Transient Expression of CCL21Chemokine in Tobacco via Agroinfiltration

Maria Beihaghi-Hasan Marashi*-AbdolrezaBagheri-MojtabaSankian

Abstract- Secondary lymphoid-tissue chemokine (SLC/CCL21) is a CC chemokine that is basically expressed in different lymphoid tissues and binds to chemokine receptor CCR7 on active dendritic cells (DCs) and increase T-cell activities. Two methods of expressing recombinant proteins in plants exist; transient expression by Agroinfiltrationand stable transformation of plant genome.each having advantages depending on the goal of study. Transient transformation of tobacco leaf is a fast technique for assessing gene before generating stable plant lines using a more time-consuming technique. Transient expression takes from 3to 7 days whereas transgenic linesare generated after 2-4 months. Soagroinfiltration as a rapid method for production presence of recombinant antigen of C-C chemokine ligand 21 (CCL21) intobacco leaves.Foreign gene expression was evaluated using semi quantitative RT-PCR, Real time PCR , protein dot blot and enzyme-linked immunosorbent assay (ELISA) and western blot,All results show that the foreign gene was transcribed in transformed leaves. Finally, the efficacy of agroinfiltration for transient expression of CCL21 recombinant protein in tobacco was illustrated in this study.

Keywords- Recombinant vaccine; Agroinfiltration; Tobacco; Transient gene expression, C-C chemokine ligand 21 (CCL21)

1 INTRODUCTION

Chemokines play essential roles in tumor biology including leukocyte recruitment, tumor cell growth and survival, angiogenesis, and metastasis (Mantovani, Bonecchi, & Locati, 2006; Muller et al., 2001). Among them, C-C chemokine ligand 21/chemokine receptor 7 (CCL21/CCR7) pair promotes growth and metastasis of many tumor types including breast, melanomas, thyroid, colon, neck cancers, and head (Ding et al., 2003; Wang et al., 2008)Using plants as bioreactors for production of recombinant proteins has emerged as a promising biotechnological tool over the past two decades.

Many investigators have conducted **extensive** experiments for the production of recombinant plant-based vaccine against a wide range of bacterial and viral disease(Boehm, 2007). However, the technique suffers from major drawbacks such as low expression level and long time required for **the** production of the recombinant protein in, plant tissues (Floss, Falkenburg, & Conrad, 2007). Many strategies have been proposed for the enhancement of recombinant protein expression including; chloroplast transformation (Daniell, Lee, Panchal, & Wiebe, 2001), use of strong promotersuntranslated leader sequences (Kang et al., 2004), signal peptide codon optimization.

The Long time required for the generation of transformed plants expressing foreign antigens is another limitation for the production of recombinant proteins (Simmons, VanderGheynst, & Upadhyaya, 2009).

Transient gene expression methods are appropriate alternatives to stable transformation because they allow for a rapid and inexpensive expression of foreign gene(s) in plant tissues. This method can be carried out in many ways including protoplast transformation, vacuum infiltration (Sheen, 2001), agroinfiltration(Leckie & Stewart Jr, 2011) and particle bombardment. Among the techniques,

Maria Beihaghi, College of Agriculture, Ferdowsi University of Mashhad, Mashhad, IRAN.Beihaghi.maria@stu.um.ac.ir.com
*Hasan Marashi*College of Agriculture, Ferdowsi University of Mashhad, Mashhad, IRAN.marashi@um.ac.ir*
AbdolrezaBagheri,College of Agriculture, Ferdowsi University of Mashhad, Mashhad, IRAN.abagheri@ferdowsi.um.ac.ir.
MojtabaSankian,BuAli Research Institute,Mashhad University of Medical Sciences, Mashhad, IRAN.sankianm@mums.ac.ir
** Author for correspondence*

agroinfiltration takes advantages of a simple, cost effective and rapid procedure In agroinfiltration, the suspension of *Agrobacterium tumefaciens* harboring the gene(s) of interest is infiltrated into plant leaves using a needle-free syringe. This technique has been carried out in a variety of plants (Zottini et al., 2008) with different experimental purposes (Zheng, Liu, Meng, Li, & Wang, 2012).

