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Advantages of Therapeutic Protein Production in the Aquatic Plant Lemna

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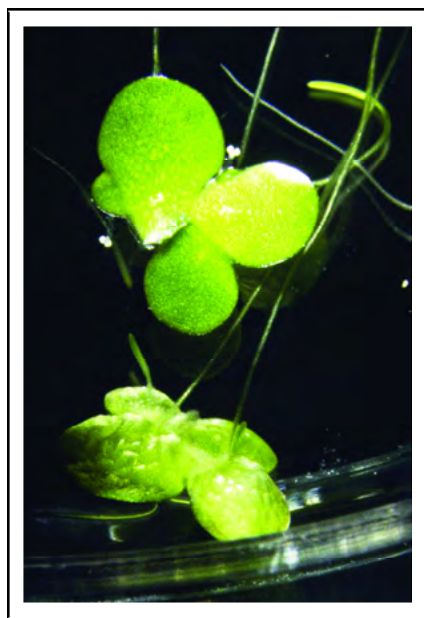
More than 130 drug and vaccine approvals for 95 entities over the last 20 years have generated roughly $30 billion in revenue for the biotech industry.[1,4] The vast majority of this revenue comes from 30 proteins that have manufacturing bottlenecks resulting from the complexities of consistent protein production. The lag times involved in constructing mammalian cell fermentation facilities keep supply of immensely successful high-volume drugs like Enbrel, Rituxan, and Remicade well below estimated demand.[2] In other cases, the complexities of peptide synthesis threaten the potential of soon-to-be-launched or recently approved drugs like Fuzeon.[3] The Pharmaceutical Research and Manufacturers of America (PhRMA) has documented more than 371 new biotech drugs in development, supporting the view that demand for many biopharmaceuticals will continue to outstrip supply.[2,4] That number does not include the multitude of biotech drugs still in research stages.



The aquatic plant *Lemna.*

Protein expression at commercial scale began with microbial systems (*E. coli*, yeast) but quickly progressed to the much more difficult art of mammalian cell culture as the complexity of the desired proteins increased. To generate the required output, facilities using mammalian cells must be constructed over a four to six year period and can cost hundreds of millions of dollars. Those capital requirements and the need to increase the speed of production scale-up have fueled the search for new protein expression systems.

Plant-based recombinant protein production systems offer a safe and extremely cost-effective alternative to traditional microbial and mammalian cell culture systems. Unlike microbial fermentation, plants are capable of carrying out post-translational modifications and, unlike production systems based on mammalian cell culture, plants are devoid of human infective viruses and prions.

Genetic engineering of the green aquatic plant *Lemna* (duckweed) has led to a recombinant protein expression platform with numerous advantages over other existing cell culture and transgenic expression systems (Table 1). Transgenic *Lemna* are grown in aseptically sealed vessels, housed in growth rooms with artificial lighting, and kept in a controlled and contained facility. The plants grow on aqueous media consisting of water and inorganic nutrients; CO_2 in the air is the only carbon source. The completely artificial and enclosed environment, combined with lack of flowering and seed production,make the Lemna System™ uniquely contained and controlled when compared to other plant and animal transgenic systems.

Lemna can secrete the target protein directly into inorganic media, considerably simplifying purification. *Lemna* does not support human or zoonotic pathogens and, because all other components of the system are defined and synthetic, viral inactivation steps in purification are unnecessary. Cost advantages result from simple media requirements for plant growth, inexpensive facilities, and simplified down-stream processing. Twelve proteins have been successfully expressed in the Lemna System™, including small peptides, Fab fragments (Fabs), monoclonal antibodies (mAbs), and large multimeric enzymes. This article describes the production of four pro-

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teins: interferon alpha 2b (IFN), human growth hormone (hGH), a Fab, and a mAb.

Materials and Methods

Plasmid construction.

