African Journal of Biotechnology Vol. 10(74), pp. 17016-17022, 23 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.418
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Expression of recombinant interferon α-2a in tobacco chloroplasts using micro projectile bombardment

Farwa Nurjis[1] and Muhammad Sarwar Khan[2*]

[1]National Institute for Biotechnology and Genetic Engineering (NIBGE), P. O. Box 577, Jhang road, Faisalabad, Postcode 38000, Pakistan.
[2]Centre of Agricultural Biochemistry and Biotechnology (CABB), University of agriculture, Faisalabad, Pakistan.

Accepted 5 October, 2011

Chloroplast-based expression of pharmaceuticals provides cost-effective benefits to the consumer. In order to establish the transplastomic biopharmaceuticals, the interferon α-2a gene along with *aadA* gene was flanked by the tobacco chloroplast inverted repeat region for two events of homologous recombination. Chloroplast transformation was accomplished upon bombardment of fully expanded 4 to 6 weeks-old tobacco leaves using helium gun. Green shoots regenerated from single antibiotic resistant cells were subjected to further rounds of selection and regeneration to develop homoplasmic clones. The molecular analysis of the antibiotic-resistant plants confirms the presence of interferon alpha-2a as well as *aadA* genes in the plastid genome. Moreover, the interferon alpha-2a protein was purified by using Ni-NTA purification columns. The presence of a fragment of 20 kDa size on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and high performance liquid chromatography (HPLC) chromatogram confirms the expression of IFNA2a in the transgenic tobacco chloroplasts.

Key words: Interferon, therapeutic, synthetic gene, tobacco, transplastomic strategy, biolistic method.

INTRODUCTION

Plant molecular farming, or "biopharming", is still a relatively new venture that combines the use of biotechnology and agricultural plants in order to produce valuable products (Webster, 2004). Plant-based pharmaceuticals, in their broadest sense, can be divided into three categories, such as single-molecule drugs (Fowler and Law, 2006), botanicals (Food and Drug Administration (FDA), 2004) and biologics (Wade et al., 2004, 2006). Both single-molecule drugs and botanicals are derived from non-transgenic plants. While the third group, biologics, are the products of transgenic plants. The technology of producing biologics is often called biopharming. The biopharming industry is growing at an annual rate of 15% (IMS Health, 2003) and projected to be worth $100 billion by the year 2020 (www. molecular-farming.com). More recently, the range of recombinant proteins made in plants has extended to include industrial enzymes (Hood et al., 2003), technical proteins that are used in research (Hood et al., 1997), milk proteins that are suitable nutritional supplements (Chong et al., 1997), new protein polymers with both medical and industrial uses (Ruggiero et al., 2000) and very high-value compounds, such as human (Lico et al., 2007) or veterinary vaccines (Dus Santos and Wigdorovitz, 2005) or interferon (de Zoeten et al., 1999), intermediates or nutraceuticals, including recombinant enzymes (Hood et al., 1999) or ω -3 and ω -6 'fish oils' (Murphy, 2006), antibodies, feed additives and hormones for human and animal health (Chakauya et al., 2006). Bio-containment of biopharmed crops is a key concern, as expressed by the US National Academies (Anon, 2004) and by numerous experts in the agbiotech and related sectors (Dunwell, 2005; Lee and Natesan, 2006). Many bio-containment strategies rely on the insertion of biological barriers that prevent transgene flow to other plants (Al-Ahmad and Gressel, 2005).

However, one of the best-known methods of transgene bio-containment is transplastomics. Transplastomics have several advantages including high expression levels

*Corresponding author. E-mail: sarwarkhan_40@hotmail.com. Tel: +92-41-2651475.

(Khan and Maliga, 1999), polycistrons are translated without processing into monocistrons (Khan et al., 2007), lack of gene silencing and position effect (Khan, 2000) and multiple genes can be expressed in operons (Khan, 2006) because of the fact that the chloroplast mechanism is basically prokaryotic (Khan, 2005a, b). Chloroplasts are maternally inherited, which helps in transgene containment as transgenes, and they are not transmitted via pollen (Khan et al., 2001). Moreover, foreign proteins synthesized in chloroplasts are properly folded with appropriate post transcriptional modifications (Khan et al., 2005a, b), including disulfide bonds (Arlen et al., 2007) and lipid modifications (Glenz et al., 2006). In this study, we plan to develop the system for expressing the interferon alpha-2a, a high value pharmaceutical protein cost effectively by using the transplastomic technology in tobacco, a non-edible plant.

MATERIALS AND METHODS

Plant material preparation for chloroplast transformation:

Nicotiana tabacum var. Petit Havana was grown aseptically on 0.7% phyta-agar-solidified MS salts (Murashige and Skoog, 1962), pH 5.8, containing 3% sucrose at 27°C under 100 µmol photons m^{-2} s^{-1} (16 h light, 8 h dark). Fully expanded leaves of plants 4 to 6 weeks old were used for chloroplast transformation.

