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Recent developments in the use of transgenic plants for the production of human therapeutics and biopharmaceuticals

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Abstract

In recent years there has been a dramatic increase in the application of plant biotechnology for the production of a variety of commercially valuable simple and complex biological molecules (biologics) for use in human and animal healthcare. Transgenic whole plants and plant cell culture systems have been developed that have the capacity to economically produce large-scale quantities of antibodies and antibody fragments, antigens and/or vaccine epitopes, metabolic enzymes, hormones, (neuro)peptides and a variety of biologically active complexes and secondary metabolites for direct use as therapeutic agents or diagnostic tools in the medical healthcare industry. As the products of genetically modified plants make their way from concept to commercialization the associated risks and acceptance by the public has been become a focal point. In this paper, we summarize the recent advances made in the use of transgenic plants and plant cell cultures as biological factories for the production of human therapeutics and biopharmaceuticals and discuss the long-term potential of 'molecular farming' as a low-cost, efficient method for the production of biological materials with demonstrated utility to the pharmaceutical industry or medical community.

Introduction

Much like the Green Revolution changed agriculture of the mid-1950's leading to dramatic improvements in crop yields worldwide, the advent of genetic engineering in the early 1970's and its application to plant biology revolutionized agriculture and contributed to the dramatic growth of the biotechnology industry. Plant biotechnology now boasts one of the largest shares of the total biotechnology market globally and has impacted agricultural crop productivity, food quality, and human nutrition and health in both developed and emerging nations. Two important discoveries form the foundation of modern plant biotechnology and together enabled plant scientists to develop new procedures for crop improvement based on genetic engineering. The first was the discovery of the natural gene transfer mechanism used by the plant-pathogenic bacterium, *Agrobacterium tumefaciens*, to introduce genetic material into the plant genome during the infection process (Van Lerebeke et al., 1974). The second breakthrough was the capacity to regenerate whole plants from single cells without changing the genetic features of the cell (Birch, 1997). Together these discoveries enabled numerous laboratories worldwide to conduct successful gene transfer into plant genomes and to generate transgenic plants with stable expression of an introduced foreign gene (Bevan et al., 1983; Zambryski et al., 1983). Since that time, plant biotechnology has become a cornerstone of the expanding biotechnology revolution, witnessed the steady development of new techniques for the identification and isolation of potentially important plant genes, an improvement of methods for the manipulation and transfer of genes into plant cells, and the development of better techniques for the recovery of transformed cells and the regeneration of transgenic plants (Jähne et al., 1995; Pauls, 1995;

Saxena et al., 1999; Newell, 2000; Veronese et al., 2001; Rakoczy-Trojanowska, 2002).

The main objectives of creating transgenic plants using these new and powerful approaches are attempts to modify metabolic pathways for the production of tailor-made low molecular weight compounds and polymers, increased resistance towards biotic and abiotic stresses, improved food quality (with altered carbohydrate, protein and lipid composition), and production of polypeptides for medical, pharmaceutical or industrial use (Ap Rees, 1995; Herbers and Sonnewald, 1999; Miflin, 2000; Ye et al., 2000; Kumar, 2001; Rohini and Rao, 2001; Veronese et al., 2001; Lessard et al., 2002; Sharma et al., 2002). The exploitation of plant biotechnology to produce diagnostic and therapeutic products has become a well-recognized and important field of biopharmaceutical science with promising economic potential.

Although the major focus during last 10–20 years has been on microbial and animal cell cultures, whole plants and plant cell cultures are now con sidered as viable and competitive systems for large-scale production of industrial, pharmaceutical recombinant proteins and secondary metabolites. Recently, various recombinant proteins have been expressed in plants including antibodies, viral and bacterial antigens and various human and animal therapeutic proteins using plant genetic engineering advanced tools (Kusnadi et al., 1998; Fischer et al., 1999; Herbers and Sonnewald, 1999; Doran, 2000; Giddings et al., 2000; Daniell et al., 2001b; Larrick and Thomas, 2001; Korban, 2002). Engineered transgenic plants have many potential advantages for the production of recombinant proteins compared with microbial and animal cell culture systems, and transgenic animals. Among these are

- production of raw material on an agricultural scale at low cost with the possibility, in some cases, of using the edible plant material directly;
- reduced capitalization costs relative to fermentation methods;
- rapid scale up of production;
- correct eukaryotic assembly of multimeric proteins such as antibodies (unlike bacteria); and
- increased safety, since plants do not serve as hosts for human pathogens, such as HIV, prions, hepatitis viruses etc. (Ponstein et al., 1996; Evangelista et al., 1998; Timko and Cahoon, 1999; Langridge, 2000; Giddings, 2001; Larrick and Thomas, 2001).

The development of new plant varieties by genetic engineering is seen by some as progress towards a more environmentally friendly agriculture, with less reliance on costly fertilizers and decreased use of toxic pesticides and herbicides. In this regard, plant genetic manipulation is often more acceptable to the general public than gene-based experimentation involving animals and gene therapy research in humans (Jorgensen et al., 1996). There remains, however, a significant portion of society that is less accepting of these new technologies and passionately argues against the production and use of genetically-modified organisms (GMOs), regardless of whether it is plants, microbes, or animals. Many among this sector view the use of transgenic plants and transgenic plant-derived products as the first step towards ecological destruction. Among the concerns raised by opponents to GMOs and GM plants in particular are the potential for transgene escape to and contamination of the natural environment, increased potential for allergic for reactions resulting from presence of the transgene(s), unexpected modifications resulting from genetic manipulation leading to introduction of novel plant antigens, and a general uncertainty regarding how the growth and testing of plants used for production of human bioactive compounds would be monitored.

The purpose of this review is to outline the recent progress that has been made in the use of transgenic plants and plant cell cultures for the production of human therapeutics and related biologics, including antibodies and antibody fragments, pathogen-derived protein antigens and subunit vaccine epitopes, hormone- and neuropeptides, enzymes of therapeutic and nutritional significance and secondary metabolites with pharmacological properties of medicinal value. We will not attempt to discuss all of the recent advances made in plant genetic engineering, but rather concentrate only on those developments that bear directly on progress towards the use of plants as vehicles to produce compounds of value as human therapeutics. Four major areas of research will be addressed:

- the production of peptides, proteins, and enzymes of industrial and pharmacological value,
- the generation of antibodies and antibody fragments in plants,
- transgenic plants as vaccine production systems, and

– the manipulation of plant metabolism for the production of novel compounds and secondary metabolites with medicinal value.

When possible, we will comment on what is known about the economic feasibility of these approaches, as well as the limitations and risks involved. For information on aspects of plant genetic engineering related to improved agronomic traits, disease resistance, and food quality the reader is referred to recent reviews by Comai (1993), Kahl and Winter (1995), Goddijn and Pen (1995), and Collins and Shepherd (1996).