IFN. The IFN coding region was derived from pRRB20IF-23 (American Type Culture Collection, Manassas, VA). The nucleotide sequence encoding either the rice α-amylase signal peptide (pBx05) (GenBank M24286) or the native human IFN signal peptide (pBx02) (GenBank M24286) was joined to the 5' end of the IFN coding region by direct PCR or overlap extension using synthetic oligos. The final PCR products were cloned into a modification of the binary vector pBMSP3 (obtained from Dr. Stan Gelvin, Purdue University), which is a derivative of pBINPLUS containing the chimeric octopine and mannopine synthase promoter region fused to intron I from the maize alcohol dehydrogenase gene.[5-7] Construct pBx09 was synthesized by Operon Technologies (Alameda, CA) by replacing the native human codons present in pBx05 with frequently used *Lemna* codons. Construct pBx10 was made from pBx09 using PCR by replacing the signal peptide with a start codon. Construct pBx53 was prepared by substituting the 5'-mas leader in pBMSP3 with the leader from the rubisco carboxylase small subunit 5B gene derived from *L. gibba*.

hGH. The hGH coding region was derived from phGH106 (American Type Culture Collection). A similar genetic construct, as described above for pBx53, was created joining the codon for optimized human growth hormone signal peptide with a synthetic, optimized hGH gene (Entelechon, Regensburg, Germany).

Fab. The Fab coding region was supplied by a collaborator. A dicistronic genetic construct was created utilizing the modified pBMSP3 backbone described for pBx53, complete with the leader from the rubisco carboxylase small subunit 5B gene derived from *Lemna gibba*. The second cassette — complete with its own promoter and terminator — for expressing the light chain was inserted into the modified pBMSP3 in a head-to-tail configuration. The optimized rice α-amylase signal peptide was joined to both heavy and light chain sequences optimized (Entelechon) for *L. minor* expression.

mAb. A humanized IgG1 mAb coding region was supplied by a collaborator. A dicistronic genetic construct was created for the heavy and light chain coding regions in a similar fashion to that described above for the Fab.

Transformation and Screening

Using *Agrobacterium tumefaciens* C58Z707, transgenic plants representing individual clonal lines were generated from rapidly growing *L. minor* nodules.[8,9] For transgenic screening, individual clonal lines were preconditioned for one week at 150 to 200 µmol $m^{-2}s^{-2}$ in vented plant growth vessels containing liquid Schenk & Hildebrandt (SH) media without sucrose.[10] Fifteen to 20 preconditioned fronds were then placed into vented containers containing fresh SH media, and allowed to grow for two weeks. Tissue and media samples from each line were frozen and stored at -70°C until assayed.

Protein Characterization

IFN. The concentration of IFN was determined by commercial ELISA (PBL Biomedial Laboratories, New Brunswick, NJ). Further immunological detection of IFN was performed by Western blot analysis using a mouse anti-human IFN antibody (PBL Biomedical Research). Media samples and tissue extracts, normalized for protein content, were separated by 4–20% Tris-Glycine SDS-PAGE gradient gel (Invitrogen) and electrophoretically transferred onto a nitrocellulose membrane. Blots were developed with the Supersignal Western Femto kit (Pierce Biotechnology, Rockford, IL) using HRP goat anti-mouse IgG conjugate (Pierce) as the secondary antibody.

Comparative antiviral activity was determined by incubating HuH7 cells with 1,000 IU/ml of either unpurified IFN from the media or Intron A for 24 hr at 37° C.[11] The IFN was then removed and the cells were washed twice. The cells were subsequently infected with either Encephalomyocarditis virus (EMCV), Vesicular stomatitis virus (VSV), or Sindbis at a multiplicity of infection of 1.0 for 1 hour at which time the virus inoculum was removed. The

Table 1. Key features of the Lemna System™ combine to produce a simple and robust protein expression system. *Lemna* is similar to mammalian cell culture systems like CHO cells in clonal proliferation, biomass doubling time, secretion and containment but are superior in pathogen safety and simplicity of control systems, leading to significant capital and operating cost savings.