Vector construction for transplastomic biopharmaceuticals

Transplastomic vector was developed to express the synthetically engineered gene of high value pharmaceutical protein (Interferon alpha-2a) in tobacco plastome. The chloroplast transformation vector was comprised of left and right border sequences flanking the IFNA2 gene along with strong light inducible *rbcL* promoter that was cloned upstream to the antibiotic resistant gene *aadA*. The marker gene *aadA* was under the control of ribosomal RNA operon (*rrn*) promoter. The untranslated 3` region of *psbA* gene was used as terminator. The promoter sequences were used to facilitate recognition by the RNA polymerase and initiation of transcription, leading to gene expression. While the terminator sequences confer transcript stability that is an unimaginably important step for hyper-expression of the integrated gene. The histidine tag was used for the purification of IFNA2a.

Transformation of tobacco leaves

Gold particles obtained from Bio Rad (50 mg of 1 µm diameter) were suspended in 1 ml of absolute ethanol. After centrifugation particles were re-suspended in 0.5 ml H_2O and then 50 µl aliquots were pipetted into sterile centrifuge tubes. Each tube contains 1 µg/µl DNA, 50 µl of 2.5 M $CaCl_2$ solution and 20 µl of 0.1 M spermidine-free base. Tubes were vortexed and centrifuges at 2,700 g for 2 s. After removing supernatant, pallet was rinsed three times with absolute ethanol and finally re-suspended in 30 µl of absolute ethanol. Fully expended dark green leaves from tissue cultured 4 to 6 weeks old plants were used for bombardment. Leaves were excised under sterile conditions and placed abaxial side up on whatman No.1 filter papers laying on MS medium in plates for bombardment as described by Khan and Maliga (1999). Bombardment was carried out under a partial vacuum (28 in Hg) with a Bio Rad PDS1000 (He) gun, using 1,100 psi rupture disk and a helium cylinder pressure of 1,300 psi. The micro projectiles coated with plasmid DNA were blasted using device PDS 1000/He (Bio Rad), as described by Khan et al., 2007. Bombarded leaves were kept for 2 days at 25°C in a growth room under dark.

Selection and regeneration of transgenic plants

Upon two days post-bombardment, leaves were chopped into small pieces and placed on the RMOP medium containing 500 mg/L of spectinomycin dihydrochloride, with abaxial side touching the medium in deep (100 × 25 mm) Petri-plates and grown under 16/8 light/dark conditions. The regenerated resistant shoots were chopped into small pieces (about 3 × 3 mm) and sub-cloned into fresh deep Petri-plates containing the same selection medium. Antibiotic resistant shoots from the second round were transferred to the rooting medium (MSO medium supplemented with IBA, 1 mg/L) for root initiation in a growth room at 28°C under standard conditions (16 h light: 8 h dark, 170 µmol photons m^{-2} s^{-1}). Rooted plants with expanded leaves were used for further studies.

Molecular and protein analysis of transgenic plants

After extracting the total cellular DNA of transgenic leaves following the protocol as described by Murray and Thompson (1980) and Cheema et al. (2010), the PCR (polymerase chain reaction) was carried out using the protocol given by Saiki et al. (1985). All the reactions for analysis of transformant DNA were carried out using Biotaq polymerase (Bioline, London, UK). A reaction volume of 50 µl was used, including 5 µl of 10X reaction buffer (Appendix 2, section 2.5) and 4 µl dNTPs (2.0 mM), 0.5 µl of each primer (30 pmol), 3 µl 25 mM $MgCl_2$ and 10 to 20 ng template DNA were added. PCR amplification was carried out using a master cycler gradiant PCR (Eppendorf AG 22331, Hamburg, Germany) with a variety of programmers. In all cases, each cycle began with 2 min at 94°C for denaturation, followed by 2 min at an annealing temperature of 56°C, which depends on the sequence and length of the oligonucleotide. A 3 min extension period at 72°C completed each cycle. A total of 30 cycles were used before cooling to a temperature of 4°C to avoid any degradation of amplified DNA. Primer melting temperatures were calculated as 4 x (G+C) + 2 x (A+T). The expressed interferon alpha-2a was purified by using the Ni-NTA purification system (from invitrogen) under denaturing condition and analyzed through SDS PAGE following the protocol as described by Laemmli (1970).

High performance liquid chromatography was performed on Shim-Pack CLC-ODS column (15 cm x 4.6 mm, 5 µm) attached to a UV detector with wavelength set at 280 nm as described by Luykx et al. (2005) with certain modifications.