Production of whole antibodies and antibody fragments in plants

Antibodies are protein molecules formed by the B lymphocytes of the vertebrate immune system that recognize and bind to specific molecular epitopes (termed antigens). A wide variety of substances can serve as antigens including proteins, carbohydrates, simple organic molecules, or even metal ions. These compounds can enter the body by themselves or may be carried in by an invading organism (Kilpatrick et al., 1995). The role of the antibody is to neutralize the antigen and target it for removal from the cell. The main serum immunoglobulin antibody classes are IgG, IgA, IgM, IgD and IgE. IgG antibodies are among the most common type of antibody formed in the cells, followed by IgM and IgA. IgG antibodies consist of two pairs of identical polypeptides termed the heavy and light chains. The two heavy chains are joined to each other in areas of little variance (Fc regions) forming a Y. The two light chains are connected to the arms of the Y by disulfide bonds. The ends of the arms of the Y contain the variable regions (Fv) of the molecule responsible for interactions with a particular antigen. Each antibody molecule is made of two identical light chains and heavy chains. One heavy and light chain combine to form the antigen binding site so each immunoglobulin monomer is capable of binding two antigen molecules. The secretory antibodies have a more complex structure than their serum counterparts, they are dimers of the serum-type antibody, the two monomers being attached by an additional component call joining chain (J-chain) and there is also a further polypeptide called the secretory component, which protects the antibodies from proteases.

The constant regions of immunoglobulins are not required for antigen recognition, so it is possible to express smaller derivatives and still retain the antigen-binding specificity (Chadd and Chamow, 2001; Fischer et al., 2003). Fab and $F(ab')_2$ fragments, which contain only the sequences distal to the hinge region, and single chain Fv fragments (scFvs) which contain the variable regions of the heavy and light chains joined by a flexible peptide chain, are often more effective as drugs than full-length immunoglobulins, because they show increased penetration of target tissues, reduced immunogenicity and they are cleared from tissues more rapidly. Other derivatives include bispecific scFvs, which contain the antigen recognition elements of two different immunoglobulins and can bind to two different antigens and scFv-fusions, which are linked to proteins with additional functions (Fischer et al., 2003).

Until recently, the commercial production of antibodies was primarily accomplished by one of two means. The first method involved eliciting an immune response in a host animal, followed by animal sacrifice and recovery of the antibody from the blood serum. The second method relied on the use of animal cell culture. In either case, the amounts of antibody recovered and cost of purification placed severe limits on the availability of these materials. Once the genes encoding the heavy and light chain polypeptides were isolated from lymphocytes, it became possible to over-express these molecules in transgenic organisms.

The first reports describing the successful expression of antibodies in transgenic plants occurred in the late 1980's and early 1990's (Hiatt et al., 1989; During et al., 1990). Since that time, the combination of two rapidly advancing technologies (immunology and plant genetic engineering), has resulted in the expression of a diverse range of antibodies (often referred to as plantibodies) in numerous vascular plant and green algal species (see reviews by Conrad and Fiedler, 1994; Kilpatrick et al., 1995; Ma and Hein, 1996; Timko and Cahoon, 1999; Fischer and Emans, 2000; Smith and Glick, 2000; Larrrick and Thomas, 2001; Stoger et al., 2002).

Based on published findings, transgenic plants and/or plant cell cultures offer a number of advantages for the production of antibodies over extraction from human or animal fluids/tissues, use of recombinant microbes, transfected animal

cell lines or transgenic animals. These advantages include
- low cost of production,
- ease of scaling up or down to meet market demand,
- a lower capital cost than mammalian cell culture and other transgenic systems, and
- freedom from possible contamination with associated blood-borne interspecific pathogens.

In addition, transgenic plant systems allow for the expression of heavy and light-chains and assembly of functional dimeric antibodies *in vivo* or *in vitro*, similar to mammalian secretory antibodies (Hiatt et al., 1989; During et al., 1990; Hein et al., 1991; De Neve et al., 1993; Ma et al., 1995; Smith and Glick, 2000). Antigen-binding fragments (Fab), single-chain binding fragments (scFv) and functional full size antibodies can be expressed in leaves and seeds of plants without loss of binding specificity or affinity (Hiatt et al., 1989; De Neve et al., 1993; Ma et al., 1995; Baum et al., 1996; Fischer et al., 1999; Smith and Glick, 2000; Xu et al., 2002). Several research groups have successfully expressed active, full-length antibodies in plants by targeting the antibodies to the apoplastic space (i.e., the space between adjacent plant cells) via the endoplasmic reticulum (ER) (Conrad and Fiedler, 1998; Smith and Glick, 2000). It appears that the apoplast provides an environment suitable for the accurate assembly of these complex molecules.

Generally, one of two different approaches has been employed to produce biologically active whole antibodies in plants: transformation of the heavy and light-chain genes separately into plants, followed by cross-pollination of the two transgenic parents to yield F_1 individuals carrying both the heavy and light chain gene (Hiatt et al., 1989; Hein et al., 1991; Ma et al., 1994) and co-transformation of the heavy and light chain genes on a single expression cassette (During et al., 1990; DeNeve et al., 1993; Voss et al., 1995). Although co-expressing the heavy and light chain genes on a single expression cassette may be less involved (see During et al., 1990; DeNeve et al., 1993; Voss et al., 1995), by using the cross-pollination strategy, it is also possible to produce a wide range of novel antibodies by combining plant lines carrying different sets of modifications in either the heavy or light chains (Ma et al., 1994).

Using the former strategy, Hiatt and his coworkers (Hiatt et al., 1989; Hiatt and Ma, 1993) reported the successful production of the IgG 6D4 antibody in plants. The IgG 6D4 antibody recognizes a synthetic phosphonate ester P_3 and catalyzes hydrolysis of certain carboxylic esters. Hiatt and his coworkers constitutively expressed the IgG heavy and light chain genes in separate tobacco plants and then cross-pollinated the transgenic parental line to produce F_1 progeny co-expressing both chains. The majority of the F_1 progeny that expressed both genes produced assembled full-length 6D4 antibodies. The most productive of the F_1 line expressed full-length 6D4 antibody as 1% of the total soluble protein. Suspension cell cultures derived from the F_1 transgenic progeny secreted up to 20 mg l^{-1} of antibody into the culture medium (Hein et al., 1991). Subsequently, Ma et al. (1995) reported the expression of the more complex IgA secretory antibodies in plants. Secretory IgA is the primary form of antibody produced in mucosal secretions throughout the gastrointestinal tract. To assemble IgA complexes, Ma et al. (1995) engineered four transgenic tobacco lines, each containing different component of the IgA complex then crossed in various combinations and obtained 200–500 μg of recombinant IgA antibody per 1 gm of fresh leaf tissue. This antibody was functionally indistinguishable from antibody elicited in mouse in its ability to recognize and bind antigen (Ma and Hein, 1996). Interestingly, synthesis of IgA antibodies normally requires the co-operation of two different mammalian cell-types (i.e. plasma and epithelial cells), while a single transgenic tobacco cell was able to assemble the antibody without requiring further engineering of assembly factors and chaperonins. Although co-expressing the heavy and light-chain genes on a single expression cassette may be less involved than using the cross-pollination strategy, it is also possible to produce a wide range of novel antibodies by combining plant lines carrying different sets of modifications in either the heavy or light chains (Ma et al., 1994).