Key Features of the Lemna System™	
Feature	**Description**
Clonal proliferation	*Lemna* proliferates vegetatively (clonal replication). No seeds or pollen are generated
Rapid proliferation	*Lemna* doubles its biomass in 36 hours
High protein content	Protein constitutes 30% of dry weight; little metabolic energy is devoted to support elements
Secretion	Transgenic proteins are secreted into a simple media (water, inorganic salts)
Containment	Plants are batch-cultured in aseptically sealed vessels under artificial light
Pathogen safety	No human or zoonotic viral pathogens are supported by *Lemna*; media is completely synthetic
Inexpensive	System inputs are water, light, air and inorganic nutrients

cells were washed three times and allowed to grow for 24 hours at 37° C in normal growth media. After harvesting, the cells were lysed by six freeze/thaw (-80° C) cycles and cell debris was removed by centrifugation. The virus present in the supernatant was assayed as the mean of eight separate determinations at each ten-fold dilution (ranging from 10^3 to 10^{10}) by determining the cytopathic effect on monkey CV1 Vero cells. For antiproliferative activity, sextuplet cultures of interferon-sensitive Daudi cells in RPMI 1640 growth medium, supplemented with 10% fetal calf serum, were established in microtiter plates at an initial concentration of 50,000 cells/ml. The cultures were either left untreated or were treated with 1,000 IU/ml of unpurified IFN, Intron A, or an equal volume of *Lemna* control media (derived from non-transgenic plants grown under identical conditions). After four days, the number of viable and dead cells was determined by a hemocytometer using the trypan blue dye-exclusion viability test.

IFN purification was accomplished by mAb affinity column chromatography.[12] N-terminal sequencing was obtained from the Keck Foundation (Yale University) while matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF/MS) was performed at Commonwealth Biotechnologies, Inc.(Richmond, VA).

hGH. The concentration of hGH in the media was determined by commercial ELISA (Roche Diagnostics, Mannheim, Germany). Immunological detection of media and tissue was determined by Western blot analysis as described above for IFN with the exception that a mouse anti-human hGH antibody (United States Biological, Swampscott, MA) was used as the primary antibody with comparison to standard hGH (United States Biological). Confirmation of bioactivity was determined by an Nb_2 cell bioassay and was performed by Orpegen Pharma (Heidelberg, Germany).[13]

Fab. Immunological detection of Fab was determined by Western blot analysis as described above for IFN using a Fab-specific goat anti-human IgG antibody (Sigma, Saint Louis, MO) as the primary antibody and a HRP-conjugated mouse anti-goat secondary antibody (Pierce). Control Fab, produced in *E. coli*, was supplied by the collaborator. Expression levels were determined by quantitative Western blotting. Functional antigen binding activity was determined by a modification of the Western blot procedure detailed above. Control antigen was separated on a 4–20% Tris-Glycine SDS-PAGE gradient gel (Invitrogen) and electrophoretically transferred onto a nitrocellulose membrane. *Lemna*-produced Fabs were used as primary antibodies with an HRP-conjugated goat anti-human Fab (Sigma) as a secondary antibody.

mAb. The concentration of mAb was determined by a commercial human IgG-Fc ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX) and confirmed by quantitative Western blotting. The blotting was carried out as described above using a goat anti-human IgG, recognizing heavy and light chains (Pierce), as the primary antibody and HRP-conjugated mouse anti-goat (Pierce) as the secondary antibody. Human IgG1 was used as the control standard (Sigma). Protein A pulldown was accomplished by incubating the *Lemna* mAb with Protein A agarose beads (Sigma). The beads were subject to heat denaturation and directly loaded onto a SDS-PAGE gel. Functional antigen binding activity was determined as described above for the Fab. *Lemna*-derived mAbs were used as a primary antibody while an HRP-conjugated goat anti-human IgG (Sigma) was used as a secondary antibody.

Table 2. Expression of IFN in *Lemna*.

