Chapter 13
Molecular Farming

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13.1 Introduction

The manufacturing of recombinant proteins in plants has emerged as a viable alternative to bacterial, fungal or animal cell-based expression systems. The use of plants for the production of such novel valuable compounds is commonly defined as molecular farming. Molecular farming presents a sustainable, green manufacturing technology for high value products of biological origin and a promising innovative hi-tech sector in agriculture and horticulture.

A number of plant species have been tested throughout the years as host organisms, both dicots and monocots and domesticated and less domesticated plants with both stable transgenic and transient expression approaches. The host tissue accumulating the recombinant proteins has varied from secretion through roots to accumulation in tubers, leafs, flowers, fruits and seeds (Yano et al. 2010; Sharma and Sharma 2009; Basaran and Rodríguez-Cerezo 2008; Stoger et al. 2005; Drake et al. 2003). Not only have the different tissues been targeted for protein accumulation, but intracellular localisation has been explored as well, such as retention in the endoplasmic reticulum; accumulation in organelles such as chloroplasts, protein bodies and cytosol and secretion out of the cell (Streatfield 2007; Kamenarova et al. 2005; Ma et al. 2005). The optimal path for the protein and its final cellular destination may vary depending on the protein, the plant species and the tissue it is accumulated in. The biological diversity of plants and their

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eucaryotic nature at the cellular level offer many strategies for the manufacturing of recombinant proteins, and different strategies have their strengths and weaknesses. The accumulation of recombinant proteins can be tissue specific or systemic in the whole plant, depending on the nature of the promoters chosen to drive gene expression. Plant cells grown in suspension culture can and have been used successfully for the production of recombinant proteins. In fact, the first plant-made pharmaceutical to be approved by the FDA is a glycosylated enzyme, glucocerebrosidase, produced by suspension culture of carrot cells (Protalix Inc.). Suspension culture of plant cells can be considered as a hybrid manufacturing technology between traditional bioreactors and whole plant approaches.

Considering whole plants, amongst the strategies to choose from are whether to harvest the protein from rapidly growing, highly metabolically active green tissue or metabolically quiescent storage tissue. Target peptides added to the protein can be used to direct the protein accumulation to various intracellular compartments that may increase stability or affect the level and nature of post-translational modification of the recombinant product. The choice of a strategy will affect protein yield, the protein environment in the host plant and the extract, the downstream process and ultimately the economics and feasibility of the molecular farming and may even affect the quality of the product.

The interest in developing molecular farming is partly based on the ample existing know-how and technology adapted to domesticated plants; the agricultural infrastructure for any scale of cultivation and harvesting is present for any crop species, and in many cases, suitable postharvest treatments such as seed processing and storage for seed-based systems are known. To put it simply, the large-scale upstream processes were honed to perfection by agricultural practices before the term of molecular farming was even phrased. Existing infrastructure with solar energy driving photosynthesis and sustainable growth of the biomass provide an attractive economical and environmental incentive to develop molecular farming into a green, high-value low-carbon footprint industry, with a potential to contribute significantly to the bioeconomy of the future.

Cereals have been intensively bred to produce large harvestable grains, accumulating polysaccharides, lipids and specific storage proteins in the stable, metabolically quiescent tissue of grains. Grains stored under proper conditions can last dormant for years, maintaining the proteins intact for an extended period of time.

The major commercially important cereals, maize, rice, wheat and barley have all been genetically transformed (Ramessar et al. 2008a, b; Fujiwara et al. 2010; Han et al. 2012; Erlendsson et al. 2010; Hensel 2011), representing major crops in both temperate (wheat and barley) and warmer regions (maize and rice). Recombinant proteins have been demonstrated to accumulate at high levels in grains of cereals (Christou et al. 2008; Boothe et al. 2010).

Molecular farming in seed-based systems is considered promising for vaccine production, and recombinant antigens produced in seeds have been demonstrated to remain stable and immunogenic in both animals and humans for over 18 months when stored without refrigeration, thus eliminating the necessity of costly cold

chain for vaccine delivery in the developing world (Penney et al. 2011; Nochi et al. 2007).

Barley has a number of qualities that make it an excellent platform for the manufacturing of recombinant proteins with molecular farming. The combination of genetics, physiology, agricultural suitability, inherent containment properties, infrastructure and long history of domestication of barley contribute to the benefits of barley for industrial cultivation. This review attempts to describe some of the developments of sustainable molecular farming industry based on grain-based molecular farming with barley.

