigens including insulin (Arakawa et al., 1998; Li et al., 2006a; Ruhlman et al., 2007), rotavirus enterotoxin (Yu and Langridge, 2001), accessory colonization factor A or P4/P6 epitopes of TCPA of *Vibrio cholerae* (Sharma et al., 2008a,b), anthrax lethal factor (Kim et al., 2004b), HIV antigen (Kim et al., 2004a; Kim et al., 2004c; Kim et al., 2004e), VP2 of parvovirus (Molina et al., 2005), porcine epidemic diarrhea virus antigen (Kang et al., 2006a; Oszvald et al., 2007), foot and mouth disease virus antigen (He et al., 2007), heat stable toxin (Rosales-Mendoza et al., 2009), immunocontraceptive epitope ZP3 (Walmsley et al., 2003) have been produced in plants as a fusion partner of CTB or LTB and have been shown to assemble in functionally active oligomers. The assembly of these chimeric proteins into biologically active oligomers in transgenic plants demonstrates the feasibility of using plants for the synthesis of various antigenic epitopes fused with CTB/LTB.

5.2. Therapeutic products

Several therapeutic products can be produced in plants which include diagnostic proteins (antibodies and enzymes), replacement proteins (Factor VIII for hemophiliacs, insulin for diabetics), immune system stimulator/suppressants (interleukins, interferons, and colony stimulating factors), biopolymers and adhesive proteins for surgical purposes or growth factors (Daniell et al., 2001b; Goldstein and Thomas, 2004; Rajasekharan, 2006; Twyman et al., 2005). Antibodies or immunoglobulins (IgGs) are serum proteins that play a central role in the humoral immune response. Immunoglobulins bind and inactivate pathogens or trigger an inflammatory response which results in their elimination. The engineering of recombinant antibodies and their successful expression in plants has opened up new opportunities, not only for the medical sciences but also for the applied and fundamental agronomic research. The production of immunoglobulin fragments and their assembly in plants was reported

in tobacco for the first time (Hiatt et al., 1989). Since then the molecular farming technology has expanded rapidly and a large number of therapeutic antibodies or their fragments have been produced in plants and are referred as "Plantibodies" (De Jaeger et al., 2000; Goldstein and Thomas, 2004; Ko and Koprowski, 2005; Ma et al., 2005c). After isolation and purification from the plant tissue, these can be used as diagnostic tools for immunochromatography or in medical therapy. The *in planta* interests include studying the function of antigen or even the epitope of an antigen in plants, change in the agronomic traits, immunization of the plants against pathogen infection or providing resistance against herbicides (De Jaeger et al., 2000). The binding affinity of single chain variable fragment (scFv) is similar to complete antibodies (Glockshuber et al., 1990; Milenic et al., 1991) but they are easy to produce in plants as compared to complete antibodies because of the problem of assembly and folding associated with full sized antibodies (Schillberg et al., 1999). The recombinant antibodies can be produced in plants in many forms which include full size recombinant antibody, chimeric antibody, secretory antibody, single chain Fv fragments (scFvs), scFv fusion, bispecific scFv, antibody fragments or heavy chain variable domains (Ma et al., 2003). The subcellular destination of the recombinant antibody is important to achieve higher expression levels. Full size antibodies are generally unstable in cytosol and high expression can be achieved by targeting them through secretory pathway (Schillberg et al., 1999). The expression of antibody in the cytosol becomes useful when the ultimate target is either to engineer pathogen resistance or to modulate the metabolic pathway.

The glycosylation pattern of the proteins varies according to the system used for their production and has direct impact on their function and efficacy (Raju et al., 2000; Sethuraman and Stadheim, 2006). Comparisons have been made for plantibody and murine produced Guy's 13 antibody and it was found that the plantibody had structurally diverse N-glycans (Cabanes-Macheteau et al., 1999). Plantibodies have also been evaluated for their function and appear to be indistinguishable for serum half life (Khoudi et al., 1999) or their binding activities (Vaquero et al., 1999; Zeitlin et al., 1998) from the antibodies produced in other systems. But non-human glycoforms raise immunogenicity and safety concerns, and their clinical use may induce certain adverse reactions (Bardor et al., 2003). Efforts are being pursued for humanizing the plant N-glycans.

