Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Molecular Biology of Tropical Plants, 2006: 17-39 ISBN: 81-308-0138-8
Editor: Claudine Franche

2 Rice and virus biotechnology: Application for the expression of an anti-*leishmania* vaccine

Siré, C.[1], Piron, F.[1], Bangratz-Reyser, M.[1], Lemesre, J-L.[2] and Brugidou, C.[1]
[1]UMR 5096-Génome et Développement des Plantes and [2]R008-Pathogénie de Trypanosomatidés, Institut de Recherche pour le Développement, 911 avenue Agropolis, BP 64501, 34394 Montpellier, Cedex 5, France

Abstract

Plant systems are showing a considerable potential for the economic production of proteins. Recent experiments of biopharmaceutical production from transient expression in plants are encouraged. We discuss here a general strategy using RYMV-based vectors (Rice yellow mottle virus) to produce an anti-leishmania vaccine in monocot (Oryza sativa L.) and dicot (Nicotiana tabacum or Nicotiana benthamiana) plant species.

Correspondence/Reprint request: Dr. Christophe Brugidou, UMR 5096-Génome et Développement des Plantes Institut de Recherche pour le Développement, 911 avenue Agropolis, BP 64501, 34394 Montpellier, Cedex 5 France. E-mail: christophe.brugidou@mpl.ird.fr

Introduction

Nowadays, many tools are available for production of heterologous proteins, such as bacteria, fungi, mammalian and insect cell systems. Nevertheless, high level of natively folded proteins is limited. Other strategies have been thus exploited to overcome such constraint.

Over the last decade, researches on plants, especially on plant models (i.e. *Arabidopsis thaliana* for dicot species and *Oryza sativa* for monocot species), lead to increase significantly our knowledge of gene regulation and protein synthesis in different plants. Similar to evolution of genomic, new biotechnological tools, with transgenic technologies, were developed to express foreign genes. However, such technologies were exploited not only to improve agronomic performances but also to achieve rapid high level production of valuable proteins (e.g. industrial or pharmaceutical products).

Thus for molecular farming, plants were described as attracting, safe and practical bioreactors.

Nevertheless, technical limitations also existed in such technologies, thus much efforts have been concentrated to overcome drawbacks inherent to transgenic technology. At present day, molecular farming in plants can be achieved by stable or transient expression systems. Moreover, concomitant to genomic studies, research on plant viruses lead to the discovery of many potential of such entities to help the production of heterologous proteins. The potential of plant viruses as tools for genetic engineering, was highlighted, especially as expression vectors for production of proteins in plants.

More recently, the discovery of viral suppressors of gene silencing enhanced knowledge and advances in plant valuable proteins production. Indeed such proteins were exploited to avoid the problem of transgene silencing inherent to transgenic technologies but also to over-expression of foreign genes also in transient systems.

As a model system for cereal genomic and biotechnology, rice could be widely used to develop such strategies in monocotyledon species. In this review, in a first part, we'll discuss about the tools available for rice genomic and biotechnology. Then, in a second part, we'll develop all the strategies described, with their advantages and drawbacks, to widely produce heterologous proteins especially in rice, with the specific aim to produce a therapeutical molecule.

I. Rice and RYMV, tools for functional genomics and biotechnology

I.1. Rice as a model for cereals

Rice belongs to the *Oryza* genus, including 20 species, of which only two are cultivated: *O. glaberrimma* Steud, endemic to Africa and *O. sativa* L.

originates from Asia. *O. sativa* comprises two groups of cultivars analogous of sub-species (*japonica* and *indica*). Asian cultivated rice (*O. sativa*), worldwide cultivated, is an important subsistence crop in tropical regions, for human consumption, providing the staple food for more than a half of the world's population.