	Top Expressing Line Media (mg/L)[c]			Construct Mean Average Values 2 Week Screening Trials (ELISA)	
	Research Scale (2 wk)	Research Scale (3 wk)	Bio-production Scale	Media (mg/L)[c]	Tissue (mg/kg tissue)[a]
pBx02	2.0	-	-	0.12	23.3
pBx05	1.1	-	-	0.13	86.7
pBx09	24.3	60	30	1.51	164
pBx10	< 0.1	-	-	< 0.001	99.3
pBx53	100	300	500	15.3[d]	-

[a]Based on 1 g of tissue yielding 20 mg of protein.
[b]Based on a recovery of 10 ml of media and 1 g of tissue per screening trial.
[c]Expressed as a pre-purification titer
[d]1 week screening data.

Results and Discussion

IFN Expression

IFN has been used as the proof-of-concept protein in the Lemna System™ and is, therefore, the system's most characterized protein expressed to date. To optimize production and accumulation of IFN in *Lemna*, we constructed and tested a series of IFN transgenes. All the genes were driven by a chimeric promoter. This consisted of a trimer of the upstream activating sequence of the octopine synthase gene linked to the activator-promoter region of the mannopine synthase gene.[6] The pBINPLUS expression cassette also contained intron 1 from the maize alcohol dehydrogenase gene, which has previously been shown to increase gene expression.[5,7] To take advantage of *Lemna's* ability to secrete recombinant proteins into its simple growth media, IFN was targeted to the endoplasmic reticulum (ER) using two different signal peptides. The first chimera was constructed by fusing the rice α-amylase signal peptide to the mature coding region of IFN (pBx05). The second analogous chimera was constructed using the native

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Table 3: Comparative Antiviral Bioactivity of Biolex IFN and Intron A

Sample	IU in Test	Virus Titer		
		VSV	EMC	SINDBIS
Control media[a]	0	9×10^8	3×10^7	3.5×10^7
IFN[b]	1,000	2.2×10^7	$< 10^3$	6.3×10^5
Intron A	1,000	2.9×10^7	$< 10^3$	3.0×10^5

[a]Control media was derived from wild-type *L. minor.*
[b]Derived from a single clonal transgenic line.

human signal sequence to determine if a human targeting sequence (pBx02) was functional in *Lemna.* Because 23% of the human IFN gene (in pBx05) contained rare or non-preferred *Lemna* codons, we optimized the entire IFN coding region to create pBx09. To evaluate a non-secreted form of IFN, pBx10 was constructed from pBx09 by removing the rice α-amylase signal peptide and inserting a methionine immediately preceding the mature IFN coding sequence. Further optimization (pBx53) was accomplished by substituting the 5'-mas leader in the basal pBMSP3 vector with the leader from the ribulose-bis-phosphate carboxylase small subunit 5B gene derived from *L. gibba.*[14] Transgenic plants expressing IFN were generated using *Agrobacterium*-mediated transformation and a minimum of 40 independent transgenic lines were screened for each construct.[9]

In a typical research feasibility study, regenerated plants are put through an initial screening trial to select the top expressing (5–10%) lines. Those top lines are then rescreened and further evaluated to monitor accumulation of recombinant protein over time. At the end of this process, the highest expressing line is then selected and moved into development for larger scale production and further optimization of expression. As is typical of a random genetic transformation, there is considerable variation in the level of transgene expression from one clonal line to the next because of variations in the number and localizations of gene insertions.[15] Therefore, it is necessary to examine a population of transgenic lines in order to evaluate the effectiveness of different expression cassettes.

Table 2 summarizes IFN expression on research and bioproduction scales for the constructs described above. When comparing constructs, a series of key conclusions can be reached based on this data. First, secretion into the media is dependent on the presence of an ER signal peptide. The only construct that lacks a signal peptide (pBx10) also shows no significant accumulation of IFN in the media. Second, *Lemna* can recognize human signal sequences with the same efficiency as plant signal sequences (comparing pBx02 with pBx05). Third, codon optimization of IFN (comparing pBx05 and pBx09) is an effective way to increase transgene expression. Polyribosome binding studies suggest that this increase was due to an increase in mRNA abundance and not polyribosome association (data not shown).