5.3. Nutritional components

Plants can provide most of the nutrients required in the human diet. However, major crops have been found to be deficient in one or the other nutrients. The advances in genetic modifications have made it possible to enhance the nutritional quality of the plants (Galili et al., 2002; Zimmermann and Hurrell, 2002). Though manipulation of the plant metabolism is possible, but identification of the target nutrients, suitable for the purpose, is difficult. The targets may include macronutrients such as proteins (e.g. it may be desirable to increase levels of essential amino acids), fatty acids, micronutrients such as vitamins A and E, carotenoids such as lycopene, flavonoids and minerals like iron, zinc etc. (Tucker, 2003). Several technical advances have been made from earlier attempts of flux redirection to simultaneously manipulating multiple steps in plant metabolic pathways and in constructing novel, multi-enzyme pathways in plant tissues (Kinney, 2006; Sandmann et al., 2006; Wu et al., 2005). In the last few years, a lot of progress has been made in the field of biofortification. Plants have been engineered to increase accumulation of β-carotene (Aluru et al., 2008; Datta et al., 2003; Naqvi et al., 2009; Paine et al., 2005; Ye et al., 2000; Zhu et al., 2008), lycopene (Fraser et al., 2002), vitamins (Díaz de la Garza et al., 2007; Nunes et al., 2009; Rocheford et al., 2002; Shintani and DellaPenna, 1998; Storozhenko et al., 2007; Van Eenennaam et al., 2004), flavonoid (Bovy et al., 2002; Butelli et al., 2008; Reddy et al., 2007; Verhoeyen et al., 2002), resveratrol (Delaunois et al. 2009; Fan et al., 2008; Liu et al., 2006; Nicoletti et al., 2007; Ruhmann et al., 2006), polyamines (He et al., 2008; Mehta et al., 2002), nutraceuticals (Kang et al., 2009; Lee et al., 2008; Park et al., 2008), amino acids (Cho et al., 2000; Dancs et al., 2008; Falco et al., 1995; Kalamaki et al., 2009; Lai and Messing, 2002; Mazur et al., 1999; Wakasa et al., 2006; Yamada et al., 2005; Zhou et al., 2009; Zhu and Galili, 2003), nutritional proteins (Altenbach et al., 1992; Altenbach et al., 1989; Chakraborty et al., 2000; Khan et al., 1996; Molvig et al., 1997; Stoger et al., 2001; Yu et al., 2005; Zhang et al., 2003), minerals (Chiera et al., 2004; Lee and An, 2009; Lucca et al., 2002; Morris et al., 2008), fatty acids (Chen et al., 2006b; Cook et al., 2002; Hoffmann et al., 2008; Hong et al., 2002; Kajikawa et al., 2008; Liu et al., 2002; Napier, 2007; Qi et al., 2004; Sayanova et al., 1997) and carbohydrates (Hellwege et al., 2000; Kok-Jacon et al., 2005b; Oakes et al., 1991; Regina et al., 2006; Schwall et al., 2000).

5.4. Industrial products

Plant molecular farming is starting to become a viable new industry. The plant-produced products are now approaching towards commercial release (Ma et al., 2005b; Spok, 2007). Moreover, even for the production of human proteins, plants are emerging as a system of choice. Whereas, bacteria cannot perform most of the post-translational modifications of proteins, plants can perform many of these complex processing steps required to produce mammalian proteins in active form.