However, there have been few reports on production of recombinant vaccines in plant systems via transient gene expression. The main reason is that transient gene expression assays are not as appropriate as stable transformation for production of recombinant vaccines (Wroblewski, Tomczak, & Michelmore, 2005).However, recombinant antigens produced via transient gene expression can be used for the production of specific antibodies, which can then be used in diagnosis and molecular detection . Moreover, transient expression assay can be carried out as a quick method to investigate efficiency of a candidate antigen for inducing immunogenic response in animal models(Sohi, Jourabchi, & Khodabandeh, 2005).

The time course required for antigen production via agroinfiltration (and other types of transient gene expression) is significantly shorter than that of stable transformation (Leckie & Stewart Jr, 2011). In this study, we investigated the production of an immunogenic recombinant protein of CCL21 by agroinfiltrationto tobacco leaves.

2. Materials and Methods

2.1. Codon optimization and DNA constructs

The sequence of Homo sapiens chemokine (C-C motif) ligand 21 (CCL21) cDNA (XM_011518004) was retrieved from GenBank data base and analyzed by DNAstarsoftware (DNASTARE, Inc.). The base composition of the IL-1β chemokine was also analyzed by using this software. 134 amino acids of CCL21 and part of IL-1β(VQGEESNDK) as adjuvantswere designed as the main part of expression cassette. A ribosome binding site, Kozak sequence GCCACC, was introduced prior to the start codon. This sequence has been reported to enhance translation efficiency(Kozak, 1989). An endoplasmic reticulum signal peptide called SEKDEL, which has been reported to increase recombinant protein accumulation in plant tissues (Haq, Mason, Clements, & Arntzen, 1995), was attached to 3' end, just before the stop codon.

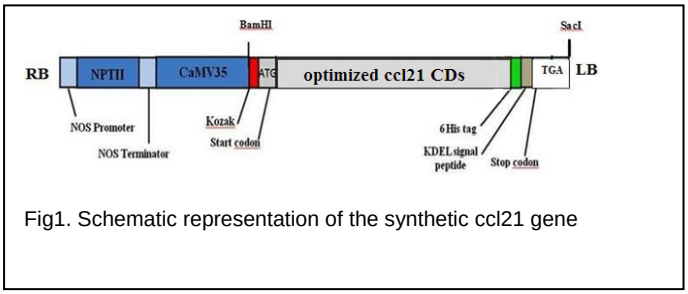


Fig1. Schematic representation of the synthetic ccl21 gene

Start codon (AUG) and stop codon (UAA) were also added into the 5' and 3' ends of the construct, respectively. Restriction enzyme sites of *Bam*HI and *Sac*I were introduced into the 5' and 3' ends of the synthetic gene, respectively. The resulted chimeric gene was optimized based on codon usage pattern of tobacco taken from "*Kazusa*" site. The optimized chimeric gene was cloned into the pGHvector (Biorey, China).

The synthetic *ccl21* gene was removed from pGHvector by digestion with *Bam*HI and *Sac*I and inserted into the binary vector pBI121, yielding pBI121-CCL21 vector (Fig. 1). The ligation reaction mixture was used to transform *E. coli* strain DH5-α kanamycin-resistant colonies were isolated after overnight incubation at 37ºC. After amplification, the plasmid was extracted from bacterial cells using alkaline lysis method. The plasmid was introduced into *Agrobacterium tumefaciens*strain GV3101 by heat shock method. Transformed cells were screened by kanamycin-resistance and PCR.

2.3. Plant Transformation via Agroinfiltration

A single colony of *Agrobacterium* containing

pBI121-CCL21 plasmid was cultured for 48h in LB medium (NaCl 10 g/L, yeast extract5 g/L, tryptone 5 g/L)

supplemented with gentamicin 10 mg/l, rifampicin 50 mg/L and kanamycin 50 mg/L. After reaching a cell density of OD600=1.5, the culture was centrifuged, supernatant discarded and the pellet resuspended in infiltration medium (10 mM MgCl2, 10 mM MES pH 5.6, and 150 μMacetosyringone), that was adjusted to OD600=0.5. The suspension was then incubated for 2 h at room temperature. Before being transferred to tobacco leaves with a needle-free syringe,Tobacco plants wereplaced in growth chamber for three to seven days under 25ºC before being analyzed.