13.2 Benefits of Barley for Molecular Farming

When considering a host organism for the production of technical proteins, or proteins of medical relevance, i.e. protein products for non-food, non-feed purposes, a number of challenges and criteria need to be considered, such as toxicity, safety, agricultural practices, containment strategies, the applicability of molecular biology and tissue culture and biochemistry and downstream processing.

The use of crop plants for molecular farming provides obvious benefits in terms of their long history of agricultural and ecological interactions with the environment, safety and efficient management under different conditions. Well-defined varieties through long and extensive breeding provide farmers with stable crop plants with a well-synchronised life cycle, manageable cultivation and efficient harvesting. Domestication is a process of increasing interdependence of humans and target plant or animal populations (Zeder et al. 2006). Domesticated crop species furnish with a long history of safety of the crop compared to poorly domesticated plants with high variation in life cycle, possible invasiveness and largely unknown potentially toxic metabolic responses to abiotic and biotic stresses. Furthermore, the reduced competitiveness and invasiveness of crop plants under noncultivated conditions and general dependency to human intervention to successfully complete their life cycle contribute to the safe use of crop plants from the point of environmental concerns.

Barley certainly is a crop plant with a long history of domestication reaching back thousands of years and has been adapted and bred to emphasise yield and other agricultural traits to accommodate the needs of humans for mainly feed and brewing purposes (see also Chaps. 1 and 3).

The relatively long life cycle, self-pollination, large, few and heavy grains that are poorly suited to wind dispersal may contribute to poor invasiveness and efficient confinement of the annual plant barley. Another benefit of barley is the possibility to obtain doubled haploids, which is useful to improve yields in molecular farming and stability of elite production lines (see also Chap. 20). In addition, barley is an annual plant that does not persist in natural habitats or fields without intervention, making cultivation of barley for molecular farming a reversible and highly

manageable operation. Molecular farming with barley can be ceased (like any other barley cultivation) with no lasting effect on the environment, positive or negative.

Production of recombinant proteins in grains of barley is, however, a time-consuming strategy, compared to cell suspension culture or transient expression, as stably transformed elite product lines need to be generated, defined and propagated before downstream processing and production can commence.

The fact that barley has a long history of domestication implies that it is a food but mainly a fodder plant. It can be argued that molecular farming should be restricted to non-food, non-fodder plants to avoid the risk of contamination of the food chain with material not intended for consumption. While this may seem a reasonable demand, it needs to be weighed against the alternative, conducting molecular farming in non-domesticated plants with limited knowledge available on the genetics, biochemistry, biology and ecological behaviour of a non-domesticated plant that by definition would not be dependent on human intervention. The risk of unforeseen events at every level is greater than with a plant with known agronomic traits and established procedures.

13.3 Inherent Containment Properties

Amongst the valuable biological containment features of barley is the high level of self-pollination that effectively bars cross-fertilisation between adjacent plants (Ritala et al. 2002; Nair et al. 2010). An indication of the containment is the short isolation distances required to maintain purity of barley cultivars. Canadian Seed Growers' Association stipulates isolation distances for certification of foundation, registered or certified non-hybrid barley seed from different varieties of barley to be 3 m, indicating the ease of maintaining barley varieties isolated and the low risk of gene flow (Canadian Seed Growers' Association 2013). Several varieties are characterised by almost complete self-pollination such as the Golden Promise variety that is preferentially used for genetic transformation, including molecular farming (Hensel et al. 2008; Erlendsson et al. 2010). Self-pollination (cleistogamy) of barley occurs where pollination has taken place before the flower opens and before the pollen can be dispersed from the flower. Thus, when the pollen is released, all flowers that are open are already pollinated and not receptive to external pollen, effectively abolishing the possibility of cross-pollination between barley plants. The underlying genetic and molecular mechanism behind cleistogamy in barley cultivars has recently been elucidated (Nair et al. 2010).

Cross-pollination of barley under field conditions has been studied by Ritala et al. (2002) and more recently by Hermannsson et al. (2010), who conducted a multiyear experiment in a subarctic climate where Golden Promise was planted in 20–30 cm distance from another developmentally compatible six-rowed variety. Hybrid offspring have a distinct phenotype, as established by forced cross-pollination. This allows fast screening of a large population of offspring for rare cross-pollination events between barley varieties grown immediately adjacent to

each other. The experiment by Hermannsson and colleagues confirmed the very low frequency of cross-pollination between Golden Promise and the six-rowed cultivar Ven. In the larger setup of the experiment, not a single hybrid was detected in the 600,000 plants screened, despite the short distance between the compatible cultivars. The results show that maintaining moderate distance from other barley is a safe and simple measure to efficiently prevent gene flow between barley crops.