RESULTS AND DISCUSSION

Development of chloroplast transformation vector

Chloroplast transformation vector was developed to target interferon alpha-2a gene into the inverted repeat region of tobacco through the homologous recombination mechanism due to the flanked border sequences that were cloned in the transformation vector along the marker gene. The presence of antibiotic resistant marker gene cloned down stream to the interferon alpha-2a was confirmed by subjecting the final transformation vector to the PCR reaction using a primer pair specific for *aadA* gene that encodes aminoglycoside-3'-adenyltransferase

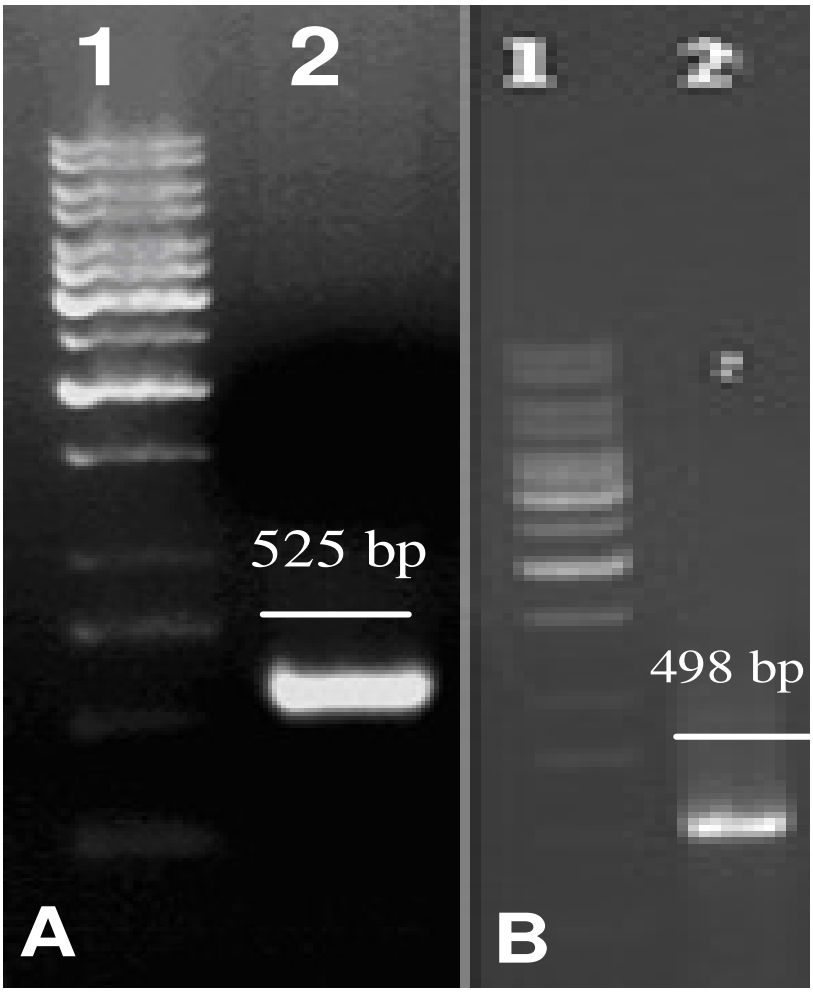

Figure 1. A: Confirmation of aminoglycoside 3` adenylyl transferase (*aadA*) presence through polymerase chain reaction strategy. Lane 1 is DNA marker of 1 kb. Lane 2 represents vector DNA containing marker gene *aadA*. B: Confirmation of presence of interferon alpha-2a gene by using the gene specific primers through polymerase chain reaction. Lane 1 is 1 kb DNA ladder. Lane 2 represents Interferon alpha-2a.

and confers resistance to spectinomycin and streptomycin as shown in Figure 1A. Marker gene is physically linked with Interferon alpha-2a for basically two reasons, firstly to give to the transformed cells a selective advantage, allowing them to grow faster and better, and secondly, to kill the non-transformed cells thereby allowing only the required gene recipient cells to grow (Khan et al., 2001). The marker gene can either be placed under the same promoter by fusing two genes transcriptionally or separately tethered to two different promoters, in either case they are physically linked together (Daniell and Khan, 2003). Moreover, the presence of interferon alpha-2a gene in chloroplast transformation vector was confirmed by amplifying the 500 bp fragment through PCR using the gene specific primers as shown in Figure 1B. The final vector was used in transformation experiments.