Its importance is not only reported at the economic scale but also at the genetic scale. Indeed, rice became a plant model for monocotyledons genomic, especially for cereals because of its genetics features. Actually, rice exhibits a high synteny with the other cereals [4,5] and its genome size is relatively small (i.e. 430 Mbp spread over 12 chromosomes). Thus, many tools have been developed to understand this species better with developing dense molecular genetics maps, YAC[1] and BAC[2] libraries [6,7]. Moreover, improved transformation techniques are now available, with biolistic delivery or explants co-culture with *Agrobacterium tumefaciens*, providing an efficient tool not only for crop improvement but also for functional genomics [8,9]. In this context, essential biological information from the rice genome will be easily assessed and will especially improve our understanding of the basic genomics and genetics of other related significant crops. In this way, a large number of genomic sequences have been generated by sequencing the entire genome that holds fundamental information for its biology, including physiology, genetics, development and evolution [5,10,11]. A large number of studies have been carried out to generate sequence analysis and also to determine complete genomic sequence [12]. Thus, completion of rice sequencing was achieved in 2003 and its genome was automatically annotated, using prediction-based and homology-based searches to identify genes [13]. Moreover several rice genome sequences have been produced, thus providing a unprecedented access to numerous genes [5]. Nevertheless, knowing DNA sequence is the first step for the elucidation of the genomes biology and efficient transformation methods contributed to the deployment of new tools for improving and studying gene function. A large number of tools have been developed to facilitate gene function discovery. Up to date, insertional mutagenesis has been widely used, in this aim [14-16]. However, a complementary method has been undertaken to identify function of genes that have not already been tagged with mutants: RNAi vectors [17]. Such vectors lead to assessment of gene function by suppressing gene expression through specific RNA-mediated RNA degradation mechanism, and can be used either in *Agrobacterium* transformation assays or in transient expression systems. Furthermore, dicotyledonous species, another original technology was used to appreciate rapidly gene function by transitively over-expressing or suppressing gene

[1] Yeast artificial chromosome
[2] Bacterial artificial chromosome

expression, with virus-based vectors. Indeed such technology has been improved for various applications in dicot, it's lacking for efficient use in monocot species.

I.2. Viral vectors: Functional genomics and biotechnological tools

During the last decade, the potential of plant virus-based vectors has been highlighted for functional genomics and study of gene expression. The study of plant viruses has generally permitted the assessment of eucaryotic biology [18,19]. Viral expression systems represent an attractive tool to complement conventional breeding or transgenic methodology. To achieve the expression of heterologous sequences in plants, the use of viral expression systems offers several advantages over stable transgenic expression particularly in regard to the expression levels that are obtained and genes can also be evaluated earlier [20,21].

Molecular genetic studies of plant RNA viruses lead to the generation of infectious RNA (i.e. upon *in vitro* transcription) [22] or directly infectious cDNAs (i.e. downstream from *Cauliflower mosaic virus* 35S promoter). Moreover, the emergence of the green fluorescent protein (gfp) from *Aequorea victoria* as a reporter molecule offers the possibility to assess plant virus infections with a non destructive assay technique [23-26]. Thus, viral infectious clones have been modified to study better different viruses but also viral protein functions by monitoring movement in infected cells, replication and virus spread [27]. Then flexibility of viruses and rapid expression of viral proteins have been exploited for fundamental research or biotechnology application [20,21,28] to produce high level of foreign genes with the development of viral expression vectors. Actually, virus-based expression vectors have number of advantages as gene expression tools including the ability to direct rapid and high level expression of foreign genes in mature and differentiated plant tissues, and have been used for a number of different applications [29,30]. In fundamental virology, fusion with reporter genes (gus or gfp) allows monitoring of viral gene expression and products *in planta* [31,32]. They are also used for production of valuable foreign peptides and proteins in plants [21,29]. Finally, virus-based expression vectors offer advantages to express foreign (or endogenous) genes for functional characterisation of ORFs (open reading frames) [29,33]. The ability of RNA viruses to trigger gene silencing has also been exploited in the construction of VIGS (virus induced gene silencing) vectors to suppress host gene expression in the aim to assign gene function [34,35]. Such vectors carry sequences that share homology with transgenes or endogenous genes and silencing might be initiated by the viral sequence whereas the maintenance step occurred on the nuclear genes targeted independently from the presence of

the virus [36]. VIGS has been validated as an efficient tool for reverse genetic to study genes involved in primary and secondary metabolism, in development, disease resistance [37]. Thus VIGS strategy is an attractive alternative to insertional mutagenesis to investigate gene function particularly in multigene families [38].

Some basis features are required for viruses used to develop plant virus-based vectors. Indeed, they need to i) be autonomous replicating systems, ii) be easily genetically manipulated, iii) have a short cycle time life and finally iv) infectious cDNA clones must be available. Many strategies have been developed for transient expression of foreign genes and two of them were mainly used [30] (figure 1).