Although tobacco leaf has been the most popular expression system for recombinant antibodies, several groups have explored the use of other plants and plant seeds for use in expression and large-scale production. For example, Stoger et al. (2000) successfully expressed the scFv antibody (ScFvT84.66) against carcinoembryonic antigen

(CEA), in the leaves and grain of transgenic rice and wheat. In general, expression levels of the ScFvT84.66 antibody were less in wheat than in rice, corresponding to about 0.1% of the total soluble protein. By comparison, expression in both of these crop plants was less than the optimized levels reported in tobacco (Conrad and Fielder, 1998). Transgenic field pea (*Pisum sativum* L.) seeds have also been used for the production of the ScFvT84.66 antibody fragments (Perrin et al., 2000). In their work, Perrin et al. (2000) observed levels of expression up to 9 μg/g tissue, comparable to the levels of expression seen in other plant systems. High levels of antibody production could also be achieved in transgenic potato tubers, as demonstrated by Artsaenko et al. (1998) for the biosynthesis of a scFv that specifically binds the synthetic hapten oxazolone. Among the more notable transgenic plant systems in use for antibody formation is the exploitation of transgenic corn seeds as a production venue. As recently reviewed by Hood et al. (2002), corn seeds have shown particular utility in the production of high levels of secretory immunoglobulin A (sIgA), the most abundant antibody class produced by the body (>60% of total immunoglobulin) and secreted onto mucosal surfaces to provide local protection from toxins and pathogens. To produce the recombinant sIgA in transgenic corn seeds four different genes encoding the four protein chains in the sIgA (i.e., humanα-herpes light-chain, humanα-herpes heavy-chain, human J-chain and human secretory component) were co-expressed. In these gene constructs the coding regions of the genes were linked to the constitutive CaMV 35S promoter and barley α-amylase signal sequence (BASS), which directs the proteins to the cell wall matrix. Using this expression scheme recombinant sIgA constituting up to 0.3% of the total soluble protein in a single seed was achieved. Based upon previous studies with transgenic corn demonstrating increases in recombinant protein expression in the range of 70–150-fold following six or more generations of recurrent selection and backcrossing, Hood (2002) projects that transgenic corn seed will be among the better commercial systems for antibody expression.

With a short period of time, the production of recombinant plant-derived antibodies has moved from theory to Phase II clinical trials. According to the Pharmaceutical Research and Manufacturers of America (see http://www.pharma.org), recombinant antibodies represent the single largest group of biotechnology-derived molecules in clinical trials occupying 20% of all biopharmaceuticals and are expected to reach five billion dollars prospective market by 2005. This is likely due to the fact that antibodies have a large diversity of potential applications including use in

– medical diagnosis and therapy,
– the detection and removal of environmental contaminants,
– the control of pathogens and (iv) industrial purification processes.

Among the products with significant potential are the following:

CaroRx^{TM}

*Caro*Rx™ is an anti-*Streptococcus mutans* secretory immunoglobulin A (sIgA) used for the prevention of dental caries (Ma et al., 1998; Larrick and Thomas, 2001). Ma et al. (1998) compared a monoclonal secretory antibody generated in transgenic plants and its parent murine IgG antibody and reported that the affinity constants for both antibodies for a *S. mutans* adhesion protein were similar. However, the secretory antibody had a higher functional affinity due to its dimeric structure. In the human oral cavity, the secretory antibody survived for up to three days, compared with one day for the IgG antibody. The plant secretory antibody afforded specific protection in humans against oral streptococcal colonization for at least 4 months (Ma et al., 1998). Thus, initial clinical studies with *Caro*Rx™ have demonstrated that topically applied anti-*S. mutans* plantibody is safe (i.e., elicits no human antimouse antibody response and no local or systemic toxicity) and effective (i.e., prevents colonization by *S. mutans*, the major cause of human dental caries). Planet Biotechnology Inc. completed United States Food and Drug Administration (FDA)-approved phase I/II confirmatory clinical trials at the School of Dentistry at the University of California, San Francisco in late 2000.

Avicidin

Avicidin is a full-length antibody specific for EpCAM (a marker of colorectal cancer) developed jointly by NeoRx and Monsanto. Avicidin

employs a mouse-derived antibody that is in clinical trials to treat patients with solid tumors such as lung, prostate, colon and ovarian cancers that no longer respond to standard therapies (Fischer and Emans, 2000). Although tumor shrinkage has occurred in some of these patients, the number of doses that can be administered is limited because mouse-derived antibodies are known to cause human anti-mouse antibodies in most patients. NeoRx represented the first time that such a protein produced in plants has been tested in humans. Unfortunately, Phase II trials were discontinued after exhibited significant gastrointestinal side effects (Fischer and Emans, 2000).

T84.66, scFvT84.66, and T84.66/GS8

T84.66 is a monoclonal antibody that has been used successfully for *in vivo* imaging and diagnosis of human colorectal carcinoma (Williams et al., 1990). It recognizes carcinoembryonic antigen (CEA), a well characterized tumor associated glycoprotein. CEA can be detected in almost all human colon cancers, 50% of all breast cancers, and in other tumors of epithelial origin (Vaquero et al., 1999). scFV T84.66 is an engineered antibody fragment (VL-VH-CH3, or 'minibody') with bivalent binding to CEA that was produced by genetic fusion of a T84.66 (anti-CEA) single-chain antibody to the human IgG1 CH3 domain (Hu et al., 1996). The T84.66/GS8 is a scFv dimer (diabody) constructed from the variable regions of the anti-CEA monoclonal antibody T84.66 using a peptide linker of only eight amino acid residues and this diabody bound antigen bivalently as well as retained a high apparent affinity for CEA (Wu et al., 1999). The full length IgG has been transiently expressed in tobacco by agroinfiltration (Vaquero et al., 1999) and the scFv has been expressed in transgenic tobacco, pea (Perrin et al., 2000), rice and wheat (Stoger et al., 2000). Fischer et al. (2003) produced a diabody, T84.66/GS8, by agroinfiltration of tobacco and Vaquero et al. (2002) reported a diabody fusion with interleukin-2 (IL-2) in transgenic tobacco plants.

Anti-HSV

The Anti-HSV IgG recognizes herpes simplex virus 2 (HSV-2). Zeitlin et al. (1998) expressed humanized anti-herpes simplex virus 2 (HSV-2) monoclonal antibody in soybean and compared it with the antibody expressed in mammalian cell culture. They reported their similarity in stability in human semen and cervical mucus over 24 h and their ability to diffuse in human cervical mucus, and their efficacy for prevention of vaginal HSV-2 infection in the mouse. The topical application of the recovered recombinant antibody has been shown to prevent vaginal HSV-2 transmission in a mouse model of the disease (Zeitlin et al., 1998).

38C13

38C13 is an anti-idiotype scFv capable of recognizing the mouse lymphoma cell line 38C13 and providing immunity against lethal challenge with the lymphoma (McCormick et al., 1999). McCormick et al. (1999) created a modified tobamoviral vector encoding the idiotype-specific single-chain Fv fragment of the immunoglobulin, infected tobacco plants and reported high levels of secreted scFv protein in the extracellular compartment. The mice were protected from challenge by a lethal dose of the syngeneic 38C13 tumor, similar to mice immunized with the native 38C13 IgM-keyhole limpet hemocyanin conjugate vaccine, hence this rapid production system for generating tumor-specific protein vaccines may provide a viable strategy for the treatment of non-Hodgkin's lymphoma (McCormick et al., 1999).