Lifetime of barley pollen in general is very short, and barley pollen is not distributed by insects (USDA 2006). The viability of the pollen released from the Golden Promise variety was studied by researchers at the MALTAgen Forschung Company indicating that the pollen was already non-viable by the time it was released from the flower (oral communication—MALTAgen Forschung). This reproductive behaviour of barley varieties like Golden Promise is important from the point of molecular farming, as it provides an extraordinary level of contained cultivation, adding to the safety, and facilitates quality control of molecular farming with barley whether in field or in greenhouse conditions.

Barley grains are heavy, on average 30 mg/grain, greatly limiting wind dispersal of barley grains. An opportunity to measure the level of wind dispersal of grain under extreme conditions rose after a severe storm hit a fully developed Golden Promise barley plot ready to be harvested. The storm speed measured 44 m/s, and grain dispersal down the wind direction was measured to be maximum 35 m from the nearest plants. According to seed counts, 99.9 % of the seeds shed by the storm were within 5 m radius from the plants despite the heavy storm (ORF Genetics, unpublished results).

This further corroborates the inherent containment properties and manageability of barley even under extreme field conditions. Barley stays on the field.

The superb biological containment exhibited by barley has not only implications for responsible contained molecular farming; it has relevance for the economy of molecular farming operations as well, both for in field and greenhouse cultivation of barley. High-density cultivation of barley lines becomes possible without the risk of contamination between lines, as the need for isolation distance is minimal, enabling efficient use of land and greenhouse space. Under greenhouse conditions, the containment attributes of barley become especially valuable; transgenic barley lines producing different proteins can be cultivated in the same greenhouse without the risk of cross-contamination. In fact, during 8 consecutive years of all-year-round greenhouse cultivation of transgenic Golden Promise lines, no examples of cross-fertilisation between thousands of GP lines have been observed despite high-density cultivation (20 cm distance between different GP lines) (ORF Genetics, unpublished results). Subsequently, greenhouse cultivation of multiple lines becomes more economical and amenable to high level of automation, and greenhouse facilities become effectively multiproduct facilities. Molecular farming with barley in hydroponic culture on conveyor belts has proven successful and economical for the production of a variety of small to moderate volumes of recombinant proteins (Fig. 13.1). The feasibility of scaleup in greenhouse or field conditions for any given product of molecular farming depends largely on the combination of the

Fig. 13.1 Molecular farming with barley in geothermally heated greenhouses near Reykjavik, Iceland, courtesy of ORF Genetics

type of recombinant protein, its intended application, and the economics and market properties of the protein.

Heavy regulatory burden associated with European regulations on GMOs that are designed to have strict control on widespread agricultural use of genetically engineered plants for food and fodder production across Europe is poorly suited to address the manageable, localised and limited scope of cultivation required by molecular farming of valuable compounds for other uses. Such poorly developed regulatory environment stifles the advancement of responsible field-based molecular farming and the R&D activities by universities, research institutes and SMEs, effectively handing over a monopoly to large multinationals that have the resources to tackle the costly and time-consuming regulatory process.

To develop further the safety aspects of molecular farming and to address concerns of possible postharvest mixing of molecular farming crops with other

Fig. 13.2 Heads of Dimma cultivar to the *left* and of the Golden Promise cultivar to the *right*

crops, ORF Genetics, in collaboration with the Icelandic Agricultural University, has set out to develop the new barley variety "Dimma" that is self-pollinating and both amenable to transformation and tissue culture but is black in colour. The resulting barley grains are easily recognisable from ordinary barley to the resolution of single grains. This makes postharvest monitoring possible and provides a tool to effectively prevent postharvest mixing of molecular farming with barley harvested for other purposes (Fig. 13.2).