Development of transgenic chloroplast plants

Fully expanded tobacco leaves were bombarded using DNA-coated gold particles. Tobacco leaf sections after bombardment were placed on RMOP medium (Svab and Maliga, 1993) containing 500 mg/l spectinomycin. Spectinomycin resistant shoots recovered on regeneration medium were subjected to further rounds of selection and regeneration to purify the transgenic plastids as shown in Figure 2. Phenotypically, these antibiotic-resistant plants were indistinguishable from wild-type plants which were growing on the same medium without spectinomycin. The regenerated shoots which survived on the antibiotic-containing medium may be due to the following reasons: integration of cassettes into chloroplast genome, or integration of cassettes into the nuclear genome due to illegitimate recombination events or due to spontaneous

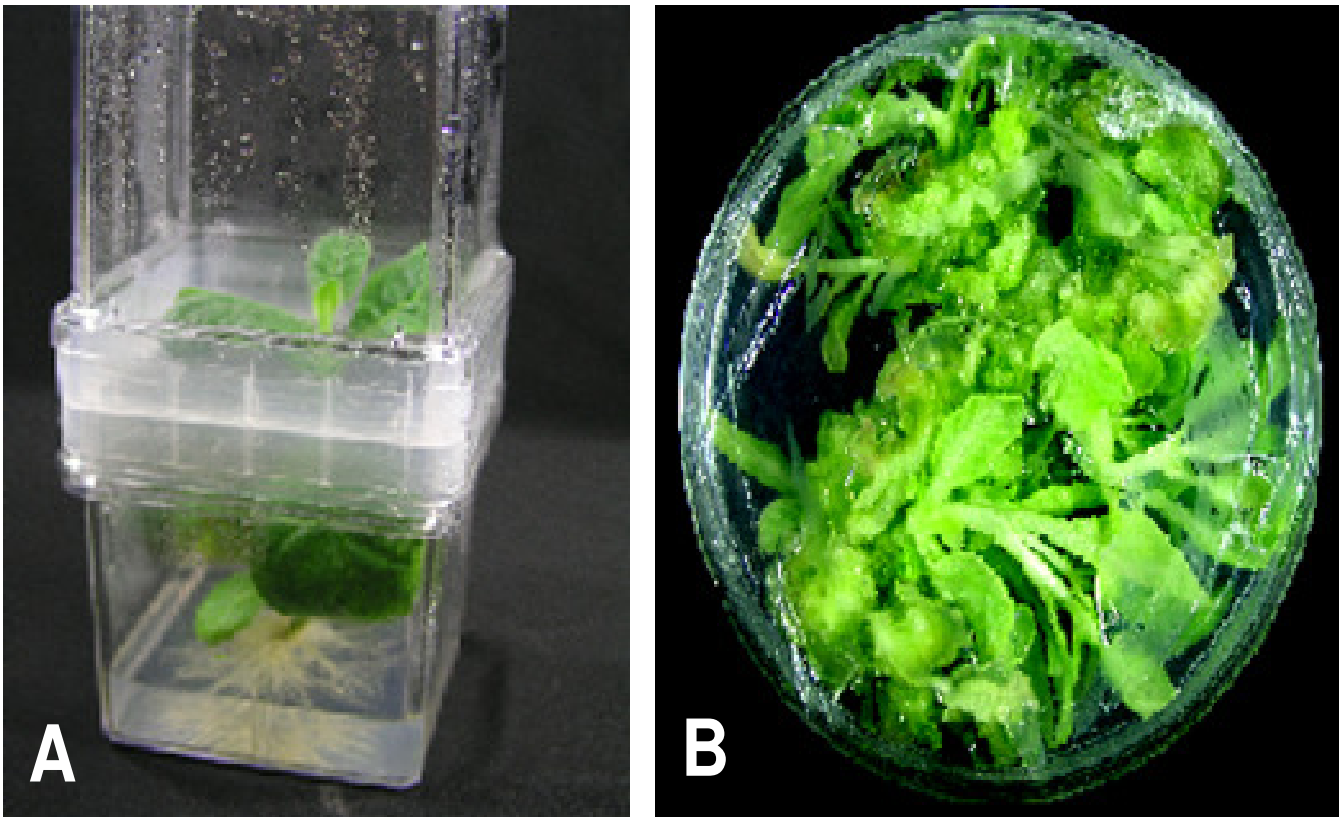

Figure 2. (A) Transgenic tobacco plant. (B) Regenerated tobacco shoots on RMOP medium.

mutation in the 16S rRNA gene that is known to confer resistance to spectinomycin (Khan and Maliga, 1999). The chloroplast transformants were sorted out from nuclear transformants and spontaneous spectinomycin resistance mutants by subjecting the spectinomycin resistant plants to different round of selection and regeneration on antibiotic containing maintenance and regeneration media. Transplastomic plants obtained after each round of selection and regeneration were subjected to genetic analysis using PCR technique by amplifying both the marker as well as interferon alpha-2a genes as shown in Figure 3A and B.