The first pharmaceutically relevant protein made in plants was human growth hormone produced in tobacco (Barta et al., 1986). Since then, several mammalian proteins and other products of industrial importance have been expressed in diverse range of plant species (Sharma et al., 2004; Streatfield and Howard, 2003). The pharmaceutical proteins of mammalian origin that have been synthesized in plants are blood products, such as human serum albumin, for which there is an annual demand of more than 500 tons, enzymes like gastric or pancreatic lipases, structural proteins, cytokines and other signaling molecules that are required in much smaller amounts. For the production of proteins, which are required in large quantities and where huge investment in other production systems is required, plants offer a huge competitive advantage. Human glucocerebrosidase (hGC) is one of the most expensive drugs and is required at large scale for enzyme replacement therapy. The dose required for a single person per year may cost thousands of dollars. Therefore, successful production of hGC in plants would be an ideal example of potential of plants as bioreactors. This enzyme has been produced in tobacco at the rate of >1 mg/g of fresh weight and plant-produced hGC has been found to be enzymatically active (Cramer et al., 1996). Protalix Biotherapeutics is using transgenic carrot cells to produce hGC. Transgenic cells are filled in plastic bags, cultured and are processed to extract the drug (Kaiser, 2008; http://www.protalix.com/glucocerebrosidase.html). Human somatotropin (hST), which is used to treat hypopituitary dwarfism in children, Turner syndrome and chronic renal failure, was produced in tobacco chloroplasts. The biologically active recombinant protein accumulated up to 7% of TSP (Staub et al., 2000). For the fortification of infant formula, an artificial substitute for human breast milk with the bioactive proteins, a synthetic human lactoferrin (HLF) gene linked to a rice glutelin 1 promoter and signal sequence was transferred in rice. In the transgenic plants, HLF was produced up to 0.5% of dehusked rice grain weight. rHLF was evaluated for iron binding, antimicrobial activity as well as resistance to protease digestion and was found to be identical to native HLF (Nandi et al., 2002). Similarly, the gene encoding human lysozyme driven by rice glutelin and globulin promoters was introduced in rice. The recombinant protein accumulated in type II protein bodies in endosperm. Also, the transgenic lines with higher expression levels exhibited morphologically different protein bodies (Yang et al., 2003). Avidin, a glycoprotein, found in

avian, reptilian and amphibian egg white, is primarily used as a diagnostic reagent. The plant-optimized avidin coding sequence was expressed in maize. The resultant avidin had properties almost identical to those of avidin from the chicken egg white. The p*I*, K_i and antigenic properties were also identical (Hood et al., 1997; Horn et al., 2004). Trypsin is a proteolytic enzyme that is used in a variety of commercial applications, including the processing of some biopharmaceuticals. Although trypsin has been expressed in a variety of recombinant systems, none of these systems has been demonstrated to be commercially viable on large scale. Expression of this enzyme at commercially viable levels in maize was possible only by expressing the enzyme in an inactive zymogen form (Horn et al., 2004; Woodard et al., 2003). The dragline silk produced by the spider *Nephila clavipes* is comparable to synthetic superfiber Kevlar in tensile strength. Further, it also shows high elasticity. Transgenic tobacco and potato plants expressing recombinant dragline silk protein were generated. The recombinant protein accumulated up to 2% of TSP in the endoplasmic reticulum of tobacco, and potato leaves and tubers (Scheller et al., 2001). Several other products have been produced in plants which include streptavidin, acetylcholinesterase, hirudin, protein C, human β casein, vegetable oils, collagen, gamma-amino-butyric acid, β-glucuronidase, cyclodextrins, enzymes like phytases, xylanases, amylase, laccase, glucanases and transglutaminases (Akama et al., 2009; Boehm, 2007; Cahoon et al., 2007; Capell et al., 2004; Goldstein and Thomas, 2004; Scheller and Conrad, 2005; Sharma et al., 2004).