Anti-human chorionic-gonadotropin (HCG)

HCG is synthesized and secreted soon after fertilization and traditionally it has been used as an index for pregnancy (Bottger et al., 1993). HCG circulates from the trophoblast to the ovaries, and is amenable to inactivation by antibodies. Since immune response to vaccines varies greatly between individuals, passive immunization using antibodies neutralizing HCG has been proposed as a contraceptive measure (Talwar et al., 1994) and other applications of anti-HCG antibodies are in the diagnosis, prognosis and immunotherapy of cancers (Okamoto et al., 2001). In order to investigate the efficacy of different antibody formats specific for HCG, Kathuria et al. (2002) engineered a chimeric full-size IgG, an scFv fragment, and a diabody derived from the original anti-hCG murine mAb PIPP and expressed them in tobacco plants. The recombinant antibody transiently

produced in tobacco leaves retained its specificity for β-HCG and HCG. Antibody efficacy was confirmed *in vitro* by inhibiting HCG induced production of testosterone by Leydig cells and blocking HCG induced rise in mouse uterine weight *in vivo*. Studies of efficacy have shown that the full-size anti-hCG antibody was 1000 times more active than either the scFv fragment or diabody derivative. These could potentially be used for pregnancy detection (emergency) contraception and the diagnosis and/or therapy of tumors that produce hCG (Kathuria et al., 2002).

The recent availability of large amounts of secretory IgA plantibodies has also opened up a number of novel therapeutic opportunities for disorders of the mucosal immune system. These include therapies for intestinal pathogens such as *Helicobacter pylori*, hepatitis virus, and enterotoxigenic *E. coli*, respiratory pathogens such as rhinovirus and influenza (Larrick and Thomas, 2001), genito-urinary sexually transmitted diseases (e.g. HSV) and contraception (Zeitlin et al., 1998).

Factors influencing use and efficacy of plant-derived antibodies

The level to which recombinant antibody (rAb) accumulates in a particular plant expression system can be enhanced by use of appropriate regulatory elements in the expression construct, by optimizing codon usage in the rAb gene to better match the usage in the plant, and by enhancing the stability of the antibody. Other considerations also exist. For example, it is important to recognize that potential differences in activity and specificity might exist between native antibodies and recombinant antibodies produced in animal or human cells and plant-based systems. Such differences could arise because of subtle differences in antibody structure caused by small changes in secondary modifications to the protein. Of these modifications, changes in glycosylation pattern between plant and animal cells are among the most likely to affect antibody activity. For this reason, recent efforts have focused on the humanization of plant glycans to reduce immunogenic potential. Cabanes-Macheteau et al. (1999) analyzed the glycosylation of a functional mammalian glycoprotein expressed in a transgenic plant. They compared N-linked glycan attached to heavy chains of Guy's 13 monoclonal antibodies produced in transgenic tobacco plants and IgG_1 of murine origin. The number of Guy's 13 glycoforms is higher in the plants than in the mammalian expressed antibodies. In addition to high-mannose-type N-glycans, 60% of the oligosaccharides N-linked to the plantibody have β- (1,2)-xylose and α-(1,3)-fucose residues linked to the core Man-3-GlcNAc-2. These linkages which are not found in mammalian N-linked glycans, are potentially immunogenic. Thus, these plantibodies may show the potential toxicity in humans. Bakker et al. (2001) reported the stable expression of human β-1,4-galactosyl transferase (most important enzyme, i.e. missing for conversion of typical plant N-glycans into mammalian-like N-glycans) in tobacco plants. Crossing a tobacco plant expressing human β-1,4-galactosyl transferase with a plant expressing the heavy and light-chains of a mouse antibody resulted in the expression of a plantibody that exhibits partially galactosylated N-glycan (50%). This level of carbohydrate incorporation is approximately as abundant as when the same antibody is produced by hybridoma cells. These results represent a major step forward in the engineering of the N-glycosylation of recombinant proteins.

While altering of the glycan structure has become an essential aspect of antibody engineering, another option is the creation of aglycosyl antibodies. This is accomplished by altering the peptide recognition sequences for N-linked glycosylation (Asn-X-Ser/Thr). High mannose-type glycosylation may be favored by the addition of a C-terminal KDEL sequence, and the subsequent targeting of the plantibodies to the proximal endoplasmic reticulum. The KDEL sequence is not cleaved during processing and the potential of a KDEL tag to alter immunogenicity, pharmacokinetics, or other properties is not known (Larrick et al., 2001).

Effects of plant developmental age and environment

There is currently little information available about the effects of age developmental stage, and physiological changes on the yield and quality of foreign proteins produced in plants. Stevens et al. (2000) showed that the levels of an IgG1 antibody from mouse heterologously expressed in tobacco paralleled total soluble protein levels and that the ratio of the heterologous IgG1 and leaf total soluble protein was constant throughout the development of the leaf. In most cases, total soluble

protein contents decrease when the plants begin to senesce. During senescence, some enzymatic processes are activated, but a majority of proteins show increased turnover rates (Smart, 1994; Buchanan-Wollaston, 1997). Proteolytic degradation is a major part of the senescence process, beginning when the plant tissue reaches maturity. Proteolytic degradation can be a serious obstacle for the production of antibodies *in planta*, by negatively affecting product homogeneity. Regulation of environmental conditions had only a moderate affect on heterologous protein expression. For example, decreasing the growth temperature altered the timing of antibody turnover/decline indirectly by controlling the rate of plant development. The accumulation of antibody in mature leaf tissue was slightly up-regulated by increased light levels, but this reflected a general increase in leaf total soluble protein content with higher irradiance (Stevens et al., 2000).

Purification

Recently, significant progress has been made for recombinant antibody production in tobacco, corn and other crops. Particularly, expression in seeds assures excellent storage properties and another advantage is the limited range of proteins in the seeds, which added flexibility in processing (Stoger et al., 2002). Commercial scale production of recombinant antibodies from plants will also benefit from the food and beverage industrial technologies (Larrick et al., 2001). There are major hurdles of purification of antibodies from plants, which can be sorted out easily. A major concern of phenolics, which alter the properties of protein dramatically and irreversibly, can be removed by ultrafiltration or diafiltration. Other concerns include secondary metabolites, endotoxins and mycotoxins, which can be minimized by rapid processing and early filtration (Larrick et al., 2001). The removal of these constituents is an important aspect for progressing towards the clinical trials of plantibodies, which is really not a major hurdle as several antibodies have been purified to homogeneity for human clinical use.