2.4. Detection of CCL21 Gene in Transformed Leaves

For PCR analysis, genomic DNA was isolated from both non-transgenic and transgenic tobacco leaf tissuesusing Parstous DNA extraction kit.The presence of the *ccl21* in transgenic plant genomic DNA was determined by PCR analysis using specificprimers,CaMV-ccl21F 5' GATGACGCACAATCCCACT 3' and CaMV-ccl21R 5'CCCTTTCCCTTCTTTCCA 3' that amplify 386 bp around the attachment region between CaMV promotor and *ccl21*gene sequences. PCR was carried out: based on the following temperature profile: 94ºC 1 min, 50º C 1 min, 72 ºC 2 min for 30 cycles.

2.5. R T- PCR Assay

RT- PCR assay was performed to analyze gene expression at transcription level. Total RNA was extracted from infiltrated leaf tissue (Parstous kit).Complementary DNA (cDNA) was synthesized via reverse transcription using oligo(dT) primer (Parstous kit). The resulting cDNA mixtures were used as templates for semi-quantitative RT-PCR technique and Real Time PCR. cDNA was determined by PCR analysis by using specific primers, ccl21F 5'GGGTTCAACAACTTATGC 3' and CaMV-ccl21R 5' CTTTCCCTTCTTTCCAGT 3' that amplify 103 bp around the attachment region between CaMVpromotore and *ccl21*gene sequences.

2.6. Protein extraction and dot blot assay

About 5 gram of transgenic and wild type tobacco plant material were ground and ground in a mortar containing liquid N2. The resulting powder was mixed with ice-cold extraction buffer (100 mMTris– HCl pH 8, 0.5 M EDTA pH 8, DTT2mM,). The extract was centrifuged at 12,000g for 20 min at 4C. The protein content of each sample was measured by Bradford protein assay protocol (Bio-Rad Inc.). 500 μl of each sample was mixed with similar amount of sample buffer (0.5 M Tris–HCl pH 6.8, 10% SDS, 20% glycerol, DTT 2mM, 0.02% bromophenol blue). Samples were boiled for 3 min and stored at -20C until required.
Production of recombinant CCL21 protein in transgenic leaves was evaluated by standard protein dot blot assay. Briefly, 10μL of protein samples from infiltrated leaves was dotted on the membrane. The membrane was then dried. The membrane was incubated with BSA 3% as blocking solution for 1 h. After incubation, the membrane was incubated with conjugated antibody His tag for 1 h in37ºC, washed three times with PBST/PBS and incubated with DAB (diaminobenzidine) substrate. A small volume of FMDV vaccine serotype O (about 5μL) was used as positive control and the same volume of protein obtained from wild type plant was used as negative control.

2.7. Soluble protein assay by dot blot

To ensure the solubility of the protein, dot blot assay was performed. About 10 gram of transgenic and wild type tobacco plant material were crushed by scalpel and7 μL of extract protein samples from the infiltrated leaves wereexamined by dot-blot assay. As it show in fig. 6, negative control were showed by dot bolt, SoFluorescent quantitative method by chemiluminescence detection kit (Parstous) used to remove stains blot of negative control(fig.7).

3. Results

Tobacco leaves were transformed via agroinfiltration. (Fig.3).

Accepted band was observed for the DNA sample of transformed leaves by PCR analysis, but not for the wild type plant.

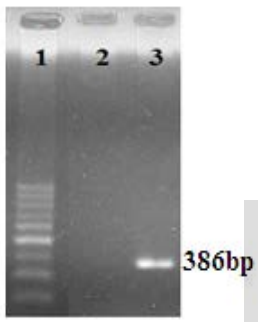


Fig4. PCR analysis for detection of *ccl21* gene in transformed leaves of tobacco.1: 100bp Size marker (parstous),2: PCR product of wild type plant (negative control); 3: transformed plant.

Expression of *ccl21* was evaluated at transcription level using semi-quantitative RT-PCR. Two samples of transformed plants were used for RT-PCR. Results showed that the foreign gene was transcribed in infiltrated leaves (Fig. 4).

