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综 述

Potato: A Favorable Crop for Plant Molecular Farming

Sunil Kumar G B, Ganapathi T R, Bapat V A*

(Plant Cell Culture Technology Section, Nuclear Agriculture and Biotechnology Division,
Bhabha Atomic Research Centre, Trombay, Mumbai 400085, India)

Abstract：Potato is one of the important food crops with a high yield potential and nutritional value. It has been used extensively for molecular farming to produce vaccines, antibodies and industrial enzymes. It has several desirable attributes as a favorable crop for the production of recombinant proteins. Potato tubers were employed for bulk production of recombinant antibodies. Vaccine production in potato has progressed to human clinical trials. Human milk proteins were successfully expressed in potato tubers. Potato hairy roots offer as another attractive system for the production of useful recombinant proteins both as intra cellular and secreted forms. This review describes the use of potato as a prospective host for plant molecular farming.

Key Words：potato; vaccines; antibodies; hepatitis B; microtubers; hairy roots

Molecular farming refers to the production of recombinant proteins in heterologous expression systems. The recent advances in genomics and proteomics resulted in identification of several new genes. These have to be functionally characterized to explore their probable use in diverse applications. It is quite unlikely that a single expression system could support the production of these proteins, therefore there is a need to identify and analyze different novel expression systems for the recombinant protein production on a large scale. Recent reports suggest that there is a growing interest and success of using plants as novel, safe and probably cost effective expression systems. An ideal plant expression system should have characteristics such as short generation times, rapid scalability and storage potential. Potato has been studied extensively as a host for plant molecular farming.

Potato (Solanum tuberosum L.) is one of the important food crops with high nutritional value and yield potential. It provides approximately half of the world's annual production of all roots and tubers, making it largest non- cereal food crop. It is a part of the diet of half a billion people in developing countries[1]. India is the fifth largest producer of potato in the world. It is grown as a short day winter crop in the plateau and plains of India, compared to its cultivation as a long day summer crop in the other countries of the world[2]. The countries of Western Europe, the United States and Japan have the highest potato yields in the world[3].

It is an herbaceous dicotyledonous plant and belongs to the family Solanaceae. The tubers are modified, thickened, and underground stems; their size, shape and color vary according to the cultivar. Eyes (dormant buds) are present on the surface of the tubers, from which growing buds arise. Potato tuber develops at the tip of the stolon as a lateral proliferation of storage tissue resulting from rapid cell division and enlargement[4]. Potato is one of the most explored systems for plant molecular farming for the production of antibodies, vaccines and other useful recombinant

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Biography：Sunil Kumar G B (1973-), male, scientific officer. Research area: plant molecular farming.

*Corresponding author：E- mail: vabapat@barc.gov.in

proteins.

1 Desirable Attributes of Potato for Molecular Farming

Potato has several desirable attributes for molecular farming, which include available micropropagation and regeneration protocols for several cultivars, amenability to genetic transformation, ability to induce microtubers and hairy roots, short generation times and ability to store the tubers at low temperature.

1.1 Micropropagation

Regeneration from different explants was obtained in potato tissue cultures. Callus and adventitious shoot regeneration from leaf segments[5], embryos from tuber discs[6], multiple shoots from shoot tips[7], direct shoot regeneration from rachis, petiole and leaflet[8] and multiple shoots from stem nodes[9] were reported.

Potato seed is produced by repeated clonal propagation of initial disease free plants. This method suffers from low multiplication rates and progressive accumulation of degenerative viral diseases. Hence availability of quality seed is a major constraint in potato production and cost of the seed alone accounts for about 40% to 60% of total production costs in many countries[10]. Production of tubers using single node cuttings[11] and microtubers[12] are employed for rapid multiplication.

1.2 Microtubers

Microtubers are miniature tubers produced under tuber inducing conditions in vitro. They are small dormant tubers, attractive for convenient handling, storage and distribution. Unlike micro-propagated plantlets, they do not require time-consuming hardening period in green houses. They may be adapted easily to large scale mechanized planting in the field. Several protocols were developed for microtubers induction in potato. Wang and Hu[13] first reported mass tuberization of potato in vitro and successful integration of this technology into virus free seed potato production program in Taiwan. Subsequently, this technique was adopted at International potato center, Peru[9] and Central potato research institute, India[14].