Production timelines

Plants offer a number of attractions over traditional pharmaceutical manufacturing for the production of simple and complex therapeutic proteins. After development of a transformed cell line, production of recombinant protein in the range of 5–20 g in mammalian cell culture systems requires about 3 months (Morrow, 2002). Scale-up from this level of protein production requires considerably more time and large capital investments. In many cases, it will cost significantly less to grow plants with the ability to mass-produce therapeutic/pharmaceutically-important proteins because plant-based expression systems are not likely to be associated with the same level of capital investment. Plant-based production schemes can also be more easily expanded or contracted to provide quantities of product reflective of demand. Depending on generation time, one or more harvesting of the transgenic plants producing the target recombinant protein(s) can be achieved within a single growing season. Moreover, plants and their seeds are a renewable resource that can be quickly scaled up to meet needs. Cline (2003), Director of Government and Industry Affairs at Monsanto Protein Technologies, estimated that an animal cell culture manufacturing facility designed to produce 500 kg of MAb per year of the type (a single product facility) currently used by the pharmaceutical industry would require up to $450 million dollars in capital and would take 4–7 years to build and approve. In contrast, the same product could be grown on 500 acres of corn, purified in a facility costing $80 million and requiring three to five years to build and approve. Cost per gram of MAbs by traditional means is $350–$1200 per gram (depending on scale). Corn would cost $80–$250 per gram (depending on scale). Similarly, it has been suggested that gram quantities of recombinant antibodies can be produced from tobacco cell cultures or transgenic plant leaf material in 3–4 months with scale-up to kilograms in less than 2 years (Hood et al., 2002). Among the other production benefits are simplified downstream processing compared to traditional production technology, the ability of transgenic plants to accommodate a wide range of protein types, including some not possible by traditional production technology, and enhanced safety. This latter factor is extremely important since transgenic plant expression systems provide no known risk of transmissible spongiform encephalopathies (prions) and no known transmission of plant viruses to humans.

Currently, more than 200 novel antibody-based potential products are in clinical trials worldwide, and market demand will certainly strain the capabilities of existing production systems (Stoger et al., 2002). In summary, plant-based systems for rAb production are highly competitive in terms of productivity, safety, cost and timelines (Humphreys and Glover, 2001).

Production of subunit vaccines

Vaccines consist of any antigenic substance(s) (proteins, peptides, attenuated living or killed organisms) capable of eliciting an immune response that precludes infection or disease manifestation upon later challenge by a pathogenic organism. Vaccination can be accomplished by introducing the antigen parenterally into the body or bloodstream, thus eliciting a serum immune response, or by oral ingestion of the antigen eliciting a mucosal immune response. For the most part, vaccines have relied upon serum responses, although there are good examples of successful oral vaccines (i.e., vaccine against polio virus). By comparison, parenteral immunization is more efficient since oral immunization appears to require a considerably large amount of subunit or soluble antigen (mg versus μg amounts) to elicit a response (De Aizpurua and Russell-Jones, 1988). For this reason at the present time there is considerable interest in exploiting the ability to overexpress antigenic proteins to human disease-causing agents in transgenic plants for use in vaccine production.

Immunization by injection (parenteral delivery) rarely results in specific protective immune responses at the mucosal surfaces of the respiratory, gastrointestinal and genito-urinary tracts. Many pathogens enter the body through the nose, mouth or other openings, and therefore, mucosal immune responses represent a first line of defense and the biggest pathogen-deterring surface in the body. When the mucosal immune response is effective, it stimulates the generation of molecules known as secretory antibodies (IgA) that dash into the cavities of those passageways, neutralizing pathogens they find. An effective reaction also activates a systemic response, in which circulating cells of the immune system help to destroy invaders at distant sites. In addition, mucosal vaccines delivered orally increase safety and compliance by eliminating the need for needles. While subunit vaccines are effective, they currently depend on capital-intensive fermentation-based technology and a 'cold chain' (refrigeration) for delivery. Both of these factors create constraints in use in the developing world, where vaccines are needed the most.

The idea for transgenic plant-derived vaccines originated in the early 1990s. At the time, Charles Arntzen and his colleagues envisaged a cost-effective vaccine production system with a safe and efficacious delivery system through the use of plants specifically engineered to deliver safe subunit preparations of candidate antigens for major diseases afflicting developing and developed nations (Mason and Arntzen, 1995; Walmsley and Arntzen, 2000). In this scenario, antigens could be produced in palatable plant organs (i.e., fruits, tubers and seeds) allowing them to be eaten directly, thereby immunization and sidestepping the demands for purification, numerous associated production and storage (refrigeration) costs and cost in time and personnel for delivery/administration of the vaccine (Langridge, 2000; Walmsley and Arntzen, 2000). In addition, transgenic plants could potentially be used to produce kg amounts of antigen utilizable in more traditional parenteral or oral vaccine preparations.

The first demonstration of vaccine antigen expression was reported in 1990 when R.I. Curtiss and C.A. Cardineau expressed the *Streptococcus mutans* surface protein antigen A (SpaA) in tobacco (Curtiss and Cardineau, 1990). After feeding the transgenic tobacco tissue to mice, the researchers observed that a mucosal immune response was induced in response to the SpaA protein. Although the mice were not subsequently tested for protection by direct challenge with the pathogen, the induced antibodies were demonstrated to be biologically active when they reacted with intact *S. mutans*. Subsequently C. Arntzen and his collaborators provided proof of concept for what is now referred to as 'edible vaccines' by showing that the surface antigen of Hepatitis B could be synthesized in transgenic plants and that animals fed the transgenic plant materials containing the immunogenic protein developed a specific immune response against the recombinant antigens (Mason et al., 1992; Mason, 2002).

Since these initial reports, numerous plant species have been used for antigen expression, including tobacco, potato, tomato, banana, corn, lupine, and lettuce (Walmsley and Arntzen, 2000;

Carter and Langridge, 2002; Sala et al., 2003) and the list of protective antigens from microbial and viral pathogens that have been expressed in plants has become increasingly larger as attention spreads from human pathogens to animal pathogens (Table 1). The development of edible oral vaccines is certainly among the most exciting current research areas (for reviews see Mason and Arntzen, 1995; Featherstone, 1996; Thanavala, 1996; Sala et al., 2003).

Vaccine candidate expression systems

Transgenic expression of candidate vaccine antigens has been accomplished primarily in two ways: the use of stable plant transformation and by transient gene expression. Stable integration of recombinant DNA into the nuclear or chloroplast genome of the plant cell, followed by regeneration of the transformed plant, results in the production of a genetic line that can be propagated either by vegetative means (e.g., stem and root cuttings) or by sexual reproduction through seed production. A foreign gene integrated into the nuclear genome will be inherited and expressed typically in a Mendelian fashion, whereas those expressed in the chloroplast will show maternal patterns of inheritance. *Agrobacterium tumefaciens*-based gene transfer is the most common way researchers have introduced sequences encoding vaccine antigens/

Table 1. Different antigens expressed in plants

Antigen	Pathogen or disease	Plant species	Reference
Hepatitis B surface Antigen (Hbs Ag)	Hepatitis B	Tobacco	Mason et al. (1992)
LT-B	*E. coli* heat-labile enteroxin B subunit	Potato, Tobacco	Haq et al. (1995)
SIgA-G	*Streptococcus mutans*	Tobacco	Ma et al. (1995)
Glycoprotein G	Rabies virus	Tomato	McGarvey et al. (1995)
Capsid protein	Norwalk virus	Tobacco, Potato	Mason et al. (1996)
CT-B	*Vibrio cholerae*	Potato	Arakawa et al. (1998a, b)
Insulin	Diabetes (autoimmune)	Potato	Arakawa et al. (1998a, b)
Structural protein VP 1	Foot and mouth disease	Arabidopsis	Carrillo et al. (1998)
Spike (S) glycoprotein	Swine transmissible gastroenteritis coronavirus (TGEV)	Arabidopsis	Gomez et al. (1998, 2000)
s-LT-B	Heat-labile enterotoxin B	Potato	Mason et al. (1998)
Structural protein VP60	Rabbit hemorrhagic disease virus (RHDV)	Potato	Castanon et al. (1999, 2002)
HBsAg	Hepatitis B	Lupin, Lettuce	Kapusta et al. (1999)
Glycoprotein B	Human cytomegalovirus (HCMV)	Tobacco	Tackaberry et al. (1999)
HBsAg	Hepatitis B	Potato	Richter et al. (2000)
F protein	Respiratory syncytial virus (RSV)	Tomato	Sandhu et al. (2000)
S-glycoprotein	TGEV	Tobacco	Tuboly et al. (2000)
Capsid protein 2L21	Canine parvovirus	Arabidopsis	Gil et al. (2001)
Hemagglutinin protein	Measles virus (MV)	Tobacco	Huang et al. (2001)
Human acetyl-choline esterase (AchE)	Organophosphate poisoning	Tomato	Mor et al. (2001)
LT-B	Heat-labile enterotoxin B subunit	Corn	Streatfield et al. (2001)
S	TGEV	Corn	Streatfield et al. (2001)
CT-A2:CFA/I-CT-B:NSP4	Cholera, enterotoxigenic *E. coli*	Potato	Yu and Langridge 2001
Protective antigen	*Bacillus anthracis*	Tobacco	Aziz et al. (2002)