5.5. Biodegradable plastics

Plastics have become synonymous with the modern life. These are difficult to dispose off and continually accumulating non-degradable wastes have become a significant source of environmental pollution (Shimao, 2001). Biodegradable plastics seem to be a viable alternative to synthetic plastics. The biodegradable materials can undergo decomposition into carbon dioxide, methane, water, inorganic compounds with the help of the enzymatic actions of microorganisms within a specified period of time (Anderson and Dawes, 1990). Polyhydroxyalkanoates (PHAs) are the biodegradable polymers which occur naturally in plants. Variety of co-polymers are produced by microorganisms but polyhydroxybutyrates (PHBs) are most common (Steinbuchela and Valentinb, 1995). Plants contain thioesters which are precursor molecules for PHA synthesis. Acetyl-CoA, precursor molecule for PHB synthesis is present in various compartments of plant cell including cytoplasm, plastids, mitochondria and peroxisomes (Moire et al., 2003; Suriyamongkol et al., 2007). Plants have been engineered to produce PHAs or PHBs in the various plant cell compartments (John and Keller, 1996; Matsumoto et al., 2009; Menzel et al., 2003; Nawrath et al., 1994; Petrasovits et al,. 2007; Poirier et al., 1992; Slater et al., 1999; Somleva et al. 2008; Wrobel-Kwiatkowska et al., 2007; Wrobel et al., 2004). For the economic feasibility of transgenic plants-derived biodegradable plastics, accumulation of at least 15% of the tissue dry weight is required (Scheller and Conrad, 2005). Various approaches used to improve the production levels have been reviewed, recently (Suriyamongkol et al., 2007). When PHB expression was targeted to the cytoplasm, these accumulated to a very low level and transgenic plants showed growth defects (Hahn et al., 1997; Nakashita et al., 1999; Poirier et al., 1992). To increase the expression level of biodegradable plastic-like compounds in plants, plastids have also been targeted (Bohmert et al., 2000; Lossl et al., 2005; Lossl et al., 2003; Nawrath et al., 1994) and expression levels ranging up to 40% of dry weight have been obtained (Bohmert et al., 2000). But defects in plant growth and metabolism were associated with their expression. In order to reduce the associated negative effects, their expression has been targeted in a tissue-specific manner to seed plastids in *B. napus* (Houmiel et al., 1999). The seed size, germination rate as well as oil production were found to be normal. Thiol esters are abundant in peroxisomes of plant cells (Goepfert and Poirier, 2007) and, therefore, peroxisomes are attractive targets for the production of PHAs. Using RAVAL residues encoding sequence at the carboxy terminal, *Pha* gene products were targeted to peroxisomes and accumulation of PHBs up to 2% dry weight was reported (Hahn et al., 1999). There is a need for further improvements in PHB production in plants, aimed at higher accumulation without any side effects on plants.

6. Regulatory aspects

Concerns have been raised about the safety of GM foods in relation to environment and human health. Although there is no scientific evidence that current modified foods involve any new or magnified risks, certain environmentalists and consumers are still not convinced (Smyth and Phillips, 2003). To some extent, a negative opinion for transgenic plants already exists among the general public and this notion that genetic engineering is against nature, makes an impact on regulators (Streiffer and Hedemann, 2005). Lack of communication among the regulatory bodies involved in research, biosafety and trade, further hampers the developments in this field (Ramessar et al., 2008b). The regulation of pharmaceutical crops is in its infancy and there are several challenges ahead for the regulatory agencies. There is a lot of pressure from pharmaceutical industry, food industry, environmental and consumer organizations against GM crops and regulations are strict and turn out to be very costly. There is a requirement to regulate the pharmaceutical crops on case by case basis. The regulatory challenges posed ahead for the molecular farming and how they are different from those for first generation transgenic crops, have been reviewed recently (Spok, 2007; Spok et al., 2008). The strategies used for risk assessment need to be reviewed. The most important issue is to segregate the GM crops from non-GM crops to prevent intermixing. A variety of approaches including physical containment as well as genetic strategies like seed sterility, maternal inheritance, male sterility, selective elimination by engineering sensitivity to chemicals, etc. have been postulated to address this question (Howard and Donnelly, 2004; Lee and Natesan, 2006; Lin et al., 2008) and threshold limits of accidental contaminations have been suggested. It is very difficult to maintain a complete segregation of GM and non-GM crops in the open fields, as has been visualized by USDA (US Department of Agriculture, 2006), even after stringent confinements. The European Parliament and the Council of the European Union has allowed the GM presence up to 0.5% in non-GM food or feed where the genetically modified material overwhelms the negative effects and its presence in non-GM is technically unavoidable (European Parliament, 2003). For the plant-made substances other than pharmaceuticals that do not pose hazardous risks, the threshold limit for contamination of non-GM crops is 0.9% (Spok, 2007). Another important issue is to label the GM products. In order to empower the consumer to select between GM and non-GM products, labeling could be an option. But, the mandatory labeling might not be economically justifiable in every situation (Smyth and Phillips, 2003) and would not provide the consumer with the required information. Alternatively, information domains can be built that can provide the essential information related to GM content of product to the consumer. Also a system that could trace the product in the market to its source and a good strategy for post-market monitoring and surveillance would be practical (Smyth and Phillips, 2003). Although there are a lot of developments in the last decade for addressing the concerns related to transgenic plants (Chen et al., 2005; Devos et al., 2008; Gomord et al., 2005; Ma et al., 2005a; Sparrow and Twyman, 2009; Stewart, 2008), a lot more is still needed to be done.

