Molecular Farming and the new frontiers of vaccinology

Genomic and proteomic approaches to the study of fundamental cell mechanisms are rapidly contributing to broaden our knowledge on metabolic pathways for the optimal exploitation of the cell as a factory. In the last few years this knowledge has led to important advances in the large scale production of diagnostic and therapeutic proteins in heterologous hosts (bacteria, yeasts, mammalian and insect cells or transgenic animals and plants), allowing the comparison of the most efficient methods in terms of costs, product quality and safety.

Vaccinology is a rapidly expanding research field and new vaccination strategies have been developed thanks to modern technologies based on the rational design of attenuated pathogens, live recombinant vaccines and protein (antigen)- or peptide (epitope)-based subunit vaccines (Plotkin 2005). The aim of these approaches is to obtain efficient and safe vaccine formulations being able at the same time to induce effective, long lasting immunity against complex viral pathogens such as HIV-1 (Nabel 2001), influenza A/H5N1 virus (Stephenson et al. 2004), SARS-coronavirus (Stadler and Rappuoli 2005). To reach this goal, targets must be found able not only to generate long-term memory B cell producing neutralizing antibodies to block free pathogens, but also T cell mediated immunity for...
the control of pathogen spreading by the elimination of infected cells (Lambert et al. 2005).

The newborn branch of plant biotechnology defined as “molecular farming” is mainly focused on the exploitation of plants of agronomic relevance as factories for the large scale production of biomolecules. The possibility to produce subunit vaccines through plants paves new ways and offers solutions to some of the problems associated to traditional production systems.

2. Antigen production in plants: advantages and perspectives

2.1 Stable nuclear and plastid transformation

The stable expression of heterologous proteins in plants can be performed by inserting the exogenous encoding gene into nuclear or plastid genomes. To obtain plants transformed in the nuclear genome the natural capability of Agrobacterium tumefaciens to transfer DNA into plant cells (Tinland 1996) is amply exploited. By this method plant tissues can be transformed either in vitro, on small leaf explants, or directly in planta (Feldmann and Marks 1987; Bechtold and Pelletier 1998; Clough and Bent 1998). But the transformation of plant species less susceptible to this pathogen (i.e. some monocots), can be also achieved by shooting DNA-coated tungsten or gold microbeads on plant tissues through a “particle gun” (biolistic method) (Taylor and Fauquet 2002).

Several antigens have been successfully expressed in plants by stable nuclear transformation with different ends. In some cases, plants were considered only as “biofactories” for massive production, while in others edible plant varieties have been chosen for the direct oral delivery of the expressed antigens (Table 14.1).

Table 14.1. Representative antigens stably expressed in plants.

| Pathogen          | Antigen          | Transgenic Plant | References                        |
|-------------------|------------------|------------------|-----------------------------------|
| Hepatitis B Virus | Surface antigen  | *Lactuca sativa* | Mason et al. 1992; Thanavala et al. 1995; Ehsani et al. 1997; Kapusta et al. 1999; Richter et al. 2000; Thanavala et al. 2005 |
|                   |                  | *Lupinus luteus* |                                   |
|                   |                  | *Nicotiana tabacum* |                                 |
|                   |                  | *Solanum tuberosum* |                                |
The use of plants can indeed represent not only a measure to reduce production costs, improve product safety and eliminate the use of needles, but also a strategy to efficiently deliver vaccines at mucosal level (Tacket et al. 1998; Kapusta et al. 1999; Tacket et al. 2000; Tacket et al. 2004; Thanavala et al. 2005). Nonetheless, antigen production in plants still suffer some limitations of the nuclear transformation technology, basically due to the low and variable expression levels of the heterologous gene among independent lines. This is particularly detrimental when the direct oral delivery of the plant tissue is foreseen, as a precise a priori establishment of the vaccine dose is impracticable. A step taken to face this problem consists in optimizing the heterologous gene sequence by using host codon usage, removing cryptic genetic signals that can negatively affect post-translational steps and targeting the heterologous protein in appropriate subcellular compartments ensuring proper folding (Sullivan and Green 1993; Koziel et al. 1996). Another step consists in identifying suitable 5’ and 3’ regulatory elements able to ensure reproducible high-level and tissue-specific transgene expression, as it has been shown that the activity of nominally constitutive promoters can vary as a function of the developmental stage, organ and plant species (Williamson et al. 1989; Malik et al. 2002; Samac et al. 2004). Recently, new plant-derived regulatory sequences, such as the promoter-terminator