Purification of interferon alpha-2a protein from transplastomic tobacco plants

The presence of interferon alpha 2a protein in transplastomic tobacco plant was confirmed by SDS-PAGE, after purification through Ni-NTA purification system (from invitrogen). The purified interferon alpha-2a was also subjected to the HPLC analysis, as compared to the standard, single peak was observed in the chromatogram as shown in Figure 4. The purified expressed 20 kDa protein as shown in Figure 5 was further analyzed by developing a reaction with anti-interferon antibodies. Nevertheless, the quantity of the expressed protein was very low. This was perhaps because of heteroplasmic nature of transgenic plants. Therefore, the eluted fraction was precipitated using acetone, which was run on the SDS-PAGE. Presence of 20 kDa protein that also made conjugates with interferon antibodies had confirmed that transplastomic (transgenic chloroplast) plants are expressing IFN alpha-2a gene. However, further purification of transgenic plants to achieve homoplasmicity is required.

A major advantage of transgenic plants for molecular farming is the comparatively low cost of large-scale production. Both capital and running costs are significantly lower than those of cell-based production systems because there is no need for farmers or the skilled personnel to run them. Because of the relative ease of scalability, the production costs of plant based pharmaceuticals are estimated to be reduced from 1000 to $5000 per g of protein for animal systems to 1 to $10 in plants (Bloomberg News, 2005), and about ten-fold less when compared to microbial fermentation production costs (Hood and Woodward, 2002). For developing countries, this significant cost saving can be used to meet other needs such as improvement of infrastructure for health care delivery and hunger alleviation. Introduction of novel proteins or enzymes into plastids can be

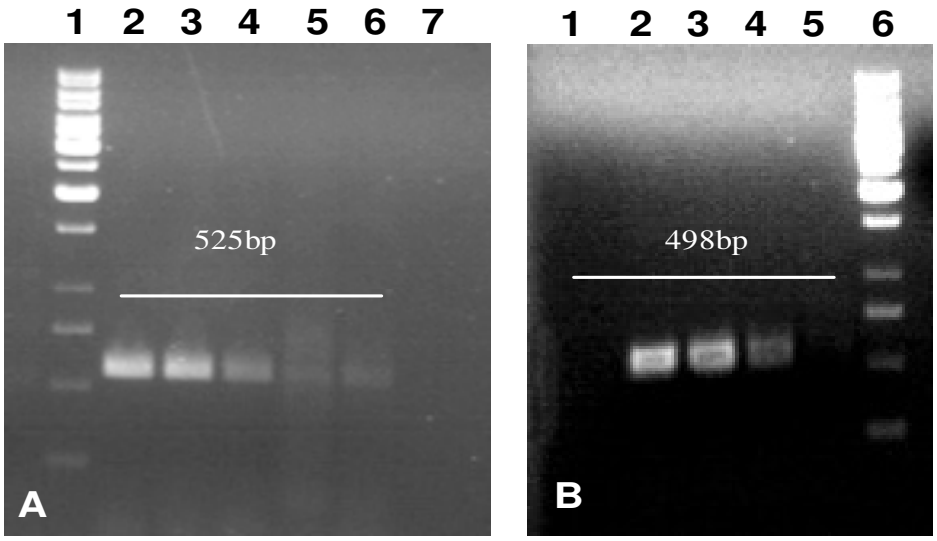

Figure 3. A) Amplification of selection cassette from putative transgenic plants using *aadA* specific primers. Lane 1 shows 1kb DNA ladder. Lanes 2 to 6 represents transgenic plants. Lane 7 represents wild type untransformed plant sample. B) Confirmation of integration of IFN alpha-2a gene cassettes into plastome using gene specific primers. Interferon alpha-2a specific primers confirm the presence of IFNA2a gene in the transgenic plant by amplifying the 500 bp fragment. Lane 1 represents wild type tobacco plant. While Lanes 2 to 5 are amplified IFNA2a gene from transgenic plant. Lane 6 illustrates 1 kb DNA marker.