The first method consists in the fusion of the protein, or protein domains, with the viral coat protein (CP) for presentation at the surface of the viral particle such as epitope presentation [39-41]. This strategy has been widely exploited for production of vaccine sub-unit [30]. The second method consist in expressing foreign genes and viral genome independently of one another by using duplication of sub-genomic mRNA promoter, gene insertion or gene replacement techniques [20,21,30]. However, whichever the strategy used the insertion of foreign genes can interfere with CP function (e.g. particle assembly, virus movement) and steric constraint limited the size of the inserted sequence in most cases. Such vectors have been improved to preserve viral genome integrity and to promote insert stability with different inoculation methods or gene trans-complementation, for example [42-44].

Transient knock-out strategy through VIGS vectors have also been improved with the assessment of insert features such as insert size [45], insert orientation or the choice of gene fragment. Furthermore viruses have evolved to develop silencing suppression properties [46], and viruses possessing strong silencing suppressors are not suitable tools for the construction of VIGS vectors. Finally in some cases strong VIGS response or symptoms apparition interfering with the assessment of gene function [47] need to be get round.

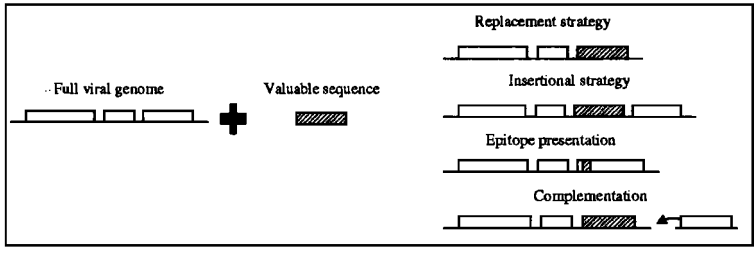

Figure 1. Different strategies to produce virus-based vectors for valuable proteins production.

There are now several plant viruses which have been converted into vectors and implemented for transient over-expression of genes of interest, and also, for VIGS of transgenes, or endogenous genes. Viruses of dicot species have been developed as vectors, but none are functional for cereals. Moreover there are no suitable vector for expression of foreign genes in monocotyledonous plants except a BMV vector [48], thus our lab is interested in developing such technology for rice based on the genome of *Rice yellow mottle virus* (RYMV) (figure 2).

Rice yellow mottle virus is a single-stranded-positive-sense RNA virus that specifically infects rice leaves and causes serious disease in irrigated rice systems in East and West Africa [49-51]. This virus, belonging to the *Sobemovirus* genus, is transmitted by chrysomelid beetles and can be artificially inoculated with sap. RYMV-genome properties or particle structure have been well described [2,3]. RYMV genome is simple and corresponds to a positive single-stranded RNA composed with four partially overlapping ORF [52] (figure 2).

Recently genetic diversity and phylogeny of the virus have been studied [52,53]. This virus thus, represents a good model for developing virus-based expression or VIGS vectors as:

i) infectious cDNA clones are available [54],
ii) RYMV highly replicates in infected cells,
iii) P1 protein involved in PTGS suppression is highly variable among different RYMV isolates [46,55],
iv) tolerant rice genotypes are available to bypass effects of the infection,
v) transgenic plant expressing viral ORF [56,57] are also available for trans-complementation of viral sequences deleted to overcome size constraint.

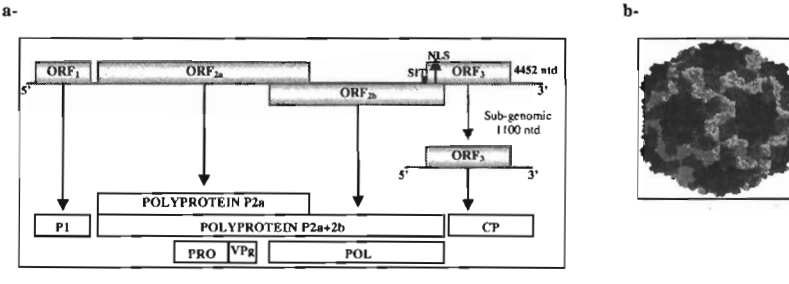

Figure 2. *Rice yellow mottle virus.* **a-** Genomic organisation [54] with SIT : transcription initiation site (GTGGGATAGGGCGAGTCTCCCACAAAGATG [1]), NLS : Nuclear localization signal $(KK(X)_{10}KRKXRR$ [2]), PRO : protease, VPg : *viral protein genome linked*, POL : RNA polymerase, CP : coat protein. **b-** Atomic structure of RYMV particle with a 2,8Å resolution [3].