The very high expression levels attained for pBx53 further show that the Lemna System™ has yet to reach its full potential. One challenge of any new expression system is to define the genetic elements that will allow the system to operate at maximum efficiency. For *Lemna*, a simple substitution in the 5'-untranslated region of the DNA cassette allows for at least a ten-fold improvement in transgene expression. It should be pointed out that the average mean values for pBx53 found in Table 2 are the result of a one week growth cycle (compared to two weeks for the other constructs). This represents our success in streamlining the system, minimizing the time it takes to go from gene to line selection and then to kg of protein. Currently, it takes less than eight months to go through this process.

Table 2 also indicates our ability to scale-up transgenic *Lemna* and duplicate or exceed research-scale expression levels. Bioproduction scale-up represents a more than 200-fold increase in biomass

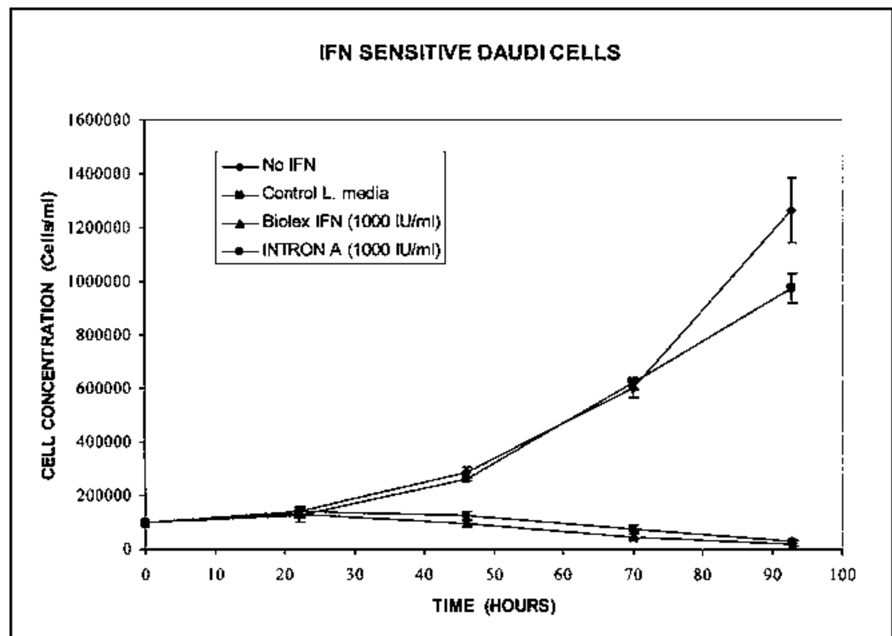

Figure 1. Antiproliferative activity of Biolex IFN. IFN-sensitive Daudi cells were incubated with control and IFN-containing samples and cell density was determined over time. Biolex IFN had the same antiproliferative activity as Intron A. No lysed cells were evident during the course of the experiment, as determined by trypan blue exclusion.