7. Economic viability

Production of recombinant proteins in plants offers many practical, economic and safety advantages as compared to more conventional

Table 1
Transgenic plant-based products commercially available in the market.

Product name	Company name	Plant system	Commercial name	Catalog no./URL
Avidin	Prodigene	Corn	Avidin	#A8706, Sigma-Aldrich
β-Glucuronidase	Prodigene	Corn	GUS	#G2035, Sigma-Aldrich
Trypsin	Prodigene	Corn	TrypZean™	#T3568, Sigma-Aldrich
Recombinant human lactoferrin	Meristem Therapeutics, Ventria Bioscience	Corn, rice	Lacromin™	#L4040, Sigma-Aldrich
Recombinant human lysozyme	Ventria Bioscience	Rice	Lysobac™	#L1667, Sigma-Aldrich
Aprotonin	Prodigene	Corn, transgenic tobacco	AproliZean	#A6103, Sigma-Aldrich
Recombinant lipase	Meristem Therapeutics	Corn	Merispase®	http://www.meristem-therapeutics.com[a]
Recombinant human intrinsic factor	Cobento Biotech AS	*Arabidopsis*	Coban	http://www.cobento.dk/?id=76
Vaccine purification antibody	CIGB, Cuba	Tobacco	–	Kaiser, 2008

[a] MERISTEM Therapeutics & SBH Sciences has announced an agreement for joint development of animal-free recombinant proteins.

systems. It has been estimated that at an expression level of 1% of the dry weight in corn and even after 50% recovery during purification, the cost of plant-produced proteins may be 2–10% of microbial systems, but in comparison to mammalian systems cost benefits may be up to 1000-folds (Chen et al., 2005; Hood et al., 2002; Twyman et al., 2003). The current demand of insulin is 5000–6000 kg/year which is projected to touch 16,000 kg/year by 2012. According to SemBioSys, in its July 2006 press release, the company is capable of producing 1 kg of insulin/ac of transgenic safflower that will reduce the product cost by 40% (http://www.sembiosys.com). MALTAgen Forshung GmbH claims the capability to produce 3 g human serum albumin (HAS) or 2 g lactoferrin or 1.5 g lysozyme/kg of barley seeds (http://www.maltagen.de/PDF/ Products.pdf). Farmacule BioIndustries is producing a high value protein "Vitronectin" in plants. Currently, Vitronectin is produced from animal serum and may cost up to US$ 5 million/gram (http://www.lifescientist.com.au/article/144678/farmacule_grows_proteins_tobacco). Plant molecular farming of diagnostic products is more realistic and their commercialization would be less stringent. Several plant-produced biomolecules have been commercialized (Table 1).

Genetically engineered foods are among the riskiest of all possible insurance exposures and therefore amount of insurance coverage available to biotech firms have declined in recent times. Many insurance companies have declared their reluctance to provide cover to genetically modified crop industry (http://www.mindfully.org/GE/2003/Biotech-Food-Business1dec03.htm). The regulating agencies have defined very strict and costlier regulations under pressure from the food industry because of which it is really difficult for industry to flourish. Even after getting the permits for growing the transgenic crops, it is difficult to actually grow them in the field due to resistance from consumers and other agencies. According to USDA (http://www.aphis.usda.gov/brs/ph_permits.html) number of field trials for the crops producing bio-pharmaceuticals or other compounds, had increased from 6 in 2003 to 13 in 2007. The number of release permits is 10 till July 2008. The area of planting crops producing pharmaceuticals as well as industrial proteins has increased from 45 ac in 2004 to 198 ac in 2006. In 2006, 797 ac area was proposed for transgenic crops but only 198 ac could be planted. For 587 ac land, permits were withdrawn. Similarly, only 176 ac land was planted with transgenic crops as against 811 ac proposed land in 2007. The risk assessment of any technology should be made scientifically but the public is more receptive to objective as well as statistical information (Peterson and Arntzen, 2004; Porter, 1995) which makes significant impact on adaptation and marketing of new technology.