epitopes into the plant nuclear genome. Several laboratories are also developing chloroplast transformation and expression systems for vaccine antigen expression (Daniell et al., 2001a; Ruf et al., 2001). Some success in improving levels of gene expression over those observed with nuclear transformation (Daniell et al., 2001a, 2002).

As an alternative to stable gene integration, a number of research groups have now demonstrated that specifically modified recombinant plant viruses can be used as vectors for the transient expression of foreign genes and their encoded proteins in plant cells (Beachy et al., 1996; Modelska et al., 1998; Nemchinov et al., 2000; Koprowski and Yushibov, 2001; Mor et al., 2003). The rationale of these studies is based on the notion that during viral replication, gene copy number becomes amplified, resulting in a much higher level of transgene expression than observed with stable transformation. Among the potential advantages of transient viral expression of transgenes over stably transformed transgenic plants are the shorter time for cloning of foreign genes in the viral genome as compared with the time required to transform plants, the ease at which antigen production can be scaled up, and the wide host range of plant viruses that allows the use of multiple plant species as biofactories (Koprowski and Yushibov, 2001).

At the present time, the prospect of developing effective edible vaccines for oral immunization is not without limitations. Expression levels obtained thus far in transgenic plants are below optimum and need to be enhanced. In addition, not all vaccine candidate proteins are highly immunogenic in plant tissues and secondary metabolites found in plants may compromise the ability of the vaccine candidate protein to induce immunity. Most of the examples discussed above commonly showed that plants accumulate foreign proteins to relatively low levels (0.01–2% of total soluble protein). Less immunogenic proteins would require even larger doses to be effective. Even with more palatable alternatives to potatoes (e.g. banana), these accumulation levels limit the practicality of edible vaccines. Two solutions to overcome this limitation are being explored. First, techniques to enhance antigen accumulation in plant tissues are being explored. A number of factors, including codon usage, the nature of the transcriptional regulatory sequences (or promoter) used, the type of 5′-untranslated sequence incorporated, the presence of specific intra- and extracellular targeting or compartmentalization sequences present, the site of gene integration into the genome, etc. affect transgene expression and ultimately vaccine epitope accumulation in plants. Optimization of coding sequences of bacterial or viral genes for transient expression, as well as defining the best subcellular compartment for product accumulation to obtain optimal quantity and quality, is also being considered. To enhance the immunogenicity of the orally delivered antigens, the use of carrier proteins may also be required, especially for small, non-particulate subunit vaccine antigens (Walmsley and Arntzen, 2000). Another approach is to use bacterial enterotoxins such as CT or LT (Mason and Arntzen, 1995), mammalian and viral immunomodulator, or plant-derived secondary metabolites (Yu and Langridge, 2001). Clearly, additional experimentation in this area is needed.

The status of human trials of hepatitis b, bacterial and norwalk virus-induced diarrhea

Human trials of edible plant vaccines are currently underway for hepatitis B and bacterial and Norwalk virus-induced diarrhea. The status of this work is described below.

Hepatitis B vaccine development

Over a decade has passed since Mason et al. (1992) reported the successful expression of the Hepatitis B antigen (HbsAg) in tobacco for the first time. Although the levels of expression were low (approximately 0.01% of the total soluble protein), the plant-expressed antigen formed a virus like particle (VLP) similar in appearance to that formed by the commercially available vaccine Rombovax[7] produced in yeast by Merck, Sharpe and Dohme and elicited immunogenic responses (Thanavala et al., 1995). Various researchers have reported the use of VLPs or non-living vaccines expressed in plants (Langeveld et al., 2001; Lauterslager et al., 2001) and in preliminary human trials (Tacket et al., 1998; Kong et al., 2001). Subsequently, the HBsAg has been expressed in potato (Arntzen et al., 1994; Richter et al., 2000), banana (May et al., 1995) and lettuce (Kapusta et al., 1999). Following initial clinical trials,

Kapusta et al. (1999) reported that oral delivery of the HbsAg stimulated development of anti-HBsAg IgG in humans. In subsequent clinical trials performed at Roswell Park Cancer Institute (Buffalo, NY), patients who had previously been vaccinated with the yeast recombinant injectable HBsAg and were fed raw potatoes expressing the HBsAg showed stimulation of antibody titres. Based on these promising preliminary results, trials continue to more forward (Mason et al., 2002).

Bacterial diarrhea

Mason et al. (1998) and Arakawa et al. (1998a, b) engineered transgenic potatoes to produce part of labile toxin B (LT-B) secreted by *E. coli* bacterium and Cholera toxin B (CT-B) secreted by *Vibrio cholerae* bacterium respectively. Both these organisms cause diarrhea with secreted toxins that bind specifically to GM1 gangliosides present on epithelial cell surfaces. Both recombinant LT-B and CT-B produced in transgenic potatoes showed antibody responses after ingestion by mice, proving that the plant-produced proteins folded correctly and assembled native GM1 binding pentameric complexes (Arakawa et al., 1998a, b; Mason et al., 1998). In the human trial (Phase I Proof-of-Concept trial) performed with 14 healthy adults, 11 were chosen at random to receive the genetically engineered potatoes and three received pieces of ordinary potatoes. The investigators analyzed blood and stool samples from the volunteers and evaluated the vaccine's ability to stimulate both systemic and intestinal immune responses. Ten of eleven volunteers (91%) who ingested the transgenic potatoes had a four-fold increase in serum antibodies at some point after immunization and six of eleven volunteers (57%) developed a four fold increase in intestinal antibodies. The potatoes were well tolerated and no one experienced serious adverse side effects (Mason et al., 2002).

Viral diarrhea

Norwalk virus capsid protein (NVCP) from the diarrhea causing Norwalk virus, expressed in transgenic tobacco and potato with 0.23% of total soluble protein, also assembled VLPs and stimulated serum IgG and gut IgA specific for NVCP when fed to mice (Mason et al., 1996). The clinical trial was conducted at the Center for Vaccine Development with NVCP potatoes (Tacket et al., 2000). Twenty adults ingested either two or three doses each of 150 g raw potato containing 215–750 μg NVCP. Nineteen of twenty adults showed significant increases in the numbers of specific anti-NVCP antibody-secreting cells of the IgA subtype, and six developed increases in IgG antibody-secreting cells. This study proved that orally delivered plant-expressed VLPs could stimulate immune responses and further that GM1 binding activities not required for oral immunization.