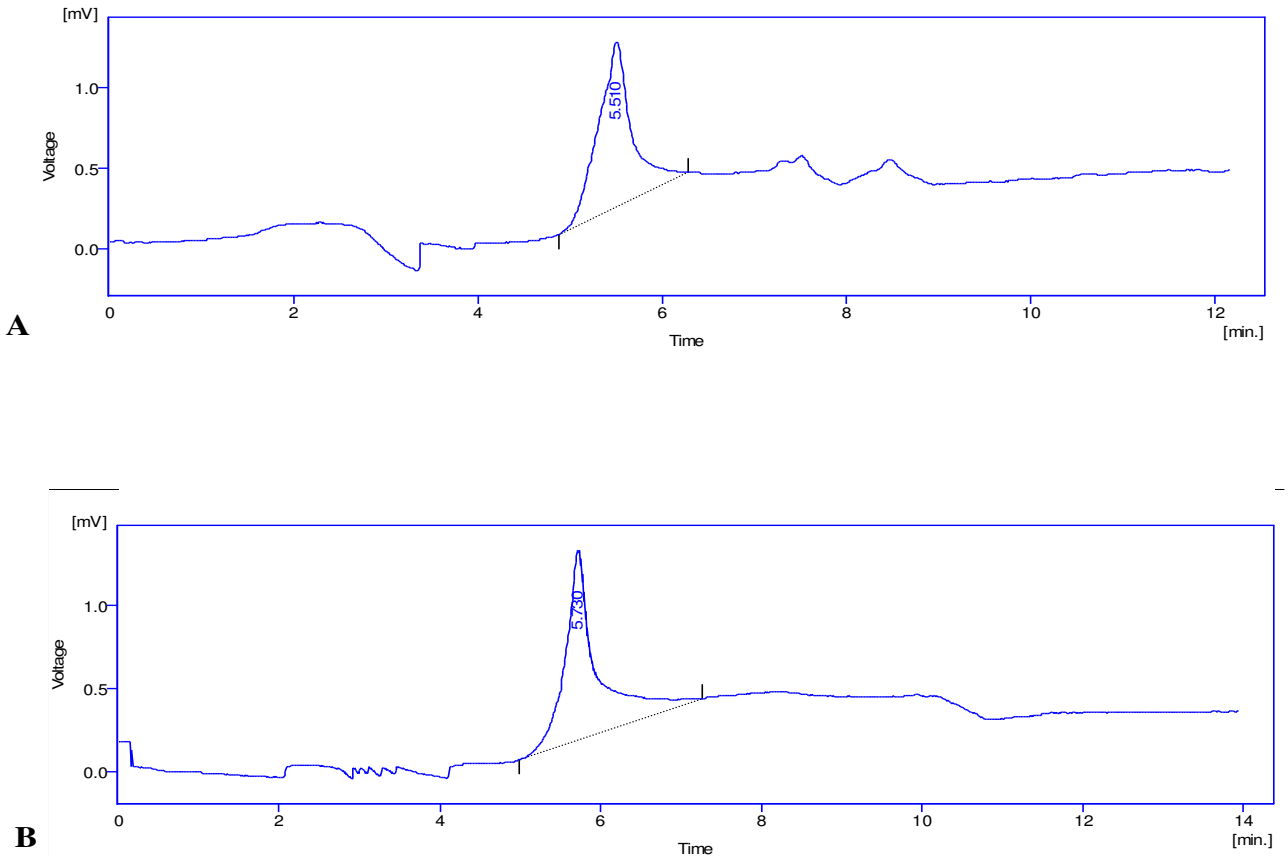

Figure 4. (A) Chromatogram shows the standard peak after passing through the HPLC column at the flow rate of 0.3 ml/min. B) Sample containing the purified IFNA2a after HPLC analysis.