8. Opportunities and challenges

Plant molecular farming appears to have bright future, because plants offer significant opportunities to produce a vast range of the recombinant molecules with desirable post-translational modifications. Many of these modifications cannot be achieved in bacterial production systems (Ma et al., 2003). Plants offer a lot of flexibility for engineering pharmaceutical proteins and products that can be used as research agents, animal vaccines, pharmaceutical drugs, animal feed, industrial enzymes, biopolymers and biodegradable plastics (Goldstein and Thomas, 2004; Rajasekharan, 2006; Twyman et al., 2005).

Some proteins are required in very large amounts like palivizumab and infliximab antibodies that are used systemically at doses of 10–15 mg/kg body weight or Guy's 13 mAb which prevents colonization of the oral cavity by *Streptococcus mutans*, required at a dose of 22.5 mg per course of treatment (Ma et al., 1998). Transgenic plants may be the feasible solution for these proteins as it is possible to manufacture protein up to 10 kg/year on an average 50 ha of cultivated land using potato as plant host (Spok, 2006, 2007). But before harnessing the opportunities displayed by molecular farming, there are several issues which need to be addressed. Technical difficulties associated with plant-based systems are immunological issues, problematic extraction and inconsistency in product quality (Bardor et al., 2003; Kirk and Webb, 2005). Immune system attack can make the drug ineffective and may trigger allergic reactions. Extraction of the desired compound from an environment where hundreds of other molecules are present is another problem. There is inconsistency in product yield and quality from plants of different genetic backgrounds or even from the identical plants grown in different environments (Bhat and Srinivasan, 2002). Various approaches to enhance the expression level of heterologous proteins have been discussed (Streatfield, 2007). The environmental concerns of public for potential recombinant molecules to enter in the food chain need to be addressed. Plastids can be targeted to enhance the expression level as well as to minimize the transgene flow, as plastids are maternally inherited (Daniell et al., 2001b; Staub et al., 2000). Further, the use of male sterile lines will check the production of pollen, and seed viability can be made dependent on exogenous stimulus. The use of non-food crops is another possibility to address the issue. The phenotypic markers like the colour of tomato fruits or fluorescent marker proteins can be used for the visual identification of transgenic products (Ma et al., 2005a). It is also felt that contamination is inevitable at commercial scale. Therefore, there is a need to define the maximum permissible levels to legalize the plant-made pharmaceutical contaminants (Spok, 2007).

In human clinical trials performed using plant-derived antigen proteins, dose escalation regimens have not been used which is actually the standard design for phase I vaccine trials (Kirk et al., 2005). Along with optimization of expression strategies for the development of therapeutic molecules, including vaccine antigens, focus should be on preclinical and clinical developments including question of basic immunology and dose definition. Oral tolerance is another issue which is associated with the oral delivery of vaccine antigens. Autoantigens have been delivered orally in the past to produce oral tolerance (Arakawa et al., 1998; Carter and Langridge, 2002; Ma et al., 2004; Snowden and Langridge, 2003). Due to oral tolerance risk, regulatory agencies are reluctant to conduct human clinical testing for many of the antigens. Although the antigen doses used for vaccination are not sufficient enough to develop oral tolerance and in the cases where plant-produced vaccine antigens have been used for oral delivery, there is no report of oral tolerance (Arntzen et al., 2005), oral tolerance model formulation is necessary

Table 2
List of the products in phase II, I or pre-clinical trials.