Expression of ccl21 was checked out at transcription level by using Real Time PCR. Three samples of transgenic plants and untransgenic plant as negative control and were used for Real Time PCR. So the result of Real Time PCR showed that ccl21 was transcribed in infiltrated leaves (Fig.5).

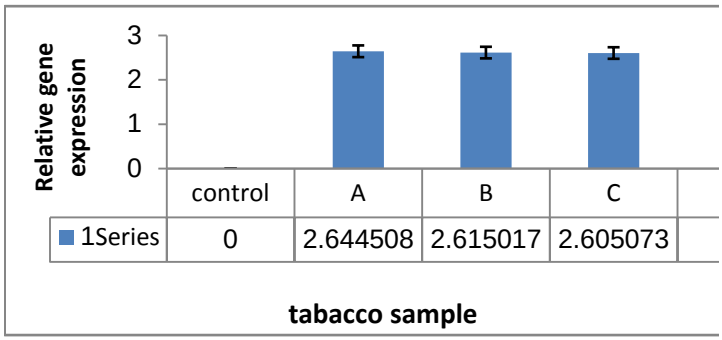
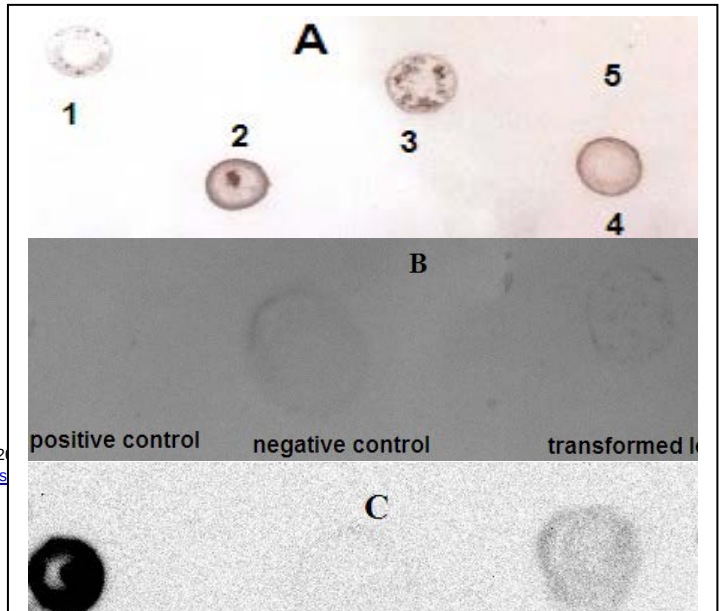


Fig.5.Quantificatin expression of ccl21 gene in transformed leaves of tobacco via Real Time PCR; Un transformed leaf used as control and Samples A,B and C are three samples of transformed leaf.

Translation of *ccl21* gene was evaluated by protein dot blot assay. Dot blot results confirmed expression of foreign gene at translation level, whereas no signal was observed for wild type plants (Fig.6).



Expression of recombinant protein of CCL21 was evaluatedby ELISA method. The production of recombinant protein in transgenic plants was so higher than non-transgenic plants. And there is no strong signal in non- transgenic plant rather than transgenic plants (Fig.9).

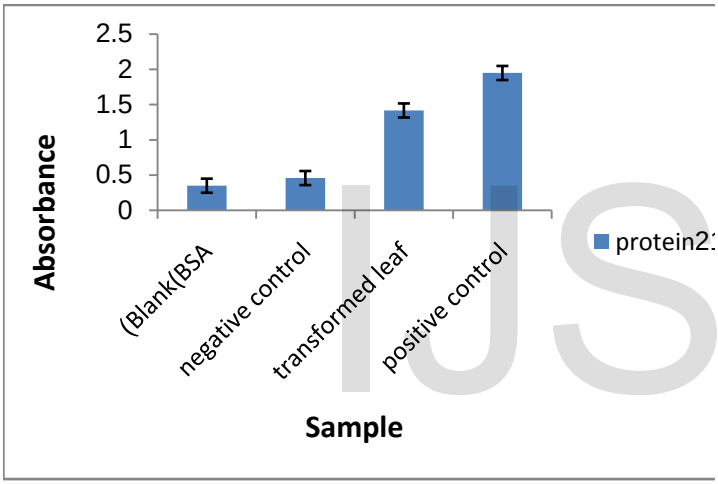


Fig.9. Quantification of recombinant CCL21 protein in transgenic plants by ELISA.