1.3 Genetic transformation of potato

Microtubers were used as explants for Agrobacterium mediated gene transfer to potatoes[15]. Sheerman and Bevan[16] reported a rapid transformation method of Agrobacterium mediated gene transfer using tuber discs as explants. Genotype independent leaf disc transformation of potato using Agrobacterium tumefaciens was achieved[17]. A one step transformation of two Andean potato cultivars by Agrobacterium mediated transformation was also reported[18]. Romano et al[19] reported transformation of potato using particle bombardment. These developments and ease of gene transfer led to the development of potato as a host system for molecular farming.

1.4 Potato as a host system for molecular farming

Potatoes offer several advantages for molecular farming, which include 1) efficiency of genetic transformation by Agrobacterium tumefaciens with relatively short transformation and generation times, 2) clonal propagation, 3) availability of tissue specific promoters and capability of microtuber production, 4) storage potential and 5) the ability to feed raw potatoes to test animals and human volunteers. Potato plants and its different tissues (leaves, tubers and hairy roots) were successfully employed for the expression of recombinant proteins and these systems are depicted in Figure 1.

Clonal propagation allows stable production of a transgenic plant lines. Putative potato transgenic plants can be induced to produce microtubers, thus they can be screened readily for tuber specific expression. Tubers are the storage organs of the potato and can be stored for a period of time before being consumed. Foreign proteins may be stable for long periods of time in tuber as long as the tuber does not sprout or become damaged. The benefits of being able to store tubers without any processing may make potatoes very desirable as a vaccine production system for those animals that do eat raw potatoes[20].