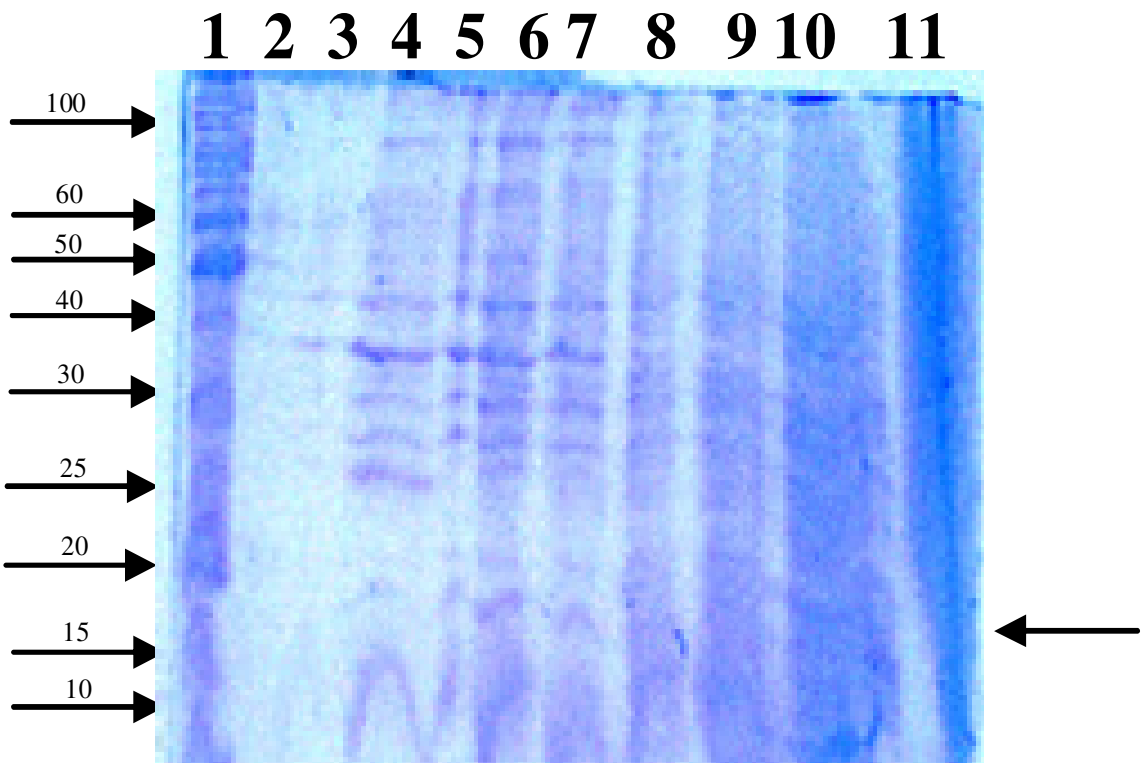

Figure 5. Purification of interferon alpha 2a protein from putative transgenic plants. Lane 1 shows protein ladder. Lane 4 shows eluted interferon alpha 2a protein. Lanes 6 and 8 represent washings at pH 7.8 and 5.3 of Ni-resin containing alpha 2 interferon, respectively.

achieved by either of the two following routes: (1) insertion of a foreign gene directly into the chloroplast genome for expression by the plastid's own protein synthesis machinery or (2) insertion of a chimeric foreign gene construct (containing the gene of interest fused to sequences coding for a plastid transit peptide plus appropriate transcriptional control signals) into the nuclear genome, hence the gene product after its synthesis in the cytoplasm, is targeted to the chloroplast (Khan et al., 2005b).

REFERENCES

Al-Ahmad H, Gressel J (2005). Transgene containment using cytokinin-reversible male sterility in constitutive, gibberellic acid-insensitive (gai) transgenic tobacco. J. Plant Growth Regul. 24: 19–27.

Anon (2004). Drugs in crops – the unpalatable truth (editorial). Nat. Biotechnol. 22: p. 133.

Arlen PA, Falconer R, Cherukumilli S, Cole A, Cole AM, Oishi KK, Daniell H (2007). Field production and functional evaluation of chloroplast-derived interferon-α2b. Plant Biotechnol. J. 5: 511–525.

Bloomberg News (2005). Syngenta, Bayer bet gene-based drugs can thwart consumer doubts, 27 May 2005. Online: http://quote.bloomberg.com/apps/news?pid=nifea&&sid=adT0ydpQc5Gg.

Chakauya E, Chikwamba R, Rybicki EP (2006). Riding the tide of biopharming in Africa: considerations for risk assessment. South Afr. J. Sci.102: 284-288.

Cheema NM, Mali MA, Qadir G, Rabbani MA (2010). Characterization of caster bean genotypes using SDS-PAGE of total seed storage proteins. Pak. J. Bot. 3: 1797-1805.

Chong DKX, Roberts W, Arakawa T, Illes K, Bagi G, Slattery CW, Langridge WHR (1997). Expression of the human milk protein beta-casein in transgenic potato plants. Transgenic Res. 6: 289-296.

Daniell H, Khan MS (2003). Engineering the chloroplast genome for biotechnology applications. N. Stewart ed. Transgenic Plants: Current Innovations and Future Trends. Horizon press UK. 5: p. 28.

Daniell H, Khan MS, Allison L (2002). Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. Trends Plant Sci. 7: 84-91.

De Zoeten GA, Penswick JR, Horisberger MA, Ahl P, Schultze M, Hohn T (1999). The expression, localisation and effect of a human interferon. Virology, 172: 213–222.

Dunwell J (2005). Technologies for biological containment of GM and non-GM crops. Defra Contract CPEC 47. URL http://www.defra.gov.uk/science/project_data/DocumentLibrary/CB02036/CB02036_3629_FRP.doc [accessed on 10 July 2007].

Dus Santos MJ, Wigdorovitz A (2005). Transgenic plants for the production of veterinary vaccines. Immunol. Cell Biol. 83: 229–238.

Food and Drug Administration (FDA). (2004). Guidance for Industry. Botanical Drug Products. Washington, DC: US Food and Drug Administration. URL http://www.fda.gov/cder/guidance/4592fnl.pdf.

Fowler MW, Law I (2006). Plant-Based Pharmaceuticals: a StrategicStudy Relating to UK Activity and Interests. York: National Non-Food Crops Centre (NNFCC). URL http://www.nnfcc.co.uk/nnfcclibrary/publications/download.cfm?id=36.