4.Discussion

In the present study tobacco leaves were transiently transformed with a chimeric construct of *ccl21* gene via agroinfiltration method. The method has been reported as an efficient and quick procedure for transient gene expression in plants (Bendahmane, Querci, Kanyuka, & Baulcombe, 2000). An approach to address these limitations is application of transient gene expression assays. Although transient expression is not the preferred method for commercial production of recombinant vaccine in plants, the antigens produced in this procedure can be used for the production of specific antibodies required in molecular diagnosis (Sohi et al., 2005). Moreover, this method makes it possible to evaluate efficacy of a potential recombinant vaccine in a short time (Schillberg, Twyman, & Fischer, 2005) .Our study showes that agroinfiltration can be a fast and confident way to produce CCL21 protein inexperimental plants. PCR assay confirmed presence of the synthetic construct in infiltrated leaves but the gene of interest is not integrated in thenuclear genome of plant cell.Thus, the transgene in plant tissue is high and PCR product band in electrophoresis is almost as sharp as that of pure plasmid as control. RT- PCR assay was be also concocted to evaluate the expression of gene. As can be seen from dot blot assay, the protein sample obtained from transformed leaves generated a strong signal which is comparable to that of positive control, whereas wild type plant protein was not detectable. Most of the works in the field of transient antigen expression in plant hosts have been conducted by means of plant viruses as vehicle for gene delivery and expression, in which the epitope of interest is usually inserted within the coat protein gene (Koprowski & Yusibov, 2001). In this study,CaMV 35S promoter, Kozak sequence, ER signal peptide were used and codons were optimized to enhance gene expression, similar to previous reports (Gil et al., 2001). Although a good level of transgene expression was achieved in our experiment, it should be mentioned that the results cannot be confidently attributed to the presence of these factors,since their effect on the foreign gene was not evaluated. Further investigation is needed in order to prove a connection between these factors and the expression level of the CCL21protein.

Acknowledgement

The authors would like to thank staff of biotechnology and plant breeding department, faculty of Agriculture, Ferdowsi University of Mashhad.

Reference

Bendahmane, Abdelhafid, Querci, Maddalena, Kanyuka, Konstantin, & Baulcombe, David C. (2000). Agrobacterium transient expression system as a tool for the isolation of disease resistance genes: application to the Rx2 locus in potato. *The Plant Journal, 21*(1), 73-81.

Boehm, Robert. (2007). Bioproduction of therapeutic proteins in the 21st century and the role of plants and plant cells as production platforms. *Annals of the New York Academy of Sciences, 1102*(1), 121-134.

Daniell, Henry, Lee, Seung-Bum, Panchal, Tanvi, & Wiebe, Peter O. (2001). Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. *Journal of molecular biology, 311*(5), 1001-1009.

Ding, Yongzeng, Shimada, Yutaka, Maeda, Masato, Kawabe, Atsushi, Kaganoi, Junichi, Komoto, Izumi, . . . Imamura, Masayuki. (2003). Association of CC chemokine receptor 7 with lymph node metastasis of esophageal squamous cell carcinoma. *Clinical Cancer Research, 9*(9), 3406-3412.

Floss, Doreen Manuela, Falkenburg, Dieter, & Conrad, Udo. (2007). Production of vaccines and therapeutic antibodies for veterinary applications in transgenic plants: an overview. *Transgenic Research, 16*(3), 315-332.

Gil, Félix, Brun, Alejandro, Wigdorovitz, Andrés, Catalá, Rafael, Martínez-Torrecuadrada, Jorge L, Casal, Ignacio, . . . Escribano, José M. (2001). High-yield expression of a viral peptide vaccine in transgenic plants. *FEBS letters, 488*(1), 13-17.