**Table 14.1.** Representative antigens stably expressed in plants.

| Pathogen                        | Antigen                        | Transgenic Plant | References                                      |
|---------------------------------|-------------------------------|------------------|------------------------------------------------|
| Norwalk Virus                   | Capsid protein                | *Nicotiana tabacum* | Mason et al. 1996; Tacket et al. 2000          |
|                                 |                               | *Solanum tuberosum* | Haq et al. 1995; Mason et al. 1998 Tacket et al. 2000 |
| Enterotoxic E. coli             | Heat-labile enterotoxin B subunit | *Solanum tuberosum* | Mason et al. 1998; Tacket et al. 2000 |
| Rabies Virus                    | Glycoprotein                  | *Lycopersicon esculentum* | McGarvey et al. 1995 |
| Cytomegalovirus                 | Glycoprotein B                 | *Nicotiana tabacum* | Tackaberry et al. 1999 |
| Vibrio cholerae                 | Cholera toxin B subunit        | *Solanum tuberosum* | Arakawa et al. 1997 Arakawa et al. 1998 |
| Foot-and-mouth disease virus    | VP1 Structural protein         | *Arabidopsis thaliana* | Carrillo et al. 1998 |
| Porcine transmissible gastroenteritis coronavirus | Glycoprotein S | *Arabidopsis thaliana* | Gomez et al. 1998 Tuboly et al. 2000 Streatfield et al. 2001 |
|                                 |                               | *Nicotiana tabacum* |                                                 |
|                                 |                               | *Zea mays*        |                                                 |

In bold characters are indicated references describing clinical trials of the plant-expressed antigens.
of the highly transcribed gene \((rbcS1)\) of the ribulose-1,5-bisphosphate carboxylase (RBC) small-subunits gene family in chrysanthemum (Ouchkourov et al. 2003), the regulatory sequences of the seed storage protein gene arcelin 5-I (arc5-I) of common bean \((Phaseolus vulgaris)\) (De Jaeger et al. 2002) or novel gene regulatory elements associated with a cryptic constitutive promoter from tobacco (Malik et al. 2002), have been identified. These sequences are currently used to improve transgene expression levels in different plant species. Turned in a similar direction are those approaches aimed to the production of transgenic plant lines stably encoding replicating viral vectors, the so-called replicons or amplicons, and expressing uniformly and synchronously a foreign gene, thanks to the efficiency of viral replicases (Palmer et al. 1999; Mallory et al. 2002; Gleba et al. 2004; Zhang and Mason 2005).

Alternatively, efforts have been made to point out preferences in \textit{Agrobacterium} T-DNA insertion target site and to evaluate whether transgene expression variability in single copy transgenic plants can be correlated with integration position (Gelvin 2000; De Buck et al. 2004; Schneeberger et al. 2005).

In a similar direction, efforts are oriented attempting to adjust gene targeting by homologous recombination in plants, to induce the integration of the heterologous gene into a pre-determined genomic location in a single copy pattern, preventing the accidental inactivation of genes fundamental for plant metabolism or the anomalous expression profiles due to post-transcriptional silencing (Hanin and Paszkowski 2003; Srivastava and Ow 2004). Progress in this sense has been achieved using Cre-\textit{lox} mediated recombination mainly in tobacco and rice, in the latter case by optimizing the design of T-DNA flanking regions (Albert et al. 1995; Terada et al. 2002; Cotsaftis and Guiderdoni 2005).