Nevertheless, we have to keep in mind the major drawback, which consists in the size constraint set by the virus icosaedral particle. Actually, even if the virus genome is highly variable, the size of the sequence and also untranslated regions are conserved among the 15 isolates fully sequenced [55].

As genome size is crucial for icosaedral viruses (e.g. RYMV), to preserve virus infectifivity and integrity, we attempted to develop RYMV-based vectors, in our lab, to highlight maximal size constraint. Thus, interesting tools both for rice functional genomics and for biotechnological applications, with the aim of large-scale production of recombinant proteins, were developed. As, a major size constraint has been demonstrated (Siré *et al.*, unpublished results), other alternative strategies will be undertaken to overcome this drawback. Indeed, the use of replicative viral system, also called amplicon, has been previously reported, in combination with silencing suppressors, to reach high level of protein production [58]. With this aim in mind, RYMV-amplicon tool was developed. Furthermore, large studies have been carried out to better understand mechanism of silencing suppression by RYMV, to improve the amplicon tool for production of recombinant proteins in rice.

I.3. Gene silencing targets viruses and viruses suppress gene silencing: Application for biotechnological tools

Both viruses and genetic invasive elements trigger and target RNA silencing. This mechanism consists in a sequence-specific RNA degradation preventing gene expression.

RNA silencing was firstly reported as PTGS (post-transcriptional gene silencing) in plants and referred to RNAi (RNA interference) in animals, is generally conserved in eukaryote cells [59,60]. Thus RNA silencing consists in an ancient regulatory and adaptive defence mechanism acting at the molecular level against different genetic invasive elements.

In cells where they are detected, double-stranded RNA (dsRNA) molecules trigger PTGS, leading to a dramatic reduction of homologous cytoplasmic mRNA accumulation [61-63]. Both highly transcribed transgenes, transgenes in inverted repeat orientation, and viruses lead to production of dsRNA molecules in higher plants [64,65]. Then the RNAseIII-like enzyme, called DICER, targets dsRNA and cleaves this molecule into small RNA duplexes of 21 to 24 ntd, reported as small interfering RNA (siRNA) [66,67]. These siRNA are then complexed with a large multicoponent RNA-induced-silencing complex (RISC), which is thought to unwind siRNA to help target the appropriate mRNA. The antisense strand of siRNA is used to target homologous cytoplasmic mRNA, which is finally degraded [68,69]. Another step in silencing mechanism has been described as the amplification and propagation steps of the signal in distant tissues, involving RNA-dependent

RNA polymerase (RdRP) activity [70]. Thus newly synthesised dsRNA, with ssRNA (i.e. single-stranded mRNA) as template and siRNA as primer, lead to intense accumulation of siRNA that move in neighbouring cells through plamodesmata [71,72].

Both in higher plants and in animals, PTGS has been reported as an immune system acting at the molecular scale to prevent viruses invasion [73,74].

To counteract such defence mechanism, plant viruses evolved through specialisation of one, or more, of their proteins, to target various steps in silencing pathway (i.e. intercellular or intracellular silencing) [75,76], as a result of diversity and multifunctionnality of viral proteins [77,78]. Silencing suppression with plant-viral proteins, thus facilitate virus replication and movement [46,75,78,79]. Up to date many viral suppressors were identified, encoded by a single ORF or by different ORF for the same virus [80,81] and were generally described as involved in viral pathogenicity and in virus spread [46,79,82]. Features of such proteins were highlighted in biotechnological applications in the aim of enhancing recombinant protein production, which is drastically limited by silencing directed against over-expressed genes [83].

Thus, with the purpose to highly produce protein of interest in rice, with or without RYMV-based vectors, behaviour of P_1 protein from RYMV in silencing suppression, was assessed. Indeed, this protein has been previously described as non autonomous cell silencing suppressor [46,70] and is dispensable for viral replication but is closely related to virus infectivity and also to virus spread [84]. Availability of a large collection of RYMV-isolates allowed an accurate and original study of silencing suppression by entire RYMV particle, and also by its P1 protein (Siré *et al., submitted*). This study characterised silencing suppression features of RYMV under natural infection on rice plants. Biolistic delivery assays on rice leaves and *Agrobacterium*-based leaf infiltration assays on *Nicotiana benthamiana*, determined that different P1 proteins undergo silencing with variable efficiency. Our results also suggested, that silencing suppression occurring under RYMV infection, is a complex mechanism, probably involving more than one viral suppressor.