13.4 Grains and Encapsulated Proteins

Grains as target tissue for recombinant protein accumulation have several advantages over protein accumulation in metabolically active green tissue: harvestability, compactness, encapsulation of the recombinant product, stability of proteins within the grains, long-term storage at ambient temperatures, low bioburden in the grain, relatively simple protein profile, scaleable processing, existing infrastructure and initial processing and operational flexibility by separation of upstream (cultivation) and downstream (protein purification) operations. The use of seeds in general for molecular farming has been discussed in a review (Boothe et al. 2010). The attenuating metabolism with reduced protein turnover and dwindling proteolytic activity and the onset of storage protein synthesis provides optimal timing and environment for recombinant protein accumulation. A way to take advantage of this in seed-based molecular farming is to have strong seed-specific promoters from storage protein genes to drive the expression of the recombinant gene. Control of transgene expression in Triticeae cereals has been reviewed recently by Hensel et al. (2011), with detailed listing of various promoter studies on target cereal species addressing both ubiquitous and tissue-specific promoters, promoters responsive to abiotic and biotic stresses and target peptides for intracellular targeting of proteins. Useful grain-specific promoters include barley *B1 hordein* and barley *D hordein* (Cho et al. 2002), barley α-*amylase* (Caspers et al. 2001), oat *globulin* 1 (Vickers et al. 2006) and rice *Glutenin B1* (Patel et al. 2000). Grain-specific expression in barley offers the possibility for controlled tissue-specific expression of a gene and accumulation of the corresponding recombinant protein in a tissue entering the quiescent state of dormancy. The recombinant proteins accumulate subsequently only in a tissue-specific manner during the late stages of the life cycle of the plant. An example of this is the endosperm-specific accumulation of growth factors listed in Table 13.1. With the grain maturation and desiccation comes the benefit of extended storage of the accumulated product under aseptic conditions inside the grain.

Steiner and Ruckenbauer (1995) verified the effectiveness of ultradry storage of cereal grains in a hermetically sealed container at ambient temperature. After 110 years of storage at 10–15 °C and ultradry conditions, barley grains of about 3.1 % moisture exhibited 90 % germination. Although there is no data on the condition of the storage proteins specifically, it must be concluded that genetic, biochemical and cellular condition of the grains must be intact for germination to occur so effortlessly.

The key to such longevity of the grain is the cessation of metabolic activity that follows the maturation and desiccation of the grain, combined with favourable (dry) storage conditions. Low metabolic and proteolytic activity provides a stable environment for any proteins that were accumulated in the seed during maturation, compared to the protein turnover in metabolically active cells. An example of metabolic activity that has relevance to recombinant protein accumulation is the activity of proteases in the host cells. The proteolytic activity of barley grain extract was compared to sonicated extracts of the BL21 strain of *E. coli*, a common strain

Table 13.1 Overview of recombinant proteins produced in barley for molecular farming purposes

Recombinant protein	Full name of protein	a.a. sequence	Protein type	Source
ANG	Angiogenin-1	Human	Growth factor	ORF Genetics
EGF	Epidermal growth factor	Human	Growth factor	ORF Genetics
EMAP-2	Endothelial-monocyte activating polypeptide-II	Human	Growth factor	ORF Genetics
FGF basic	Fibroblast growth factor	Human	Growth factor	ORF Genetics
Flt3-ligand	FMS related tyrosine kinase 3 ligand	Human	Growth factor	ORF Genetics
G-CSF	Granulocyte-colony stimulating factor	Human	Growth factor	ORF Genetics
GDNF	Glial cell line derived neurotrophic factor	Human	Growth factor	ORF Genetics
HB-EGF	Heparin binding EGF	Human	Growth factor	ORF Genetics
IFN alpha 2a	Interferon alpha 2a	Human	Growth factor	ORF Genetics
IFN gamma	Interferon gamma	Human	Growth factor	ORF Genetics
IL-16	Interleukin-16	Human	Growth factor	ORF Genetics
IL-1α	Interleukin-1 alpha	Human	Growth factor	ORF Genetics
IL-2	Interleukin-2	Human	Growth factor	ORF Genetics
IL-3	Interleukin-3	Human	Growth factor	ORF Genetics
IL-4	Interleukin-4	Human	Growth factor	ORF Genetics
IL-5	Interleukin-5	Human	Growth factor	ORF Genetics
IL-6	Interleukin-6	Human	Growth factor	ORF Genetics
IL-7	Interleukin-7	Human	Growth factor	ORF Genetics
IL-9	Interleukin-9	Human	Growth factor	ORF Genetics
IL-21	Interleukin-21	Human	Growth factor	ORF Genetics
KGF	Keratinocyte growth factor	Human	Growth factor	ORF Genetics
LIF	Leukemia inhibitory factor	Human	Growth factor	ORF Genetics

(continued)

Table 13.1 (continued)