An interesting alternative to nuclear stable transformation is the insertion of antigen encoding genes in the plastid genome (Tregoning et al. 2003; Koya et al. 2005; Molina et al. 2005; Glenz et al. 2006). Fundamental difference between plastid and nuclear transformation is that in the plastid homologous recombination of the foreign sequence is feasible, eliminating problems linked to “positional effect”. Moreover, the plastid genome present in several copies in a single cell, rapidly replicates ensuring high yields of recombinant protein production (Daniell et al. 2005). Despite these advantages, up to now no data are available regarding the oral delivery of transplastomic plant tissues, probably because fertile edible transplastomic plants are still under construction (Ruf et al. 2001; Lelivelt et al. 2005).
2.2 Transient transformation

To get round the difficulties encountered in obtaining high rate of expression of the heterologous gene in nuclear transformed plants, transient transformation strategies have been devised. In this case, the gene encoding the antigen of interest is inserted in the genome of a pathogen that is used as a vector for expression during plant infection. The two major techniques used to get transient transformation are based on plant viruses- or Agrobacterium-mediated infection of fully developed plants.

The use of plant viruses (typically ss(+)RNA viruses such as Tobacco Mosaic Virus (TMV), Cowpea Mosaic Virus (CPMV), Potato Virus X (PVX), and Alfalfa Mosaic Virus (AlMV)) to transiently express heterologous genes, has been greatly favored by the development of expression vectors harboring the cDNA of the complete viral genome (Pogue et al. 2000). Using these tools, the foreign gene can be easily inserted as an additional Open Reading Frame (ORF) or, when possible, by replacing unessential viral functions (Lacomme et al. 1998). By this way many antigens have been successfully produced able to induce in animal models good antibody responses, both when delivered in extracted and purified forms or directly per os using the infected plant tissues (Table 14.2).

Table 14.2. Representative antigens and epitopes transiently expressed in plants through viral vectors.

| Pathogen         | Antigen                                | Viral Vector | References               |
|------------------|----------------------------------------|--------------|--------------------------|
| P. falciparum    | Several epitopes                       | TMV          | Turpen et al. 1995       |
| Influenza virus  | Hemagglutinin epitope                  | TMV          | Sugiyama et al. 1995     |
| HIV-1            | Capsid epitopes                        | PVX          | Porta et al. 1996 ;      |
|                  |                                        | CPMV         | Yusibov et al. 1997 ;    |
|                  |                                        |              | Marusic et al. 2001      |
| S. aureus        | Fibronectin binding protein epitope    | PVX          | Brennan et al. 1999      |
| FMDV             | VP1 Structural protein                 | TMV          | Wigdorovitz et al.1999   |
| Hepatitis B virus| Mimotope                               | TMV          | Nemchinov et al. 2000    |
| Rabies virus     | Chimeric peptide                       | AlMV         | Yusibov et al. 2002      |
| HPV              | E7 Oncoprotein                         | PVX          | Franconi et al. 2002     |
| BHV-1            | Glycoprotein D                         | TMV          | Pérez Filgueira et al. 2003|
| Colorectal       | GA733-2 Antigen                        | TMV          | Verch et al. 2004        |
| cancer           |                                        |              |                          |
| Hepatitis C virus| Mimotope                               | CMV          | Natilla et al. 2004      |
Up to now agroinfiltration technique has been used mainly to predict expression efficiency of constructs before their use for nuclear stable transformation. However, recent approaches converted this procedure into a valuable alternative expression system. For example, Icon Genetics Inc. (recently acquired by Bayer) has developed a system (magnICON) for the overexpression of foreign genes that conjugates the efficiency of *Agrobacterium*-mediated transformation with viral rate of expression (Marillonnet et al. 2004, 2005; Gleba et al. 2005; Gils et al. 2005). The strategy is based on the optimization of the TMV genome (by the removal of cryptic sequence that, if recognized by the plant machineries, could affect viral replication/spreading) and on the split of the improved genome into 5’ and 3’ modules of expression. The 5’ module includes the viral polymerase and the movement proteins genes while the 3’ module carries the gene of interest in substitution of the viral coat protein gene. These two modules, used to co-infiltrate plants, are linked together by a recombinase encoded by a third co-infiltrated vector that recognizes specific recombinase target-sequences, artificially added to each module. This “deconstructed” virus system that lacks the coat protein gene, is unable to spread throughout the plant and in the environment, is not influenced by the dimension of the inserted gene due to the absence of the packaging process, while leads the plant cell machinery to the production of the heterologous protein. Using this strategy high levels of different *Yersinia pestis* antigens have been expressed in *Nicotiana benthamiana* plants. These plant-produced antigens are efficient in inducing protective immune responses against the pathogen when administered subcutaneously to guinea pigs (Santi et al. 2006).