Habibi-Pirkoohi, Maziar, Malekzadeh-Shafaroudi, Saeid, Marashi, Hasan, Moshtaghi, Nasrin, Nassiri, Mohammadreza, & Zibaee, Saeid. (2014). Transient Expression of Foot and Mouth Disease Virus (FMDV) Coat Protein in Tobacco (Nicotiana tabacom) via Agroinfiltration. *Iranian Journal of Biotechnology, 12*(3), 28-34.

Haq, Tariq A, Mason, Hugh S, Clements, John D, & Arntzen, Charles J. (1995). Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science, 268*(5211), 714-716.

Kang, Tae-Jin, Han, So-Chon, Jang, Mi-Ok, Kang, Kui-Hyeon, Jang, Yong-Suk, & Yang, Moon-Sik. (2004). Enhanced expression of B-subunit of Escherichia coli heat-labile enterotoxin in tobacco by optimization of coding sequence. *Applied biochemistry and biotechnology, 117*(3), 175-187.

Koprowski, Hillary, & Yusibov, V. (2001). The green revolution: plants as heterologous expression vectors. *Vaccine, 19*(17), 2735-2741.

Kozak, Marilyn. (1989). The scanning model for translation: an update. *The Journal of cell biology, 108*(2), 229-241.

Leckie, Brian Michael, & Stewart Jr, C Neal. (2011). Agroinfiltration as a technique for rapid assays for evaluating candidate insect resistance transgenes in plants. *Plant cell reports, 30*(3), 325-334.

Mantovani, Alberto, Bonecchi, Raffaella, & Locati, Massimo. (2006). Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nature Reviews Immunology, 6*(12), 907-918.

Muller, Anja, Homey, Bernhard, Soto, H al, Ge, NF, Catron, Daniel, Buchanan, Matthew E, . . . Wagner, Stephen N. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature, 410*(6824), 50-56.

Schillberg, Stefan, Twyman, Richard M, & Fischer, Rainer. (2005). Opportunities for recombinant antigen and antibody expression in transgenic plants—technology assessment. *Vaccine, 23*(15), 1764-1769.

Sheen, Jen. (2001). Signal transduction in maize and Arabidopsis mesophyll protoplasts. *Plant Physiology, 127*(4), 1466-1475.

Simmons, Christopher W, VanderGheynst, Jean S, & Upadhyaya, Shrinivasa K. (2009). A model of Agrobacterium tumefaciens vacuum infiltration into harvested leaf tissue and subsequent in planta transgene transient expression. *Biotechnology and bioengineering, 102*(3), 965-970.

Sohi, Haleh Hashemi, Jourabchi, Esmat, & Khodabandeh, Mahvash. (2005). Transient expression of human growth hormone in potato (Solanum tuberosum), tobacco (Nicotiana tobacum) and lettuce (Lactuca sativa) leaves by agroinfiltration. *Iranian J Biotechnol, 3*(2), 109.

Wang, Jun, Seethala, Raja R, Zhang, Qing, Gooding, William, Van Waes, Carter, Hasegawa, Hitoshi, & Ferris, Robert L. (2008). Autocrine and paracrine chemokine receptor 7 activation in head and neck cancer: implications for therapy. *Journal of the National Cancer Institute, 100*(7), 502-512.

Wroblewski, Tadeusz, Tomczak, Anna, & Michelmore, Richard. (2005). Optimization of Agrobacterium-mediated transient assays of gene expression in lettuce, tomato and Arabidopsis. *Plant Biotechnology Journal, 3*(2), 259-273.

Zheng, Lei, Liu, Guifeng, Meng, Xiangnan, Li, Yanbang, & Wang, Yucheng. (2012). A versatile Agrobacterium-mediated transient gene expression system for herbaceous plants and trees. *Biochemical genetics, 50*(9-10), 761-769.

Zottini, Michela, Barizza, Elisabetta, Costa, Alex, Formentin, Elide, Ruberti, Cristina, Carimi, Francesco, & Schiavo, Fiorella Lo. (2008). Agroinfiltration of grapevine leaves for fast transient assays of gene expression and for long-term production of stable transformed cells. *Plant cell reports, 27*(5), 845-853.