Recombinant protein	Full name of protein	a.a. sequence	Protein type	Source
LIF	Leukemia inhibitory factor	Human	Growth factor	ORF Genetics
M-CSF	Macrophage colony stimulating factor	Human	Growth factor	ORF Genetics
NRG1 (HRG-beta-2)	Neuregulin 1	Human	Growth factor	ORF Genetics
PDGF-BB	Platelet-derived growth factor-BB	Human	Growth factor	ORF Genetics
RANKL	Receptor activator of NF-kappaB ligand	Human	Growth factor	ORF Genetics
SCF	Stem cell factor	Human	Growth factor	ORF Genetics
SCF	Stem cell factor	Human	Growth factor	ORF Genetics
SF20	Stromal cell-derived growth factor	Human	Growth factor	ORF Genetics
TNF alpha	Tumor necrosis factor, alpha	Human	Growth factor	ORF Genetics
TNF-beta	Tumor necrosis factor beta	Human	Growth factor	ORF Genetics
VEGF	Vascular endothelial growth factor	Human	Growth factor	ORF Genetics
VEGF	Vascular endothelial growth factor	Mouse	Growth factor	ORF Genetics
FaeG F4	Fimbrial adhesion	*E. coli*	Vaccine	Joensuu et al. (2006)
	Anti glycophorin scFv-HIV epitope fusion	Synthetic	Diagnostic	Schünmann et al. (2002)
Antithrombin III	Antithrombin III	Human	Proteinase inhibitor	Stahl et al. (2002)
α1-Antitrypsin	α1-Antitrypsin	Human	Proteinase inhibitor	Stahl et al. (2002)
HAS	Serum albumin	Human	Carrier protein	Stahl et al. (2002)
Lactoferrin	Lactoferrin	Human	Carrier protein	Stahl et al. (2002), Kamenarova et al. (2007)
Lysozyme	Lysozyme	Human	Enzyme	Stahl et al. (2002), Huang et al. (2006)
Glucanase	(1,3-1,4)-β-glucanase	Bacillus species	Glucan modifying enzyme	Horvath et al. (2000)
Hb	Haemoglobin	Vitreoscilla	Carrier protein	Wilhelmson et al. (2007)

(continued)

Table 13.1 (continued)

Recombinant protein	Full name of protein	a.a. sequence	Protein type	Source
Thaumatin	Thaumatin	*Thaumatococcus daniellii*	Sweetener	Stahl et al. (2009)
rCla1	Collagen 1a	Human	Structural protein	Ritala et al. (2008), Eskelin et al. (2009)

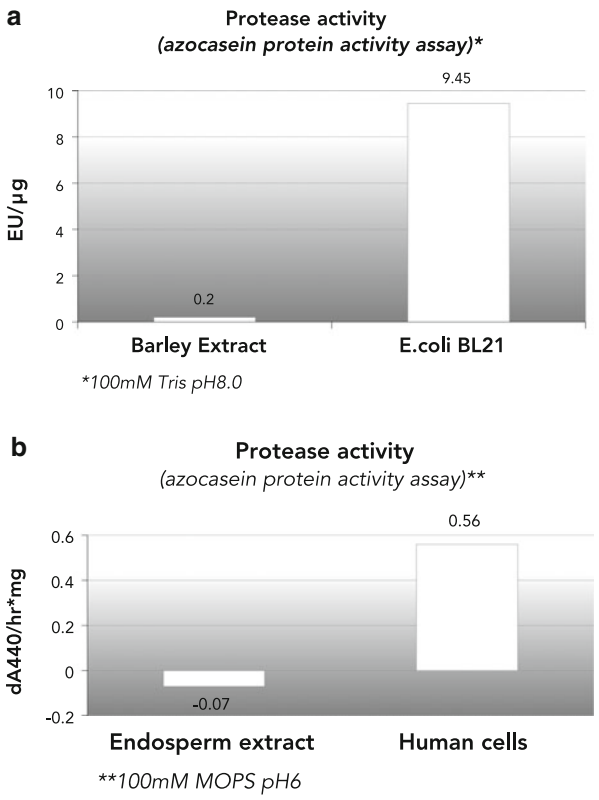

Fig. 13.3 Proteolytic activity of barley, bacteria (**a**) and human cells (**b**). Azocasein-containing solutions were incubated with clarified extracts of milled grain and lysates of *E. coli* (BL21) and human neutrophil cells. After precipitation, the absorbance of the released soluble azopeptides in the supernatant indicates the extent of proteolytic activity in the samples

used for the expression of recombinant proteins, and lysed human mononuclear cells using the azocasein proteolytic assay (Millet 1977) (Fig. 13.3). The results confirm very low proteolytic activity in the barley grain extract which is minimal compared to metabolically active bacterial and human cells. This suggests that a recombinant protein accumulated in barley grains is less likely to be exposed to proteolytic activity than in metabolically active bacterial or mammalian cells. This would contribute to the stability of recombinant proteins stored within the grains.