Product name	Company/institute name	Plant system	Reference/URL
Human serum albumin (HSA)	Chlologen Inc.	Tobacco	Currently technology is with Dow AgroSciences
38C13 (scFv)	Large Scale Biology Corp.	Tobacco	http://www.lsbc.com[a]
RhinoX™	Planet Biotechnology Inc.	Tobacco	http://www.planetbiotechnology.com/
Alpha inteferon 2b	Biolex Therapeutics Inc.	*Lemna*	http://www.biolex.com/
DoxoRX™	Planet Biotechnology Inc.	Tobacco	http://www.planetbiotechnology.com/
α-Caries MAb (CaroX™)	Planet Biotechnology Inc.	Tobacco	http://www.planetbiotechnology.com/
Aprotonin	Large Scale Biology Corp.	Tobacco	http://www.lsbc.com[a]
Collagen	Medicago Inc.	Alfalfa	http://www2.medicago.com/en/product/
	Meristem Therapeutics	Tobacco	http://www.meristem-therapeutics.com[b]
Lipase	Meristem Therapeutics	Maize	http://www.meristem-therapeutics.com[b]
Lactoferrin	Meristem Therapeutics	Tobacco	http://www.meristem-therapeutics.com[b]
	Ventria Bioscience	Rice, maize	http://www.ventriabio.com/
Lysozyme	Ventria Bioscience	Rice	http://www.ventria.com/;
BLX-301 (anti-CD antibody)	Biolex Therapeutics Inc.	*Lemna*	http://www.biolex.com/blx301.htm
GLA rich safflower oil	Sembiosys Genetics Inc.	Safflower	http://www.sembiosys.com/
DHA rich safflower oil	Sembiosys Genetics Inc.	Safflower	http://www.sembiosys.com/
Human intrinsic factor	Cobento Biotech AS	*Arabidopsis*	http://www.cobento.com/
Human glucocerebrosidase	Protalix Biotherapeutics	Carrot suspension cells	http://www.protalix.com/glucocerebrosidase.html
Parvovirus vaccine	Large Scale Biology Corp.	*Nicotiana*	http://www.lsbc.com[a]
Anti-F4 fimbriae of ETEC antibody	Novaplant	Pea (field trials completed 2007)	http://www.novoplant.de/fileadmin/redaktion/seitenspezifische_inhalte/news/pr_FieldTrial_210907.pdf
Protein A reagent (Stratocapture™)	Sembiosys Genetics Inc.	Safflower	http://www.sembiosys.com/

[a] LSBC filed bankruptcy in 2006.
[b] Meristem Therapeutics & SBH Sciences has announced an agreement for joint development of animal-free recombinant proteins.

before going ahead. Interaction between different partners is needed for accelerated development of plant-based vaccines. There is an urgent need for networking among biotechnology industry, food processing industry, government departments and other regulatory agencies to develop strategies for GM product development, marketing, monitoring and surveillance.

9. Commercial status

The molecular farming industry is still in its infancy, but is growing very fast. This industry comprises just a few dozen companies, most of which are involved in early research and development. A few have taken lead in producing therapeutic and other important proteins in transgenic crops. Major players that are developing transgenic plant-based products include Biolex Therapeutics Inc. (http://www.biolex.com/), Cobento Biotech (http://www.cobento.com/), Dow Agro Science (http://www.dowagro.com/), Greenovation (http://www.greenovation.com/), Protalix Biotherapeutics (http://www.protalix.com/), Medicago Inc. (http://www2.medicago.com/en/), Meristem Therapeutics and SBH Sciences, Joint venture (http://www.sbhsciences.com/news.asp), Metabolix (http://www.metabolix.com/), Nexgen Biotech (http://www.nexgenbiotech.co.kr), Phytomedics (http://www.phytomedics.com/), Planet Biotechnology (http://www.planetbiotechnology.com/), Sembiosys (http://www.sembiosys.com/), and Ventria Bioscience (http://www.ventria.com/). Basaran and Rodríguez-Cerezo (2008) have prepared an exhaustive list of institutions and organizations involved in plant molecular farming research and development worldwide. A number of transgenic plant-derived products are undergoing clinical trials or have reached at advanced stage of development (Table 2). Some products have already been commercialized and are listed in Table 1. ProdiGene Inc. was the first company to develop and commercialize products from transgenic plant systems. GUS (β-glucuronidase) was the first commercialized product. It is a homotetrameric hydrolase that cleaves β-linked terminal glucuronic acid in mono and oligosaccharides and phenols. The transgenic corn derived GUS protein is commercialized and is sold in the market by Sigma-Aldrich (product number G2035). The plant-produced human therapeutic protein, aprotonin has been commercialized with the trade name AproliZean™. Avidin is a glycoprotein and primarily a diagnostic reagent. The transgenic corn derived avidin has been commercialized and the product is being sold by Sigma-Aldrich, product number A8706 (Horn et al., 2004). Lipases are digestive enzymes necessary to assimilate lipids and their deficiency impairs nutritional status with a severe impact on life expectancy and life quality. Meristem Therapeutics has developed Merispase®, a recombinant mammalian gastric lipase for the treatment of lipid malabsorption, which is currently undergoing formulation optimizations. The products commercialized from Ventria Bioscience include Lacromin™ and Lysobac™. Lacromin™, a recombinant human lactoferrin, is a strong growth factor which outperforms Transferrin, an animal-derived growth factor. The product is commercialized and is available with InVitria (product number 777LAC015) and Sigma-Aldrich (product number L4040). Lysobac™, a plant-derived recombinant human lysozyme, is a bacterial cell lysis agent, which is also used in diagnostic applications, bioprocessing and life science research. It is four times more active than chicken lysozyme, a common animal-derived cell lysis agent for lysing *Micrococcus* and *E. coli*. The product is available with InVitria, with trade name Lysobac™ (product number-777LYS016) and Sigma-Aldrich (product number L1667). A vaccine based on HN protein of Newcastle disease virus expressed in plant has been approved by USDA (http://www.dowagro.com/newsroom/corporatenews/2006/20060131b.htm) for vaccination of chickens. Many more products are in pre-clinical trials and their number in development pipeline is increasing day by day.