II. Strategies to produce recombinant proteins: Application for production of anti-*leishmania* vaccine

Many biotechnological applications, like production of vaccines, antibodies, human blood products, hormones and growth factors, require high-level expression of transgenes. In the past few decades, several different systems have been developed, for the production of recombinant proteins at

low cost. However, all these systems are not perfect because in some cases they can be unsafe, or they can lead to production of biologically inactive material. So, production of recombinant proteins in mammalian cells results in products that are identical to those of natural origin but this culture is very expensive and can be carried out on a limited scale. The use of microorganism system allows production on a large scale, but exhibits the major drawback to introduce structural variations in the protein. Plant system is an economical system, where the contamination risks, with human pathogens, are minimised. Furthermore, this system can be developed at an industrial scale. Finally, plant system offers a good compromise with an eukaryotic protein modification machinery allowing subcellular targeting, proper folding and post-translational modifications at low cost. Thus, today all advantages of plant system make it the most attractive technology for the production of recombinant proteins [85,86] such as an anti-*leishmania* vaccine.

Protozoa of the genus *Leishmania* are obligatory intracellular parasites of mammalian macrophages. They are transmitted to vertebrate hosts by sandfly vectors of the genus *Phlebotomus*. They cause a wide spectrum of human diseases in many tropical and subtropical regions of the world that range from a self-healing cutaneous ulcer to a potentially fatal visceral infection (figure 3). Zoonotic visceral leishmaniasis is one of the most important emerging diseases. Wild canids and domestic dogs are the main reservoirs of *L. infantum*

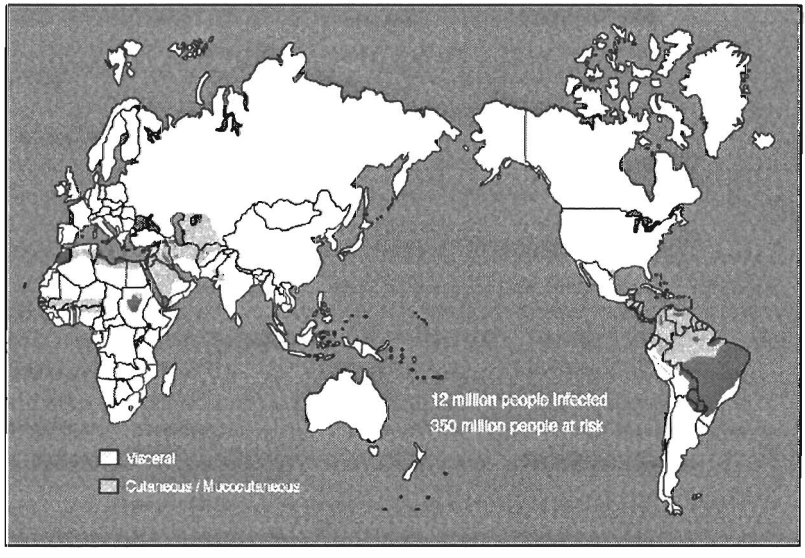

Figure 3. Geographical distribution of leishmaniasis in the world.

in the Mediterranean Basin, extended to several Middle-East and Asian countries, and of *L. chagasi* in South and Central America. Visceral leishmaniasis (VL) due to *L. donovani* is the most severe form of leishmaniasis. Approximately 500,000 new cases of human VL occur annually and the disease is mainly found in Brazil, East Africa and on the Indian sub-continent where devastating outbreaks have occurred and from where most VL cases are reported world wide [87]. In India, millions are at risk, the state of Bihar accounts for nearly 90% of cases, followed by West Bengal and Eastern Uttar Pradesh. Neighbouring countries like Nepal and Bangladesh also report a significant number of VL cases. Affected populations are among the poorest in the world and are not much aware/informed of existing preventive measures. Furthermore miss-use of the first-line drug in these communities is widely spread [88] and the lack of response to the first line drug (pentavalent antimonials) has been increasing sharply these last years in India up to more than 50% of the patients in hyper endemic areas of Bihar [89-91].

There is now an urgent need for new low cost drugs and/or new therapeutic interventions such as a vaccine for the control of this parasitic disease.