Recombinant growth factors purified from barley grains have been observed in cell proliferation bioassays to fully maintain their activity in a 2-year-old grain

material (ORF Genetics, unpublished results), indicating the stability of recombinant proteins stored in grain tissue.

It is, therefore, not unreasonable to expect that recombinant proteins accumulated amongst grain storage proteins can remain stable for decades if intact grains are stored under optimal conditions.

13.5 Safety and Quality of Products

Endotoxins are bacteria-derived molecules, e.g. lipopolysaccharides and lipooligosaccharides, that induce signalling cascades for pro-inflammatory cytokines in mammalian cells causing damage and stress to cell cultures. Depending on the cell line, this may jeopardise the cell culture and affect research results and cell-based production of biologicals (Epstein et al. 1990; Lieder et al. 2013). Endotoxins are, thus, a major concern for contamination of bacterially produced recombinant proteins used in cell culture. For pharmaceutical production, it is essential to verify that the recombinant product is not contaminated with bacterial endotoxins that may otherwise cause inflammation and disease, or even endotoxic shock in patients. Plants do not produce endotoxins, and recombinant proteins produced within grains should therefore be void of endotoxins. To verify the expected low endotoxin content of purified, freeze-dried plant-derived recombinant human growth factors, VEGF and IFN-gamma, they were subjected to endotoxin measurements by a validated commercial laboratory (Associates of Cape Cod, Inc.) (Fig. 13.4).

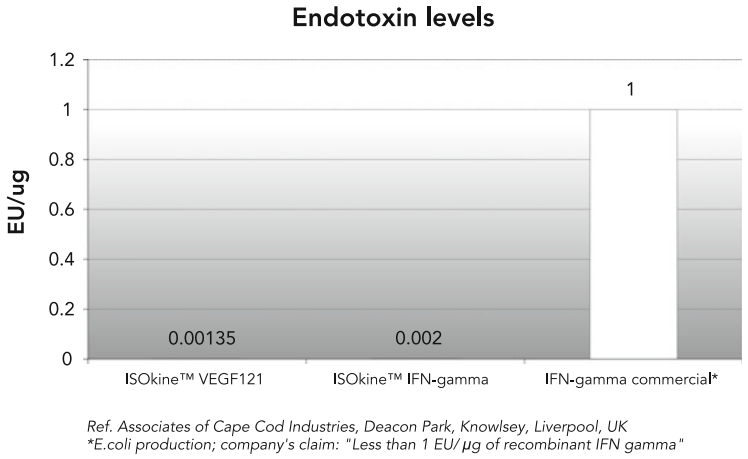

Fig. 13.4 Endotoxin measurements. *Limulus* amebocyte lysate (LAL) tests detect and quantify bacterial endotoxins extracted from the outer membrane of gram-negative bacteria. The samples were subjected to turbidimetric LAL testing (Associates of Cape Cod, Intl, Inc., Deacon Park, Moorgate Road, United Kingdom). In short, the results showed that endotoxin content in barley-derived products was hardly detectable with the most sensitive endotoxin assays