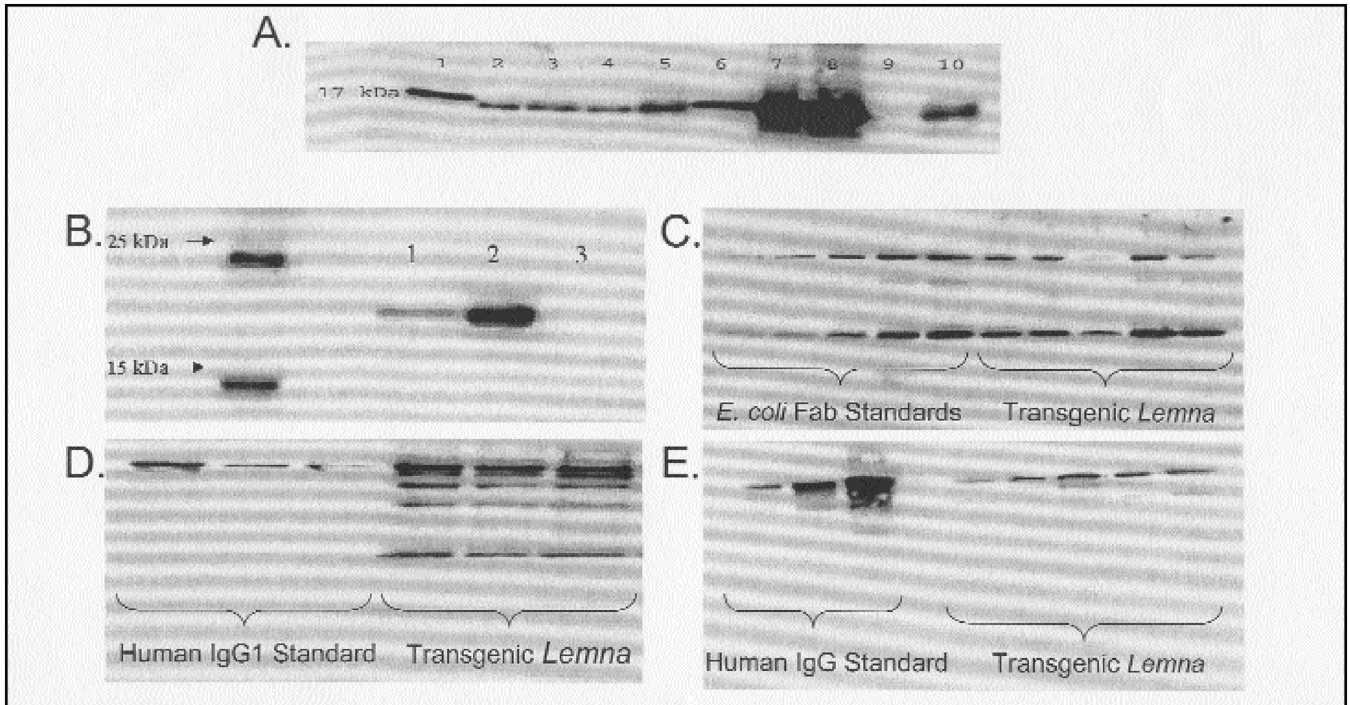

Figure 2. Western blot detection of *Lemna*-produced recombinant human proteins. (A) IFN: Lanes 2, 4, 7, and 9 represent media samples (10 μl) and lanes 3, 5, 8, and 10 represent tissue homogenates (20 mg protein). Lanes 2 and 3 represent pBx02, lanes 4 and 5 represent pBx05, lanes 7 and 8 represent pBx09, and lanes 9 and 10 represent pBx10. Lanes 1 and 6 represent *E.coli* standard IFN (PBL Biomedical). The highest expressing line was used for each construct. (B) hGH: Lane 1 is *E.Coli* standard hGH, lane 2 is Biolex IFN, and lane 3 is *Lemna* control media.(C) Fab: (D) mAb:(E) Protein A pulldown of Biolex mAb.

per vessel. One of the challenges of the Lemna System™ has been to equate our expression levels with those found in other systems. *Lemna* is unique in that it grows hydroponically in a very dilute inorganic media with very low protein content. This puts it in a very different category from typical fermentation systems or tissue-derived recombinant sources. To normalize expression values with other systems, we have defined the protein accumulation in Table 2 in terms of a pre-purification titer. This titer allows for a 50x concentration of crude *Lemna* media and would equate to the actual feed stream for commercial purification. *Lemna* media contains only 30 μg/ml of target protein and host plant proteins, which after a 50x concentration would afford a purification feedstream of only 1.5 mg/ml — still lower than most other systems. In the case of pBx53, IFN would represent over 30% of the total media proteins, resulting in considerably lower downstream purification costs.