2 Vaccine Production in Potato

Prophylaxis in the form of vaccines plays an impor-

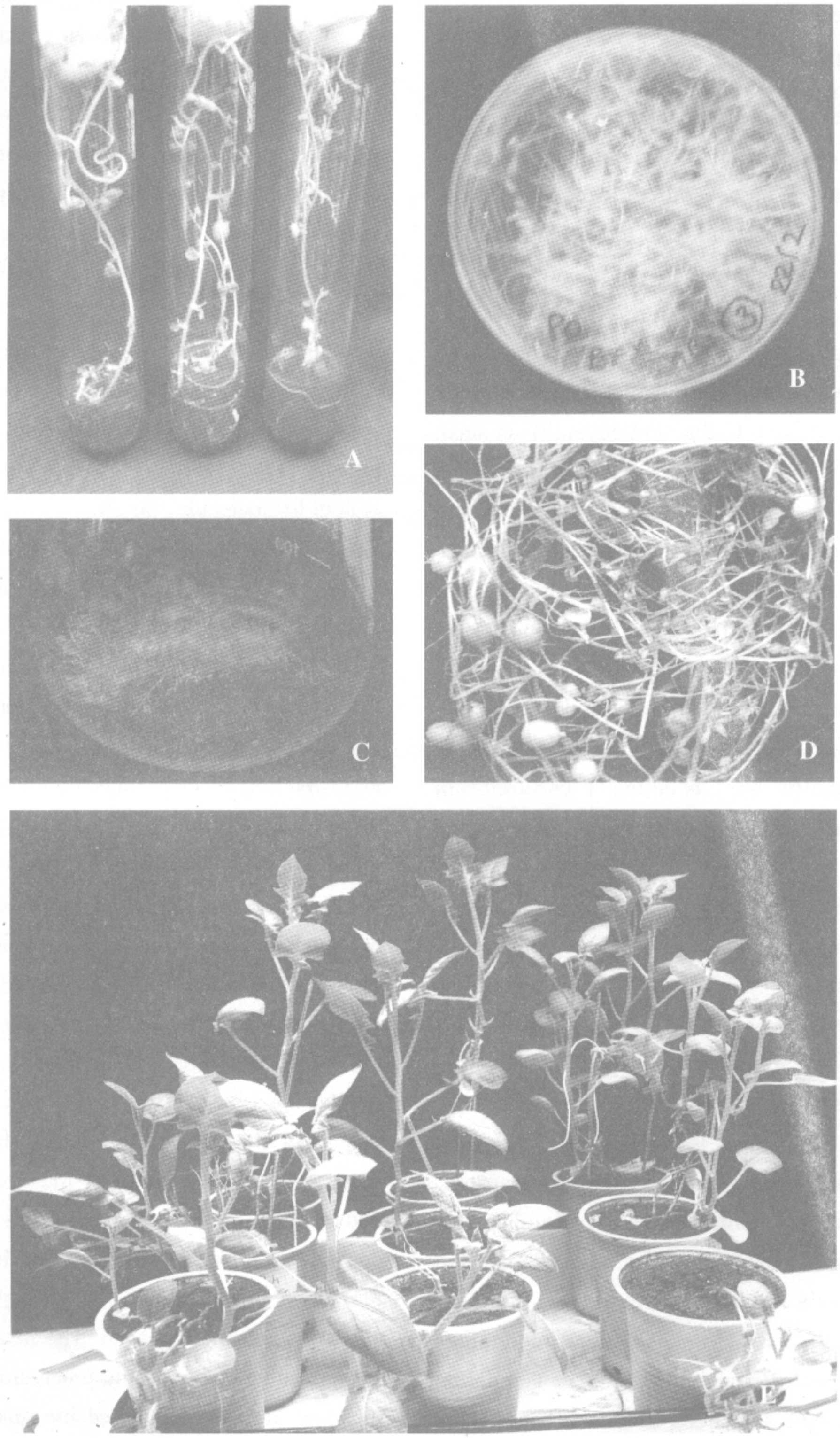

Figure 1　Potato tissues used for molecular farming.

A. Transgenic plants *in vitro*, B. Hairy roots, C. Photosynthetic hairy roots, D. Microtubers and E. Hardened plants tant role in the management of infectious diseases, especially in the developing countries. However, the prohibitive cost of several recombinant vaccines limits their use in mass immunization programs. Plant based vaccine production has proceeded rapidly in the last few years employing different plant systems[21-22]. Potato has been extensively used for this purpose. Several antigens were successfully expressed and reported to be immunogenic when they are produced and delivered in potatoes.

Diarrheal disease continues to be a major cause of death especially among children in under developed countries. Travelers to this area are also frequently exposed to the bacteria and viruses that cause diarrheal disease. Vaccines to prevent this disease could play an effective role in the control of these diseases. Rotavirus, enterotoxigenic E. coli and Norwalk virus are the major causative agents of this disease. Rotavirus VP6 capsid protein was produced in transgenic potatoes for vaccination against acute viral gastroenteritis [23].

Enterotoxigenic E. coli produces heat labile enterotoxin (LT), which has two subunits, toxic LT-A, which is a monomer and a pentameric non-toxic LT-B. LT-B is a potent oral immunogen, when administered orally elicits a strong mucosal immune response without any symptoms of the disease. Recombinant LT-B expressed in potato, when fed to mice developed antibodies in the serum and mucosa and the biological activity of LT was neutralized by these antibodies[24]. Further this has been used in first human clinical trials of edible vaccine; LT-B antigen delivered through transgenic potato resulted in serum and /or mucosal immune response[25]. This first trial is a milestone on the road to create inexpensive vaccines that might be particularly useful in immunizing people in developing countries.

Norwalk virus (NV) causes acute epidemic gastroenteritis in humans. Norwalk virus like particles lacking viral RNA are reactive with sera from NV infected humans. Thus production of recombinant Norwalk virus capsid protein (NVCP) could be used as a vaccine for gastroenteritis. The NVCP was expressed in transgenic tobacco leaves and potato tubers. The plant-derived vaccine was orally immunogenic in mice. Extracts of tobacco leaf expressed vaccine, when administered to mice developed both IgG and secretory IgA specific for rNV[26]. Subsequently, in a human clinical trial, 150 g of transgenic potatoes containing 15-750 μg of NVCP, when ingested by 20 volunteers resulted in significant numbers of anti-NVCP antibody secreting cells of IgA subtype[27].