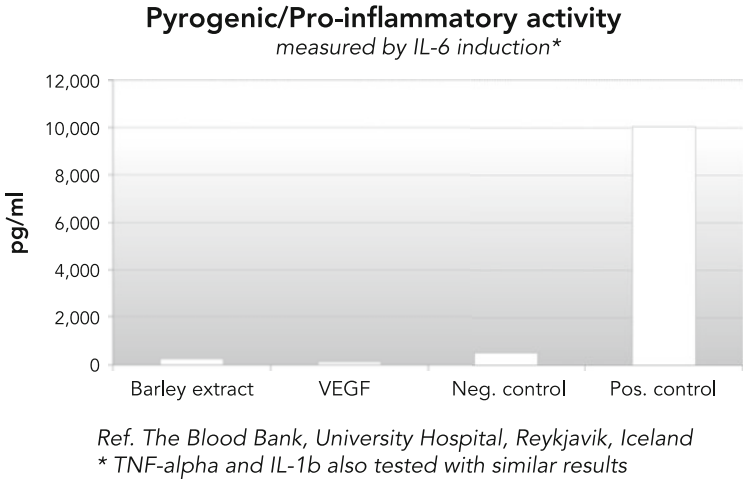

Fig. 13.5 Monocyte activation test (MAT assay) was performed with crude barley extract and a mock sample of non-transgenic barley extract that underwent the full purification protocol for a growth factor (VEGF). Human mononuclear cell cultures of density 1×10^6 were supplemented with various concentrations of the barley samples, negative control (cells without supplement) and positive control cell culture supplemented with 50 ng lipopolysaccharide (LPS). The release of the pyrogenic marker interleukin-6 (IL-6), shown above, along with eight other cytokines (data not shown) was assayed using the Procarta Cytokine Assay Kit, Human 10-plex (Affymetrix, 3420 Central Expressway Santa Clara, CA 95051, USA) monitored with a Luminex 100 instrument (the Blood Bank, Snorrabraut 60,105 Reykjavík, Iceland)

The possibility of pyrogenic, pro-inflammatory compounds being present in barley grain extracts was examined. The Monocyte Activation Test (MAT assay) measures the induction of pyrogenic marker cytokines in human monocytes. To further validate barley as a suitable host for cytokine production, the barley background was studied by the MAT assay (Fig. 13.5).

Neither barley extract nor purified barley grain-derived VEGF mock fraction induced any pyrogenic response indicating strongly that barley grain as a host tissue for expression of recombinant proteins does not carry inherent pyrogenic compounds detrimental to cell culture. This is yet another indication that barley grain as a source for recombinant proteins can be considered and generally recognised as safe.

To determine the biological activity of recombinant protein purified from barley grain, the proteins are subjected to cell-based bioassays.

The Flt3 ligand produced in barley endosperm and purified from transgenic barley grains was previously shown to exhibit yields comparable to bacterial expression systems and to be biologically functional in cell-based assays (Erlendsson et al. 2010). Fibroblast growth factor (FGF basic) was produced in a barley endosperm and purified from transgenic barley grains, and the activity was assessed with a cellular proliferation assay on FGF basic responsive, mouse 3T3 cells. Serial dilutions of the recombinant human FGF basic proteins in assay media

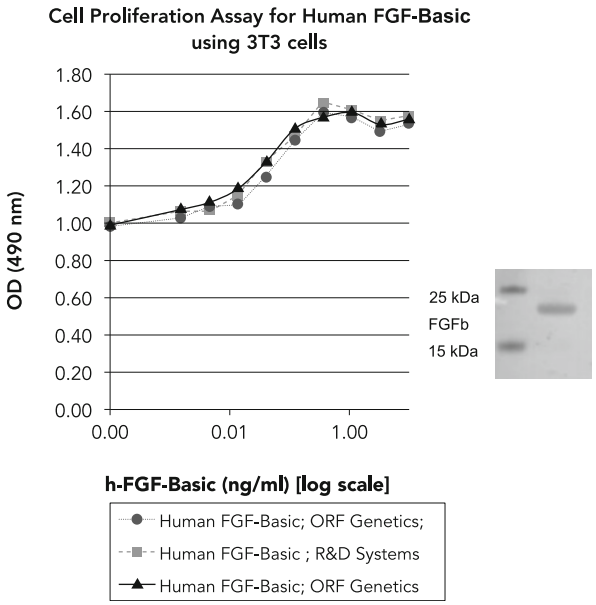

Fig. 13.6 Results of bioactivity assay of plant-derived recombinant human FGF basic growth factor purified from the grains of transgenic barley. The graph shows a cellular proliferation bioassay exposing FGF basic responsive mouse 3T3 cells to dilution series of the FGF basic protein (SBH Sciences, Natick, MA, USA). Two samples of plant-derived FGF basic (ORF Genetics, Víkurhvarf 3, 203 Kópavogur, Iceland) are compared to *E. coli*-derived recombinant human FGF basic (R&D systems; Cat# 233-FB/CF). Next to the graph is a Coomassie-stained gel showing purified plant-derived FGF basic next to molecular size markers 15 kDa and 25 kDa to the *left*

were added to cell culture and incubated for 42 h at 37 °C before staining and measuring the optical density at 490 nm (SBH Sciences, Natick, MA, USA) (see Fig. 13.6).