IFN Characterization

Type I interferons are characterized by their ability to induce antiviral activity in cells of the homologous species against a wide range of unrelated viruses.[16] We used two methods to determine the bioactivity of our IFN. First, we compared the antiviral activity of unpurified media-derived IFN against pharmaceutical grade *E. coli*-derived IFN (Intron A™) in two human cell lines, HuH7 and WISH. We used three different RNA viruses: EMCV (a Picornavirus), Sindbis (the prototype Alphavirus) and VSV (a Rhabdovirus). Table 3 indicates that our IFN and Intron A exhibit similar antiviral activity. Second, Type I interferons are characterized by their ability to inhibit the proliferation of a variety of cell lines of both neoplastic and normal phenotypes.[16] Therefore, we tested the ability of IFN to inhibit the proliferation of the human Burkitt lymphoma-derived cell line Daudi.[17] Figure 1 shows that unpurified IFN was as effective as pharmaceutical-grade Intron A at inhibiting the proliferation of Daudi cells. Media derived from wild-type L. minor had no effect on cell growth. We saw no evidence of lysed cells indicating the effect was truly inhibition of proliferation and not apoptosis.

To determine the immunoreactivity and molecular weight of IFN, Western blots of media and tissue-derived protein from the top expressing line from each construct were performed using anti-human IFN antibodies. Media-derived IFN appeared as a single band at 17 kDa compared to a 17.5 kDa band observed for *E. coli* produced IFN (Fig. 2). The absence of an immunoreactive band in the media from plants expressing pBx10 (no signal peptide) further confirmed that the media accumulation of IFN depends on the presence of a signal peptide and thus the secretion process. Affinity purification and N-terminal sequencing confirmed the correct signal peptide processing of pBx09, leaving an N-terminal cysteine as the first amino acid.[18] Specific activity was determined to be $2x10^8$ IU/mg, which is consistent with reported values for commercial IFN.

Lemna-produced media and tissue IFN appear to undergo an additional processing step that is typically not

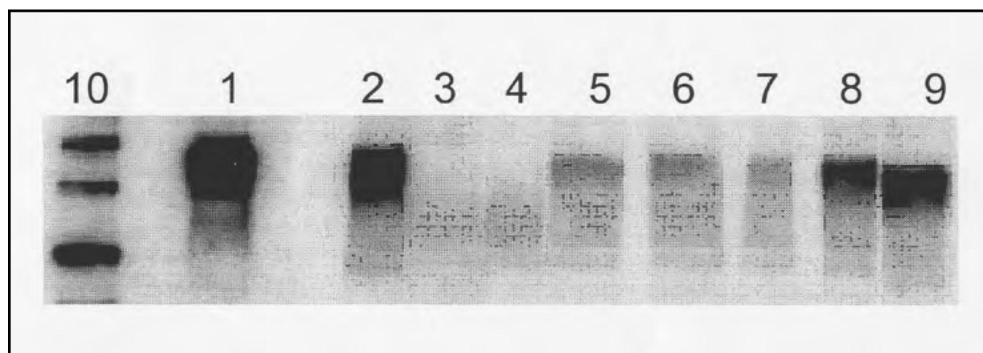

Figure 3. Antigen-binding activity of Biolex Fab and mAb. Antigen is run on a 4-15% SDS-PAGE gel and blotted onto a nitrocellulose membrane. Transgenic Biolex Fab (lanes 5-9) and mAb lanes (lane 1) were then used as primary antibodies to probe the blot. Lane 2 represents control *E. coli* Fab. Lanes 3 and 4 represent non-transgenic *Lemna*. Lane 10 represents a molecular weight marker.