**Table 14.2.** Representative antigens and epitopes transiently expressed in plants through viral vectors.

| Pathogen | Antigen            | Viral Vector | References        |
|----------|--------------------|--------------|-------------------|
| RSV      | G protein          | AlMV         | Yusibov et al. 2005 |
| HIV-1    | Tat protein        | TMV          | Karasev et al. 2005 |
| Y. pestis| F1 and V proteins | MagnICON     | Santi et al. 2006  |

*HIV-1:* Human Immunodeficiency Virus type 1; *FMDV:* Foot and Mouth Disease Virus; *HPV:* Human Papilloma Virus; *BHV-1:* Bovine Herpes Virus type 1; *RSV:* Respiratory Syncytial Virus; *TMV:* Tobacco Mosaic Virus; *PVX:* Potato Virus X; *CPMV:* Cowpea Mosaic Virus; *AlMV:* Alfalfa Mosaic Virus; *CMV:* Cucumber Mosaic Virus. In bold character is indicated the reference describing clinical trial of a plant-expressed chimeric peptide through a viral vector.
3. Peptide-based vaccine production in plants: new solutions to old problems

Epitopes are the most important parts of an antigen because, by interacting with antigen receptors of B and T cells, they are responsible for the induction of the immune response. The use of synthetic peptides corresponding in their sequence to linear epitopes has been considered for the development of safe vaccines. Unfortunately, the efficacy of peptides in the induction of an immune response is limited mainly because of their very short half-life in the serum. Several delivery systems have been designed to circumvent this limitation.

An attractive approach to produce epitope-based plant vaccines is the construction of chimeric plant viruses displaying on the surface of the assembled particles peptides of interest for vaccine formulations (Pogue et al. 2002). By this approach, plants are employed as biofactories and large scale “reservoirs” of chimeric virus particles (CVPs) carrying the epitope. To construct CVPs, the sequence coding for the heterologous peptide is fused to the viral coat protein (CP) gene in a position known to be exposed on viral surface (Johnson et al. 1997; Porta and Lomonossoff 1998). To guarantee the production of large quantities of CVPs, the structural and functional characterization of the CP is essential to place the foreign peptide avoiding the interference with viral assembly, stability and/or spreading through the plant. To this aim, fundamental are the studies that define the biochemical factors governing viral movement and CP/virus particle structures by mutational, immunological and X-ray diffraction/spectro-metry analysis (Carrington et al. 1996; Callaway et al. 2001; Bendahmane et al. 1999; Porta et al. 2003).

The efficacy of CVPs in inducing antibody responses specific to the displayed epitope have been extensively demonstrated. Purified CVPs administered to animal models intranasally, intraperitoneally, or orally by direct delivery of virus-infected plant tissues (in this case also in humans) have been able to induce strong specific neutralizing immune responses (Table 14.2).

Recent data indicate that CVPs could be able to induce also the activation of HLA class I restricted T cell responses and of Natural Killer cells (Yusibov et al. 2005). Endorsement of these findings envisages a significant expansion of plant derived CVPs in the field of vaccinology.
4. Efficient delivery of subunit (plant-derived) vaccines: will plants offer the solution?

Although subunit vaccines offer great perspectives, their development is somewhat impaired due to the fact that peptides and proteins are poorly immunogenic and/or unable to be properly presented to cytotoxic T cells (Sette and Fikes 2003). For this reason, aside from the identification of essential targets, most of the efforts are focused on the improvement of vaccine efficacy.

As far as the recombinant antigen-based formulations is concerned, a fundamental role is played by substances able to enhance the immune response against the antigen and fundamental to strengthen the efficacy of the active principle in the vaccine (adjuvants) (Cox and Coulter 1997). Research is currently in progress in this area. Nonetheless, aluminium salts, the first adjuvant described (Glenny et al. 1926), still remain the standard for human use.