Novel vaccine targets

Potential applications of edible vaccines include therapies using auto-antigens, the body's own proteins recognized as foreign by the immune system. Autoimmune diseases include arthritis, myasthenia gravis, multiple sclerosis and type I diabetes. Ma and Jevnikar (1999) expressed glutamic acid dehydrogenase in potatoes and fed them to non-obese diabetic mice, in which the reduced pancreatic islet inflammation suggested immuno-tolerization of cytotoxic T-cell-mediated autoimmune disease. As usual an appropriate oral dose of a plant-derived auto antigen will inhibit development of the autoimmune disease (Carter and Langridge, 2002; Sala et al., 2003).

Edible vaccine development for the prevention or treatment of cancer is difficult since tumor antigens are also auto-antigens (Zhang et al., 1998). Recently, a poly-epitope isolated from a human melanoma tumor was integrated into the nuclear and chloroplast DNA of tobacco in an attempt to develop a plant-derived melanoma vaccine (Sala et al., 2003). McCormick et al. (1999) expressed a scFv antibody fragment of the immunoglobulin from a mouse B-cell lymphoma in tobacco with a viral vector and showed that mice injected with this vaccine were protected from challenge by a lethal dose of tumor. Another scFv fused to the potato virus X coat protein generated protection against lymphoma and myeloma (Savelyeva et al., 2001).

Peptides, proteins and enzymes of pharmacological value

In addition to plant-derived antibodies and edible vaccines, various other pharmaceutically

important peptide, proteins and enzymes have been produced in transgenic plants, offering numerous biological and economical advantages as compared to other industrial systems (i.e., extraction from human fluids or tissues, recombinant microorganisms and microbial fermentation, transgenic animals and animal cell culture) (Cramer et al., 1996; Timko and Cahoon, 1999; Giddings, 2001). For example, the cost of producing large numbers of recombinant proteins in plants and generating large amounts of biomass as a starting point for protein or enzyme purification is relatively inexpensive compared to the cost of large scale microbial fermentation and animal cell culture.

Unlike many organisms, plant cells are quite amenable to accepting and expressing genetic information from a wide range of organisms, including genes from prokaryotic and eukaryotic sources (Walden and Wingender, 1995). Over the past decade a large number of laboratories have reported the successful expression of a variety of heterologous genes into plant cells that are capable of exhibiting, proper temporal and spatial regulation, either through stable integration into the nuclear or organellar genomes, or by transient expression. More importantly, gene transfer methods with properly defined transcriptional and translational control signals, the levels of transgene expression and heterologous protein biosynthesis fall well within the range necessary for industrial scale production. As might be expected, expression levels vary among the introduced genes as a result of a number of factors mentioned earlier in this review.

Transgenic plants also have an advantage over genetically engineered microorganisms for the production of some proteins, as they are able to produce mammalian proteins that contain appropriate post-translational modifications (e.g., glycosylation, prenylation, formation of sulfhydryl bridges) often necessary for proper protein or enzyme function. However, there are some problems, notable among these is proper glycosylation, which can adversely affect protein function. This potential problem is likely to be overcome as we learn more about the requirements for these processes in both plant and animal cells (Bakker et al., 2001).

Another concern is about the potential contamination of plant-derived recombinant proteins with potentially toxic factors due to the fact that some plant species contain numerous toxic alkaloids and other secondary metabolites. Careful selection of appropriate plant materials for heterologous expression can help alleviate this potential problem. Finally, one can not overlook the fact that genetic engineering of plants does not raise the same ethical issues within the public as does the use of transgenic animals for experimental research and industrial production. As noted above, plant genetic engineering, like all aspects of gene research and biotechnology, has its proponents and detractors. As with all new advances, time and careful scrutiny of costs and benefits will determine whether or not wide-spread use and public approval are gained.

Plants have been tested for a range of therapeutic proteins to be used either directly in foods or after purification. Expression of milk proteins, such as lactoferrin and β-casein, in plants may contribute the therapeutic values of these protein to other food products (Arakawa et al., 1999; Chong and Langridge, 2000). Earlier, Vandekerckhove et al. (1989) reported the successful heterologous expression of leu-enkephalin, a pain-killing neuropeptide in plants when fused to part of the *Brassica napus* 2S seed storage protein.

Recently, Staub et al. (2000) reported the production of correctly processed human somatotrophin (hST) in transgenic tobacco plants, with an expression level in chloroplast of up to 7% total soluble protein, while Daniell et al. (2002) have reported that 0.02–11.10% of correctly folded hST of total soluble protein in choloroplasts. The growth hormone ST is used for the treatment of hypopituitary dwarfism in children, and has possible future uses in treating Turner syndrome, chronic renal failure and HIV wasting syndrome (Staub et al., 2000). This is interesting because no plastid-encoded proteins are known to have disulphide bonds, and it was not previously established whether the chloroplast could correctly process proteins requiring disulphide bonds for biological activity. Although hST was one of the first proteins to be available from recombinant *E. coli*, transgenic plants have the advantage of being able to produce somatotrophin that needs no post-extraction chemical processing. Another recent example is, adenosine deaminase (ADA) production, demonstrated in transgenic maize, as part of the development of a selectable marker system to possibly be used for

the treatment of ADA-deficient severe combined immunodeficiency disorder (Petolino et al., 2000). Other proteins that have potential therapeutic applications after expression and purification from plants are listed in Table 2.

Secondary metabolites of pharmacological value

Being photoautotrophic, plants have an almost unlimited capacity for growth and development. Along with this inherent capacity for unlimited growth, plants also possess a relatively plastic metabolism that allows them to rapidly adapt to changing environmental factors, pathogens, and other biotic and abiotic challenges. The plasticity of plant metabolic activity is most evident in the wide variety of secondary metabolites accumulated by plants in their leaves, roots, and other organs. Despite the fact that their biosynthetic origin and role in the plant are poorly understood, they are of considerable interest because of their potential industrial, pharmacological, and medicinal value.

The use of whole plant preparations or plant extracts for medicinal purposes extends well before recorded history. Within recent times, many plant-derived products for treating human disorders have reached the market place as useful drugs including atropine, hyoscyamine, scopolamine, taxol (anticancer), vinblastine/vincristine (anti-cancer), artemisinin (antimalarial), reserpine (antihypertension), and quinine (antimalaria).

The ability to genetically engineer plant genomes has allowed for the direct manipulation of plant metabolism and the potential for manipulating the content and nature of plant secondary metabolites of commercial value. Plants are now being considered as potential factories for the production of a variety of useful compounds (Kishore and Somerville, 1993; Ap Rees, 1995; Kahl and Winter, 1995).