reported with human normal leukocyte IFN. Analysis of purified IFN by MALDI-TOF shows a mass of 18.4 kDa, which is consistent with the removal of seven C-terminal amino acids. The difference between exact mass and electrophoretic mobility has been reported elsewhere.[19] The loss of the amino acids explains the slight mobility shift seen by Western blotting (Fig. 2). This highly efficient processing occurs in both media and tissue-derived samples. A similar post-translational C-terminal cleavage has been reported from a leukemic cell line in which the majority of interferon produced was missing 10 C-terminal amino acids. Like the processing of transgenic *Lemna* IFN, the loss of the 10 C-terminal amino acids has no effect on the biological activity of this naturally occurring IFN.[20] This natural susceptibility to additional C-terminal processing may also account for the lower molecular weight entities in commercial preparations derived from human cell lines.[21] The other three proteins presented in this article show no evidence of C-terminal truncation, indicating this process may be specific for IFN.

hGH expression

The hGH coding region, including the signal peptide, was codon-optimized for *Lemna*-preferred codons. Secreted levels in the plant growth media were 609 mg/L (pre-purification titer). Similar to IFN, approximately 50% of the hGH remained in the tissue. A total of 0.81 g/kg dry weight was produced. Human growth hormone co-migrates with the *E. coli* produced on SDS-PAGE as detected by Western blotting (Fig. 2B). In addition, the *Lemna*-produced hGH in a unpurified media sample exhibited significant biological activity similar to the specific activity of the purified *E. coli*-produced hGH in a cell growth assay (data not shown).[13]

Fab expression

A Fab fragment was expressed in *Lemna* from a dicistronic vector containing codon-optimized heavy and light chains of the Fab fragment. As measured by a quantitative Western blot, the Fab fragment was up to 4% of the total soluble protein, which translates into 8.62 g of Fab/kg of dry weight. The majority of the Fab fragment was retained in the tissue. The Fab co-migrates on a SDS-PAGE gel with the *E. coli*-produced Fab, primarily as a dimer with some tetrameric species. Antigen binding by the *Lemna*-produced Fab is demonstrated in Figure 3.

mAb expression

A mAb was expressed in *Lemna* from the same dicistronic vector used with the Fab fragment. The heavy and light chains were both codon-optimized with *Lemna*-preferred codons. The total mAb, measured by a quantitative Western blot, was up to 2.8% of total soluble protein which is equivalent to 5.60 g/kg dry weight. The mAb expression level was similar to the Fab in quantity and the majority of the mAb was distributed in the tissue instead of the media. However, there are ongoing advancements in releasing the tissue-retained mAb and Fab into the surrounding media. The crude preparation of *Lemna*-derived mAb contained a high proportion of fully assembled mAb with a representation of partially assembled fragments much like those seen in CHO- and *E. coli*-derived mAb preparations (Fig. 2D).[22] The *Lemna*-produced mAb co-migrated with a CHO-derived mAb (IgG1) on a Western blot of a Protein A pulldown mAb fraction (Fig. 2E). The *Lemna*-derived mAb was also shown to bind antigen (Fig. 3).

Conclusions

The expression of these four different recombinant proteins is quite encouraging for therapeutic protein production in *Lemna*. Stable transgenic plants were generated in as few as six weeks. At least 50% of IFN and hGH was secreted into asceptic growth media with pre-purification titers as high as 609 mg/L. Both proteins were found to be biologically active with IFN shown to be at least equal to the commercial source, Intron A. Efficient secretion of these recombinant proteins into an inorganic media with no need for viral inactivation offers substantial cost advantages in downstream purification.

Overall, *Lemna* expression of a Fab fragment and a full-length mAb is very good in terms of both quantity and quality. The majority of *Lemna*-expressed mAb was full length and both the Fab fragment and mAb exhibit antigen binding. We are actively pursuing even higher expression levels through further genetic and bioprocessing refinements. In addition, preliminary experiments that alter the plant growth media to more efficiently release the Fab fragment and mAb are yielding encouraging data. These promising results indicate that *Lemna* will serve as an ideal production system for large volume mAbs as well as other therapeutic proteins in a contained and low cost format.

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