Plants have also been considered as possible source of adjuvants. The most known plant-derived adjuvants are saponins and in particular the QS21 acylated 3,28-o-bisdesmodic triterpene saponin extracted from the bark of the South American tree *Quillaja saponaria* Molina (Jacobsen et al. 1996). Basically, the interest to this natural surfactant derives from its high water solubility and the stability (hours if not days) of the mixture with the antigen preparation. Delivered in combination with different antigens not only in several animal models, but also in human trials, QS-21 has been able to enhance both Th1 and Th2 responses (Moore et al. 1999) and to favor the activation of cytotoxic T cells (Newman et al. 1997). Besides QS-21 and new variants of natural or semi-synthetic saponins (da Silva et al. 2005; Marciani et al. 2003), a broad range of different plant-derived compounds, such as the Neem tree Leaf Preparation (NLP) (Baral et al. 2005), a compound of Chinese herbal medicinal ingredients (cCHMIs) (Wang et al. 2005) and the Rb1 fraction of ginseng (Rivera et al. 2005), is under investigation to evaluate adjuvanticity. Some of these compounds together with molecules, such as triterpenoids (Squalene is at the moment the sole adjuvant, together with alum, approved for human use) (Singh and O’Hagan 1999; Stephenson et al. 2005) are as effective as Freund’s complete or incomplete adjuvant, and able to induce balanced Th1 and Th2 immune responses and active immunity even towards tumor antigens (Baral et al. 2005).
Aside the identification of immune response potentiator, new routes of delivery, or alternative delivery strategies, are also considered as key factors to improve subunit vaccines efficacy. Mucosal immunizations, for example, represent an efficient alternative to traditional parenteral immunization, inducing not only secretory IgA responses, but also systemical antibody production and T-cell mediated immunity (Holmgren and Czerkinsky 2005). Moreover, this non-invasive, easy-delivered immunization procedure is performed without the use of needles thus preventing cross-contamination. Plant-derived antigens both stably and transiently expressed in edible tissues/organs have been delivered orally and the efficiency of this immunization procedure has been evaluated in terms of antibody production. In this context, chimeric plant virus particles offer interesting solutions to the problem of peptide-vaccine efficacy for two main reasons. They are functional carriers for peptide-delivery and have been demonstrated to be efficient immune response-inducers without the need of adjuvant both when delivered in purified form (Marusic et al. 2001) or when orally administered in infected plant tissues (Yusibov et al. 2002). Studies have been carried out in order to define the fate of CVPs in vivo, by investigating the distribution in mice of CPMV particles orally or intravenously delivered (Rae et al. 2005). The conclusion was that these icosahedric virions, that are the best characterized to be used as nanoparticles (Wang et al. 2002a, 2002b; Chatterji et al. 2004), are stable during transit in the gastrointestinal tract being able to disseminate systemically thereafter. CPMV particles were found in several tissues throughout the body, supporting the idea that they can be used also as orally bioavailable nanocapsule for the delivery of vaccines and more generally of therapeutics.

5. Conclusions

More than two centuries have passed from Edward Jenner first vaccination against smallpox. Since then, many advances and scientific conquests have been done in the field of vaccinology. However, despite this progress a lot of delicate issues still subsist. First of all, the biological safety of the product as the adverse effects of vaccine delivery are unfortunately very frequent. A second but not less important socio-economical issue is that an excessive cost of the available vaccines prevents the application of WHO guidelines tending to cancel the disparities in health rights still existing among industrialized and developing countries. The use of plants as “biofactories” could offer the solution to some of these problems.
Up to now, plants have been mainly considered attractive as alternative systems only for the production of subunit vaccines by giving emphasis to their advantages in terms of cost-reduction and intrinsic biological safety of the product. However, many indications suggest that efforts should be now concentrated to define how plants could be exploited in novel strategies for subunit and epitope vaccine delivery. This could not only enhance the efficiency of vaccination but, above all, improve global health equity favouring the diffusion of modern vaccines to the world’s poorest countries.

The obvious question now is why this “friendly” and promising technology has difficulties to take off and capital investment in this sector is still so limited.

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