Recently, we have developed a vaccine involving *Leishmania* Excreted Secreted Antigen (LESA) [92-94] which was proved efficient both experimentally and in naturally *Leishmania infantum* infected dogs of southern France [95]. Access to a serum-free system for culturing promastigotes of *Leishmania* has improved the feasibility of large-scale production of well-defined parasite material. Using this methodology, it has been possible to easily purify naturally excreted secreted antigens from culture supernatant of *L. infantum* promastigotes successfully cultivated in a completely defined CDM/LP medium [92-94] and to investigate their biochemical properties. LESA only contained few excreted secreted polypeptides and mainly concentrated a major immunogenic protein belonging to the Promastigote Surface Antigen (PSA) family (figure 4).

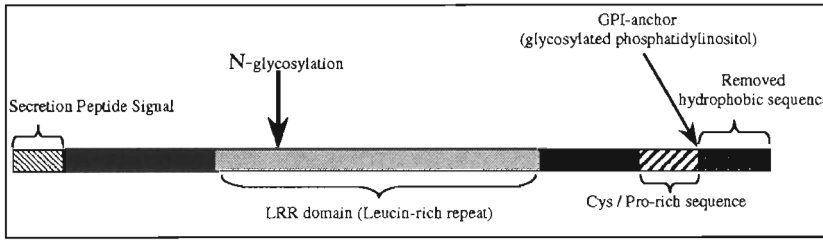

Figure 4. Diagrammatic representation of the structural features of Promastigote Surface Antigen (PSA) of *Leishmania*.

Vaccination with native promastigote surface antigen 2 (PSA-2) of *Leishmania* major has been demonstrated to protect mice from leishmaniasis through a Th1 mediated response. But recombinant PSA-2 purified from *E. coli* was unable to induce protective immunity [96]. These results have important implications for the design of vaccines against leishmaniasis. They strongly suggest that the generation of protective immunity is dependent not only of the induction of a Th1 response, but also indicate that some *Leishmania* antigens may require a near native conformation to be protective [97]. Correct posttranslational modifications and protein folding of antigens may therefore be important not only for the induction of neutralizing antibodies but also for the development of protective CD4+ T cell responses. Finally, the conformation of antigen may play a more major role for the induction of T cell mediated immunity than originally considered.

Thus, expression of the PSA is a good target to test our production system and to demonstrate that is possible to over-express recombinant proteins with the right folding and a good biological activity in cereals.

II.1. Strategies

Up to date, there is not yet a consensus for the best plant species, or tissue, for large-scale recombinant protein production. However, it would be preferable to choose a plant whose genetic manipulation is relatively easy with a large seed production [85]. Then, tobacco seems to represent suitable host for recombinant protein production in sufficient quantities. However, tobacco is not the only plant species used for biopharmaceuticals production. Thus, most antibodies expressed to date have been produced also in potatoes, soybean, alfalfa, rice and wheat. For example, a single-chain Fv antibody (ScFvT84.66) against carcinoembryogenic antigen (CEA) was successfully expressed in the cereal crops rice and wheat [98].

In our study, we decided to focus on two species: rice (*Oryza sativa* L.) and tobacco (*Nicotiana benthamiana* and *Nicotiana tabacum*).

There are currently two methods for protein production from plants: stable transformation and transient transformation. To date, the most common of the methods, stable transformation, has produced all the products available in the marketplace. This system requires a method for transferring the foreign genes into the plant cells, usually using *Agrobacterium tumefaciens* or particle bombardment, in which the genes are taken up and incorporated into the host nuclear genome in a stable manner [99]. This method of transformation presents advantages when performed in a crop species such as grains. Then, the protein product is normally accumulated in seeds that can allow the protein to exist without degradation for at least two years [99].