Plant cells produce far more chemical compounds than are necessary to meet their basic functions (i.e., growth, differentiation, and reproduction). By and large, basic, primary metabolism is shared by all cells, while secondary metabolism generates diverse and seemingly less essential or non-essential byproducts called secondary metabolites, which give plants their various colors, flavors, and smells. These products are sources of fine chemicals, such as insecticides, dyes, flavors, fragrances, drugs and phytomedicines found in medicinal plants. Secondary plant products have played an essential role in medicine for thousands of years and represent tremendous resources for scientific and clinical researchers and new drug development. Their pharmacological value is increasing due to constant discoveries of their potential roles in the healthcare and as lead chemicals for new drug development. Despite the

Table 2. Biopharmaceutical proteins produced in transgenic plants

Protein(s)	Plant host	Reference
α-Trichosanthin	Tobacco	Kumagai et al. (1993)
Hirudin	*Brassica* seeds	Parmenter et al. (1994)
α-Interferon	Rice	Zhu et al. (1994)
Erythropoietin	Tobacco	Matsumoto et al. (1995)
Glucocererbosidase	Tobacco	Cramer et al. (1996)
Protein-C	Tobacco	Cramer et al. (1996)
Avidin	Maize	Hood et al. (1997)
Ribosome-inactivating protein	Tobacco	Francisco et al. (1997)
Granulocyte-macrophage colony stimulating factor	Tobacco	Lee et al. (1997)
β-Glucuronidase	Corn	Evangelista et al. (1998)
Lactoferrin and β-casein	Potato	Chong and Langridge (2000)
Human collagen I [Pro1α (I)]	Tobacco	Ruggiero et al. (2000)
Phytase	Canola	Zhang et al. (2000)
Thermostable endo-1,4 β-D-glucanase	Arabidopsis	Ziegler et al. (2000)
Human α-antitrypsin	Rice	Terashima et al. (2002)

fact that their biosynthetic origin and role in plants are poorly understood, they are of considerable interest because of their potential applications (Table 3).

Genetic engineering of a secondary metabolic pathway aims to either increase or decrease the quantity of a certain compound or group of compounds (Verpoorte and Memelink, 2002). To decrease the production of a certain unwanted (group of) compound(s) several approaches are possible. An enzymatic step in the pathway can be knocked out, for example, by reducing the level of the corresponding mRNA via antisense, co-suppression or RNA interference (RNAi) technologies, or by over expressing an antibody against the enzyme. Mahmoud and Croteau (2001) reported improved quality of essential oil by expressing an antisense derivative of the methanofuran synthase gene to down-regulate synthesis of the undesirable constituent methanofuran in mint plants.

Other approaches being employed for the production of novel compounds in plants include metabolite diversion into a competitive or alternate pathways, increased catabolism of target compounds, changing the expression of one or a few genes to overcome specific rate limiting steps in a specific pathway, and transferal of all or part of a metabolic pathway for other organisms into plants. Success in these endeavors has been mixed.

In an attempt to modify terpenoid metabolism, the gene encoding 1-deoxy-D-xylulose phosphate synthase (DXPS), the first enzymatic step of terpenoid biosynthesis, has been constitutively over-expressed in *Arabidopsis*. Increased gene expression coupled to increased enzyme activity was correlated with increased accumulation of plant terpenoids, indicating that DXPS is rate limiting (Estevez et al., 2001). Similarly, Muir et al. (2001) reported a 78-fold increase of flavonoid levels in tomato fruits over-expressing the *Petunia* chalcone isomerase (CHI) gene. Flavonoids and anthocyanins are known antioxidants with human health benefits and key targets for genetic improvement. In another example, Verpoorte and Memelink (2002) showed that one could specifically alter alkaloid levels in *Catharnthus roseus* cell cultures by altering the expression of the genes encoding tryptophan decarboxylase (TDC) and strictosidine synthase (STR). Over-expression of TDC resulted

Table 3. Important plant-derived secondary metabolites of pharmaceutical value (after Raskin et al., 2002)

Active compound	Plant source	Proposed therapeutic use
Alkaloids		
Atropine, hyoscyamine, scopolamine	*Solanaceous* spp.	Anticholinergic
Vinblastine, Vincristine	*Catharanthus roseus* L.	Antineoplastic
Nicotine	*Nicotiana* spp.	Smoking cessation
Codeine, morphine	*Papaver somniferum* L.	Analgesic, antitussive
Quinine	*Cinchona* spp.	Antimalarial
Quinidine	*Cinchona* spp.	Cardiac depressant
Terpenes and steroids		
Artemisinin	*Artemisia annua* L.	Antimalarial
Diosgenin, hecogenin, stigmasterol	*Dioscorea* spp.	Oral contraceptives, hormonal drugs
Taxol and other taxoids	*Taxus brevifolia* Nutt.	Antineoplastic
Glycosides		
Digoxin, digitoxin	*Digitalis* spp.	Cardiotonic
Sennosides A and B	*Cassia angustifolia* Vahl.	Laxative
Others		
Ipecac	*Cephaelis ipecacuanha* (Brot.) A. Rich	Emetic
Podophyllotoxin	*Podophyllum peltatum* L.	Antineoplastic

higher levels of the immediate product tryptamine, but did not affect total alkaloid levels, whereas over-expression of STR led to increased total alkaloid contents higher levels of alkaloids were noted (Canel et al., 1998). In contrast, over-expression of ORCA3, a transcription regulatory factor that controls several steps in alkaloid biosynthetic pathway in *C. roseus* did not result in increased alkaloid production (van der Fits and Memelink, 2000).

The early successes of these gene-expression based strategies coupled with increased application of proteomic and metabolomic strategies to understanding secondary metabolism and its control bodes well for the future manipulation of plant genomes for the production of wide variety of secondary compounds with anti-viral, -bacterial, and -fungal activities or with human therapeutic value.

Conclusions and perspective

Within a very short time span the use of genetically modified plants for the production therapeutic compounds has moved from being an experimental system with significant potential to a commercially viable process poise to deliver products in useful in animal and human therapies. Inroads have been made not only in more traditional areas of therapeutic development (e.g., the identification and isolation of bioactive secondary metabolites), but also in relatively uncharted areas such as the production of novel bioactive peptides and proteins, antibody production for passive immunization therapy, and edible oral vaccines.

The rapid pace of development witnessed thus far is likely to accelerate in the very near future as additional, novel uses of transgenic plants as production systems for human therapeutics are explored. The limitations for the use of genetically modified plants will likely arise from our still somewhat unsophisticated knowledge of how plant gene expression is controlled and how various metabolic pathways within a plant interact and regulate themselves. The use of plants as production factories is already seen as an economically attractive alternative for the production of some compounds. As our level of understanding of the factors that impact transgene expression in plants improves, we will see improvement in levels of production of target molecules (peptide, proteins, antibodies), decreased costs of production, and greater overall exploitation of plant based production systems.

The use of plant-derived recombinant molecules for human therapeutic agents will likely not reach fruition without addressing the same regulatory issues that surround the use of any recombinant molecule, namely safety risks versus public benefit. Here, a close cooperation will be necessary between experts in the medical and plant engineering communities to address potential concerns about the purity and safety of plant-derived therapeutics and to demonstrate their reliability and cost-effectiveness relative to conventional approaches. It would be unfair to generalize upon the limited number of products now in use, but the fact that recombinant plants and plant products are already commercially available indicates that for the most part the public views these technologies favorably. Public acceptability of these initial products is associated with the fact in some cases that they clearly provide a more environmentally friendly alternative (i.e., decreased use of herbicides and pesticides) to current agricultural practices. Whether this will also be true of products associated with human therapeutics is yet to be determined.

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