Hepatitis B is one of the major infectious diseases and responsible for persistent viraemia in humans. The expense of the available recombinant vaccine restricts its inclusion in the expanded program of immunization. In the last few years, rapid progress has been made in the development of plant based vaccines for hepatitis B and more success was achieved with potato. The surface antigen of hepatitis B virus (HBsAg) was found to be a protective antigen to develop an effective vaccine for hepatitis B. Intramuscular injection of serum derived or yeast-derived recombinant HBsAg in healthy individuals result in effective immunization and protection from viral infection[28]. The HBsAg expression was optimized using different expression cassettes in potato and the maximum expression of 16 $\mu g \cdot g^{-1}$ tuber was obtained[29]. The transgenic potato derived HBsAg showed priming and boosting of serum anti-HBsAg IgG responses, when administered orally to mice[30]. A double-blind placebo-controlled human clinical trial with oral administration of transgenic potatoes expressing HBsAg at~8 $\mu g \cdot g^{-1}$ of tuber and a dose of 100 g tuber resulted in increase of serum anti-HBsAg titers by 62.5% in 10 of 16 volunteers. This result demonstrated that potato based orally delivered vaccine should be considered as a viable component of a global immunization program against hepatitis B[31].

A cholera toxin B sub unit-insulin fusion protein was expressed at the levels of up to 0.05% of total soluble protein (TSP) in potato leaves. Tubers accumulated the fusion protein at the levels of 0.1% TSP. Nonobese diabetic mice when fed with transgenic potato tuber tissues showed a substantial reduction in pancreatic islet inflammation and a delay in the progression of clinical diabetes [32]. These results demonstrate that potato tubers could be used as delivery vehicles for both infectious and autoimmune diseases. In addition to vaccines, antibodies constitute the important therapeutic proteins to be produced on a large scale.

3 Antibody Production in Potato

Potato tubers have a great potential for the production and storage of antibodies. These specialized organs can be used to accumulate large amount of proteins. Agronomical practices to cultivate, harvest, store and process potato tubers for starch production are well established and can be easily transferred for antibody production. It is the first agriculturally important plant species to be exploited for applicability as a biofactory[33]. Potato tubers were used as platform for the bulk production of recombinant antibodies. Different constructs were made to accumulate full size IgGs, Fab fragments and single chain variable fragments (ScFvs) in the cell apoplast or endoplasmic reticulum. Expression levels of up to 0.5% TSP were obtained for antibodies targeted to ER whereas five fold lower accumulation levels were found when they were targeted to secretory pathway. Transgenic tubers could be stored for up to six months without significant loss of antibody amount or activity. No significant variations in antibody accumulation levels were observed in tubers that are derived from the same transformant. Processing to green house or field trials including in vitro propagation of a selected transformant required only nine months from the start of transformation. This time frame allowed production of hundreds of kilograms of transgenic potato tubers. IgG produced in potato tubers was purified using standard laboratory techniques. Thus potato tubers could be employed as a viable production system for economical production of clinically important macromolecules such as antibodies[34]. High level expression of antiglycophorin single chain antibody fused to an epitope of HIV virus was achieved in potato tubers[35]. Nutrition influences the health condition of humans, especially for growing infants. Potato was also investigated as a host system for the expression of proteins of nutritional requirement.

4 Expression of Human Milk Proteins in Potato

Milk is considered as wholesome food and regarded as a best nutrition for growing infants. There is a scope for milk substitutes market as many mothers are unable to nurture their infants by breast-feeding due to unfavorable physical, psychological or socio-economic factors. The available infant formulas are based on bovine milk or soybean, which differ substantially from human milk in both protein content and composition. There are several reports demonstrating a possible link between consumption of cow milk and the development of insulin dependent diabetes mellitus. Soy based formulas have been recommended as hypo allergic alternatives for non-breast fed infants. However, many infants allergic to bovine milk are also allergic to soy proteins. Food plants engineered to produce human milk proteins can be considered as an alternative to deliver these nutritious proteins to people in all the age groups[36]. Chong et al[37]engineered potato plants to produce human milk protein β casein. Lactoferrin is another protein with antimicrobial, antiviral and antineoplastic properties found in human milk. It also has iron binding capacity. Human lactoferrin was expressed in potato plants and tested for its antimicrobial activity[38]. Potato based infant formulas with human milk proteins may provide required nutrition and immunity to the growing infants.

5 Other Useful Proteins Expressed in Potato

Industrially useful proteins were also produced in potato. Among them, endoglucanases and spider silk protein were successfully expressed in potato. Endoglucanases are useful enzymes for hydrolyzing cellulose to sugar for simultaneous or later fermentation into alcohol. An endoglucanase of Acidothermus cellulolyticus expression was optimized in transgenic potato leaves using leaf specific promoter. Endoglucanase activity was higher when it was targeted by a chlorplast signal peptide and an apoplast signal peptide than those using native signal peptide and a vacuole signal peptide. This endoglucanase accumulated up to 2.6% of TSP. This enzyme was expressed in both leaf and tuber tissues using a hybrid promoter of mannopine synthase and cauliflower mosaic virus 35S promoter enhancer region[39].