Nevertheless, regenerating transgenic plants from transformed cells is both labour intensive and time consuming. Moreover, after this hard work,

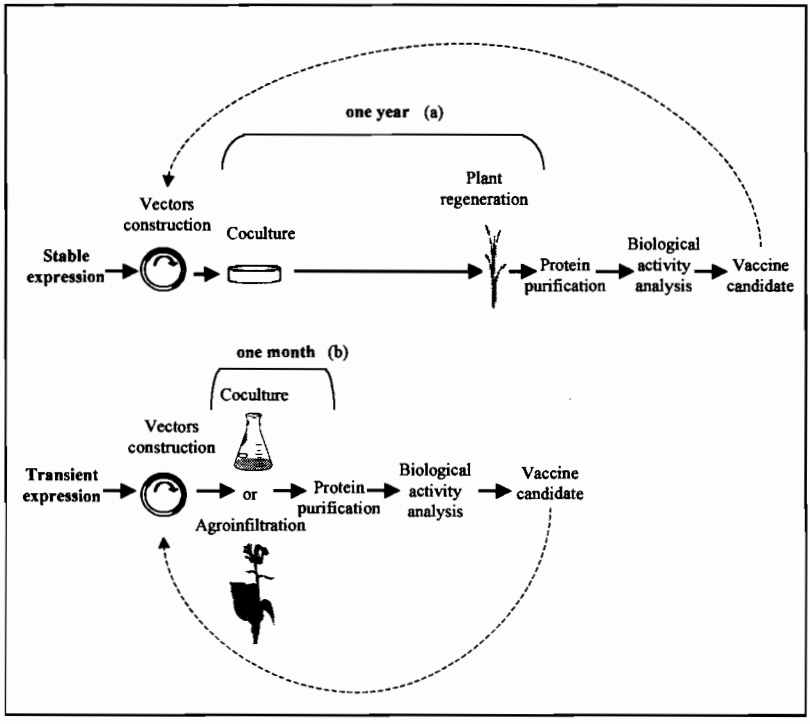

Figure 5. Comparison of time consuming between stable and transient expression systems. (a) Constitutive system is labour intensive and time consuming. To purify recombinant proteins, one year is necessary from the calus coculture to the second-generation plants. (b) Whereas transient expression is a flexible and rapid system for the production of proteins. Only one month is necessary to collect the recombinant proteins with a transient system. This is ideal for verifying functionality, integrity and stability of gene product before large scale developing stable transformed plants.

production of a correct folding protein and a biologically active protein is not guaranteed (Figure 5).

In contrast, transient protein expression using virus-based vectors allows rapid production of recombinant proteins. Thus, this flexible and changeable system is ideal to analyse the structure and the activity of protein produced in plants before developing a constitutive expression system. Transitory system is suitable not only for testing and scoring plant expression constructs and predicting their performance in transgenic plants, but also for purifying the recombinant protein [100]. It was demonstrated that the tobacco agroinfiltration take to the production of functional recombinant proteins [100].

Expression level of recombinant proteins can be variable. Avidin (i.e. immunological regeant) at 3% of extractable protein was produced in transgenic maize seeds [101] whereas only 0.1% of aprotinin [102] and 0.5% of β-glucuronidase [103] was generated with the same plant species. Apoprotinin, the bovine pancreatic trypsin inhibitor which affects known serine proteases such as trypsin, chymotrypsin, plasmin and kallikrein, has been widely used in biochemical research and as a therapeutic agent [102]. It has been hypothesised that the differences between proteins in terms of size, charge and localisation, could play a crucial role in the expression level [102].

Moreover, different levels of protein expression were published according to the use of different tools. Thus, transgenic plants (~0.5 mg/kg) revealed a lower yield of recombinant proteins than agroinfiltrated leaves (~1.5 mg/kg) due to a higher promoter activity and gene dosage during the transient expression [100].

II.2. Production enhancement

Recently, viral vectors have been generated from a large number of different viruses in the purpose to improve speed and yield of expression. In this system, viral vectors were designed to serve as over-expression tools.

RNA viruses can multiply to very high titres in infected plants, which makes them ideal vectors for protein expression. For vector construction, viral RNA genomes are reverse-transcribed *in vitro* and cloned as full-length cDNAs or only amplicon-cDNAs (replicating system of plant virus) in vectors [104]. The idea was that transcription of the amplicon and of the transgene, result in very high levels of the recombinant protein. *Nicotiana benthamiana* leaves, inoculated with *in vitro* transcripts of recombinant tobacco mosaic viral vector, accumulated recombinant protein to level of at least 2% of total soluble protein [105].

Transient expression method depends on the ability of recombinant plant viruses to infect plants and then transiently express a target protein in plant tissue [99]. Moreover, target genes are expressed at high levels consequently to the high level of virus replication [104]. The non-integrated T-DNA (Transferred-DNA) copies remain transitory present in the nucleus, that can be transcribed, leading to transient expression of the T-DNA genes [106]. Efficient of transient expression system has been shown by production and purification of His_6-tagged diabody from a scaled-up agroinfiltration tobacco leaves [100].