10. Conclusion

Biopharms producing valuable pharmaceuticals and industrial enzymes can be a fantasy. However, increasing number of major drug companies is utilizing transgenic plants as an essential component of their business models (Kaiser, 2008; http://www2.medicago.com/en/, http://www.sembiosys.com). Transgenic plants offer several advantages over other technologies, including the ability to increase the scale of production affordably and storage of the transgenic grains for extended periods of time without any significant loss of activity (Fischer et al., 2004; Ma et al., 2003; Stoger et al., 2000). Production of drugs in plants avoids the risk of spreading animal pathogens or contaminants that may trigger an allergic response otherwise. Some technical problems remain to be solved. Glycosylation pattern of plant-produced proteins is slightly different from that of transgenic animals or animal cells (Chen et al., 2005). The antibodies produced in plants having different glycosylation patterns may not activate the complement system completely. Other challenges that need attention are increasing yield, removing processing bottlenecks and addressing bio-safety and acceptability issues. Facing a looming crisis in the production capacity for monoclonal antibodies and other protein-

based therapeutics, the industry is concentrating to solve the technical problems associated with molecular farming. Several approaches have been developed and rapid progress has been made towards addressing these issues (Bakker et al., 2001; Basaran and Rodríguez-Cerezo, 2008; Corrado and Karali, 2009; Fujiyama et al., 2009; Ko et al., 2009; Mio et al., 2008; Strasser et al., 2004, 2008; Torrent et al., 2009). As plant cell cultures are grown in confined environments, their use for the production of foreign bio-molecules, addresses the issues related to the release of genetically modified organisms (James and Lee, 2001; Shih and Doran, 2009). In the near future, it is expected to provide a captive new market for many agricultural products. A few transgenic plant-derived products like GUS enzyme and avidin have already been commercialized (Table 1) and their commercial success at a lower cost than the native proteins shows the potentials of molecular farming in plants to compete in an already established market. Many more products are on the verge of commercialization and a large number are in the pipeline of development (Basaran and Rodríguez-Cerezo, 2008; Kaiser, 2008). Key for success of transgenic plants derived products in the future will be the level of expression. It is very important with regard to economics, because it affects cost of growing, processing, extraction purification and waste disposal. It is clear that attempts would be made towards higher levels of expression. If the technical hurdles can be overcome, soon it might be possible to make protein-based pharmaceuticals available to needy at affordable cost. The full realization and impact of the aforementioned developments, however, depends not only on consistent, successful and innovative research and developmental activities, but also on a favorable regulatory climate and public acceptance. Overall production of plant-derived biologics is going to be an important methodology for the future.

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