Glenz K, Bouchon B, Stehle T, Wallich R, Simon MM, Warzecha H (2006). Production of a recombinant bacterial lipoprotein in higher plant chloroplasts. Nat. Biotechnol. 24: 76–77.

Hood EE, Woodward JA (2002). Plants as Factories for Protein Production. (Eds) Kluwer Academic, Dordrecht. p.209

Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, Bailey M,

Flynn P, Register J, Marshall L, Bond D (1997). Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. Mol. Breed. 3: 291-306.
Hood EE, Bailey MR, Beifuss K, Horn M, Magallanes-Lundback M, Drees C, Delaney DE, Clough R, Howard JA (2003). Criteria for high-level expression of a fungal laccase gene in transgenic maize. Plant Biotechnol. J. 1: 129-140.
Hood EE, Kusnadi A, Nikolov Z, Howard J (1999). Molecular farming of industrial proteins from transgenic maize. In: Chemicals via Higher Plant Bioengineering (Shahidi F, Kolodziejczyk P, Whitaker JR, Munguia AL and Fuller G eds), New York: Kluwer/Plenum. pp. 127–148.
Khan MS (2000). Development of Polyclonal Antibodies against Membrane Protein of Higher Plant Plastids. Int. J. Agric. Biol. 3: 169–174.
Khan MS (2005a). Unraveling the complexities of plastid transcription in plants. Trends Biotechnol. 23: 535-538.
Khan MS (2005b). Engineered male sterility. Nature, 436(11): 783-785.
Khan MS (2006). Hybrid transcription-mediated transgene regulation in plastids. Trends Biotechnol. 24: 479-482.
Khan MS, Khalid AM, Malik KA (2005)a. Intein-mediated protein trans-splicing and transgene containment in plastids. Trends Biotechnol. 23: 217-220.
Khan MS, Khalid AM, Malik KA (2005b). Phage phiC31 integrase: a new tool in plastid genome engineering. Trends Plant Sci. 10: 1-3.
Khan MS, Hameed W, Nozoe M, Shiina T (2007). Disruption of the psbA gene by the copy correction mechanism reveals that the expression of plastid-encoded genes is regulated by photosynthesis activity. J. Plant Res. 120: 421-430.
Khan MS, Zafar Y, Malik KA (2001). Plastid genome engineering: a new road to develop environment friendly transgenic plants. Proc. Pak. Acad. Sci. 38: 47-58.
Khan MS (2001). Utilizing heterologous promoters to express green fluorescent protein from jellyfish in tobacco chloroplasts. Pak. J. Bot. 33: 43-52.
Khan MS, Maliga P (1999). Fluorescent antibiotic resistant markers for tracking plastid transformation in higher plants. Nature Biotech. 17: 910-916.
Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature, 227: 680-685.
Lee D, Natesan E (2006). Evaluating genetic containment strategies for transgenic plants. Trends Biotechnol. 24: 109–114.
Lico C, Baschieri S, Marusic C, Benvenuto E (2007). Molecular farming for antigen (vaccine) production in plants. In: Improvement of Crop Plants for Industrial End Uses (Ranalli P ed.). Berlin: Springer. pp. 417–434.
Luykx DMAM, Goerdayal SS, Dingemanse PJ, Jiskoot W, Jongen PMJM (2005). HPLC and tandem detection to monitor conformational properties of biopharmaceuticals. J. Chromatography, 821: 45-52.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
Murphy DJ (2006). Molecular breeding strategies for the modification of lipid composition. *In Vitro* Cell. Dev. Biol. Plant, 42: 89–99.
Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight DNA. Nucleic Acids Res. 8: 4321-4325.
Ruggiero F, Exposito JY, Bournat P, Gruber V, Perret S, Comte J, Olagnier B, Garrone R, Theisen M. (2000). Triple helix assembly and processing of human collagen produced in transgenic tobacco plants. FEBS Lett. 469: 132-136.
Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheimm N (1985). Enzymatic amplification of beta-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science, 230: 1350-1354.
Svab Z, Maliga P (1993). Exceptional transmission of plastids and mitochondria from the transplastomic pollen parent and its impact on transgene containment. Proc. Natl. Acad. Sci. 104: 7003-7008.
Wade D, Makela P, House H, Bateman C, Robson P (2006). Long-term use of a cannabis-based medicine in the treatment of spasticity and other symptoms in multiple sclerosis. Mult. Scler. 12: 639–645.
Wade D, Makela P, Robson P, House H, Bateman C (2004). Do cannabis-based medicinal extracts have general or specific effects on symptoms in multiple sclerosis? A double-blind, randomized, placebo-controlled study on 160 patients. Mult. Scler. 10: 434–441.
Webster P (2004). Biopharming in plants- a future method of biopharmaceutical production? Frost Sullivan. http://www.frost.com/prod/servlet/market-insight-top.pag?docid=25148491