The results show that the recombinant human FGF basic protein produced in barley is as active as the leading *E. coli*-derived recombinant human FGF basic available on the market. A benefit of the eucaryotic plant expression systems is that human proteins are expressed, folded and processed in the same way in plants and humans, maintaining structure and function with only minor differences in post-translational modifications of proteins. In comparison, bacterial expression systems are unable to do most post-translational modifications and frequently discard recombinant human proteins into inclusion bodies requiring costly and cumbersome refolding of the denatured proteins to regain activity. The lack of post-translational modifications such as glycosylation can affect the assembly, stability, half-life and activity of recombinant proteins, as shown by the analysis of Runkel et al. (1998) on the activity and stability of glycosylated and deglycosylated forms of the growth factor IFN-beta. The deglycosylated form showed less activity and was more prone to thermal denaturation. Post-translational modifications can thus

be important for the proper function of biologically active proteins. Although plants are able to glycosylate proteins in a similar manner as mammalians, subtle differences exist. Plants lack sialic acid from their glycan structures, while the plant-specific glycans β1,2-xylose and core α1,3-fucose residues are absent in mammalian glycoproteins. Both xylose and α1,3-fucose have been suggested to have allergenic properties and might therefore be expected to be problematic to plant-based pharmaceuticals (Bardor et al. 2003). Advancements in glycoengineering may bring solutions to such problems (Gomord et al. 2010).

Such concerns are specifically addressed in safety studies during clinical trials. In fact, the first plant-made pharmaceutical glucocerebrosidase is glycosylated and carries both xylose and fucose and has not been observed to cause adverse effects beyond what is to be expected in enzyme replacement therapy (Zimran et al. 2011). The fact that it passed the clinical trials and received market authorisation as an injectable drug indicates that suggested allergenicity of plant glycans is at least not a general phenomenon, but is to be studied on a case-by-case basis, as is the case with any new pharmaceuticals in development.

Molecular farming with barley as host system has become quite extensive in terms of recombinant products and is the result of intensive research and development within academia research institutes and companies during the last two decades. Barley today is probably the molecular farming platform that has delivered the highest number of plant-made recombinant proteins to the market. Table 13.1 gives an overview of recombinant proteins that have been produced in barley for molecular farming purposes. Growth factors are prominent on the list as a result of the focus of one company on the family of growth factors and cytokines as mentioned. They are used in just about every aspect of cell biology, immunology, stem cell research and medical research that involves cell culture, such as regenerative medicine as well as biopharmaceutical development. Recently published results by Ritala et al. (2014) describe the expression and accumulation of an antibody (IgE) in grains. A recent review of the use of barley for production of recombinant proteins can be found in Magnusdottir et al. (2013).

Conclusion

Barley is, in many respect, extraordinary well suited for contained molecular farming operations; the agricultural features and human dependency with the limited fitness, containment through self-pollination and heavy grains provide for management of cultivation. Although time-consuming, once stable transformant elite lines have been established, the grain-specific expression together with the protein storage properties of grains offers flexibility in production operations, such as stockpiling of harvest and just-in-time processing catering to the demand. Barley grain-based products are inherently of higher quality than products obtained with traditional expression hosts, i.e. bacteria and mammalian cells, being animal-free, serum-free and

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endotoxin-free. They are void of human or animal infectious agents and low in pyrogenic and pro-inflammatory activity, the proteins are naturally folded by the plants eucaryotic protein-folding mechanism and there is no risk of contamination by other endogenous mammalian proteins.

Molecular farming is, in many aspects, already competitive with more traditional expression systems that have been pressed to their limits in efficiency as a result of decades of intensive optimisation. The prospect of all the unlocked potential and optimisation that this novel green manufacturing technology has in reserve for the future to further improve expression levels, purification yields, and process optimisation is truly encouraging. Ongoing research efforts in barley genomics, proteomics and glycomics will pave the way for further improvements of molecular farming with barley. Some of the unharnessed potential undoubtedly includes improving further the expression levels with identification of even more efficient tissue-specific promoters, postharvest inducible promoters, regulatory sequences, responsive elements and stabilising elements. Tailoring of post-translational modification and intracellular protein targeting are likely to add to the versatility of the barley system. Advances in genetic transformation of barley varieties and cultivation of elite barley lines of selected barley varieties under optimised conditions for protein accumulation will help harness the potential of upstream processes, while advances in downstream processes and protein purification will continue to improve the yields and efficiency of molecular farming in barley.

The time is ripe to weld the efforts of basic and applied science to strengthen the foundation of a green, sustainable manufacturing technology and secure the harvest of some of nature's most complex and valuable compounds—proteins.

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