The dragline silk of the spider has a high tensile strength that is comparable to that of a synthetic super fiber Kevlar, but it has additional higher elasticity. These biomaterials could be useful for industrial and medical applications. Attempts were made to design synthetic genes encoding spider silk proteins and to express them in microorganisms. However, as spider silk proteins consist largely of Glycine and Alanine, an extensive pool of these amino acids has to be provided to microorganisms if spider silk has to be produced by them. Another difficulty in bacterial production is genetic instability due to recombination resulting from the highly repetitive genes encoding the repetitively composed spider silk proteins. To overcome these limitations, plant based production system was used for the production spider silk protein in potato tubers using ER retention. Spider silk protein accumulated up to 2% TSP in the ER of potato tubers and exhibited extreme heat stability that is used to purify them by simple and efficient procedure[40]. Apart from tubers and leaf tissues of potato, hairy roots of this plant were also employed for the expression of recombinant proteins.

6 Potato Hairy Roots as a Novel Expression System

Agrobacterium rhizogenes causes hairy root disease in dicotyledonous plants. These roots are characterized by the rapid growth and they have ability to grow in hormone free media. They have been used in wide range of research applications such as plant improvement, secondary metabolism studies and plant interaction with the environment[41]. The genetic stability and fast growth rates of hairy roots makes them an attractive system for recombinant protein production. Recombinant proteins when targeted to secretory pathway could be secreted into the spent medium, which is referred to as rhizosecretion. This is an additional advantage offered by the hairy roots.

Recently, we have reported the initiation of hairy roots from inter-nodal segments of transgenic potato and optimized the growth conditions. Potato hairy roots have very short doubling time of 2.32 d. We have also demonstrated higher levels of HBsAg expression in hairy roots compared to the transgenic plant from which they were derived[42]. The higher levels of recombinant protein expression in hairy roots could be attributed to the active protein synthesis going on in rapidly multiplying hairy roots. We could also obtain photosynthetic hairy roots by stepwise reduction of sucrose in the medium and by continuous exposure to light (unpublished results). Photosynthetic potato hairy roots offer dual advantage of exploiting the active protein synthesis of hairy roots coupled with chloroplast engineering. Higher levels of recombinant protein expression could be obtained using chloroplast transformation[43]. Staub et al[44] reported 300-fold increase in human somatotropin in chloroplast transformed tobacco plants compared to nuclear transformed plants. Further, research in this direction is required to engineer potato chloroplasts for recombinant protein expression followed by the induction of photosynthetic hairy roots.

7 Conclusions

Transgenic plants are increasingly used for the production of recombinant proteins, especially proteins of therapeutic interest. They have advantages of safe, rapid scale-up and probably cost effective systems. Among the different plant systems explored for molecular farming, potato has a unique position to be used for the production of proteins with diverse applications. Potato tubers are attractive tissues as high value recombinant proteins like antibodies could be stored for a period of six months without the loss of biological activity. Molecular farming in potatoes allows rapid scale-up and production of tubers for evaluating the viability of this technology for commercial applications.

Potato hairy roots serve as novel expression systems for the production of recombinant proteins. The hairy roots need to be evaluated further to develop it as a system for the production of recombinant proteins under controlled conditions. It also offers the advantages of containment of the transgenic material, implementation of good manufacturing practices and probably allows easier regulatory approvals. However, the scale up and bioreactor design for the growth of hairy roots has to be worked out.

Potato has been successfully used for the production of vaccines, antibodies, human milk proteins and other industrially useful proteins. The availability of tissue specific promoters such as patatin promoter for tuber specific expression of recombinant proteins helps in the accumulation of protein of interest in the tubers without any significant effect on the growth of plants and as tubers are harvested prior to flowering the concerns about gene flow could be avoided. There is a scope to further increase the expression levels in potato using different strategies like chloroplast transformation, regulatory sequences and targeting signals. The developments achieved so far indicate that potato has all the potential to become a favorable crop for molecular farming.

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