As plant viruses have a wide host range, the same vector construct is compatible for different plant species [107]. Plant virus vectors have the potential for becoming a useful tool to express foreign proteins in plants, especially when plant-specific folding and glycosylation of the recombinant proteins are of importance [107].

Then, to increase the level of anti-*leishmania* vaccine expression in tobacco and rice, we used an amplicon system based on the RYMV.

Study on a series of *Tobacco mosaic virus* (TMV)-based hybrid vectors for transient gene expression showed differences in the amounts of recombinant protein produced. These results demonstrated that building an effective vector from a virus is not a trivial exercise. An effective expression vector should contain a combination of *cis*-acting elements that appropriately partitions the limited replicase activity among the various promoters to ensure adequate replication and movement while providing the maximal level of foreign gene expression. It has been demonstrated that the most effective vector based on TMV contained sequences encoding the coat protein subgenomic mRNA promoter, coat protein ORF, and 3' UTR (untranslated region) from *Tobacco mild green mosaic virus* U5. Thus, the recombinant protein (i.e. GFP) accumulated up to 10% of total soluble protein in leaves [108].

However, gene expression in plants is influenced by posttranscriptional controls, known as posttranscriptional gene silencing (PTGS). It has been reported that plant infectious virus or transgene can induce gene silencing in absence of any known homology between viral genome or transgene and host genes [109,110]. Reduced levels of the specific mRNA encoded by the suppressed gene characterise the gene silencing phenotype. But, as a counterdefensive strategy, viruses have evolved proteins that suppress various steps of the RNA silencing mechanism.

In order to suppress PTGS induced by the transgene and to increase the transgene expression, the RYMV P1 and the TBSV (*Tomato bushy stunt virus*) p19 pathogenicity factors were co-infiltrated with the gene of interest. These two proteins have been identified as silencing suppressors of transgene [46]. It has been described two types of silencing suppressors having different actions [70]. Thus, the P1 protein could prevent systemic silencing but not its limited movement at the edge of infiltrated patches: it was the non-autonomous cell action. On the contrary, the TBSV p19 silencing suppressor have autonomous cell action. The effect of p19 was estimated to enhance 50-fold enhancement the abundance of the protein [83]. Our first studies on different RYMV P1 silencing suppressors showed that using the appropriate P1 silencing suppressor, level of expression protein could be strongly increase.

It was reported that high level expression could be achieved by pairing the amplicon approach with the use of a viral suppressor of PostTranscriptional Gene Silencing. Leaves co-expressing Hc-Pro from TEV (*Tobacco etch virus*) and a PVX (*Potato virus X*)/Gus amplicon accumulate GUS to about 3% of total proteins [58].

Free cell suspensions is generally regarded to be the best suitable for large-scale applications in the biotechnology industry. A number of plant species, like *Arabidopsis*, rice, soya bean, alfalfa and tobacco have been used for

generation and propagation of cell-suspension cultures. Moreover, plant-cell suspensions can be cultivated using conventional fermenter equipment. Large-scale fermentations up to a volume of 100 000 litres have been performed successfully [111]. The major advantages associated with *in vitro* plant systems include the ability to manipulate environmental conditions for better control over protein levels and quality, the rapidity of production compared with agriculture, and the use of simpler and cheaper downstream processing schemes for product recovery from the culture medium [112]. Using tobacco BY-2 cell line for fermentation, the cultivation of transgenic suspension cells was scaled-up to a working volume of 40 litres. With a 10% (v/v) inoculum, fermentation times of 150h resulted in a yield of 7.5kg of fresh cell weight, corresponding to 0.4kg dry weight [111].

Moreover, correct processing of protein was demonstrated in this system. For example, correct folding erythropoietin was produced in cultured tobacco BY2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) by introducing human Epo cDNA *via Agrobacterium tumefaciens*-mediated gene transfer [113].

The plant-cell-suspension cultures exhibit the advantage that recombinant proteins can be produced under certified conditions (i.e. certified Good Manufacturing Practice and certified Good Laboratory Practice) [111].

In any systems of heterologuous production, the recombinant molecule must be extracted and purified selectively from total endogenous proteins. Eighty percent of the recombinant protein production cost correspond to this purification step [114].

In whole plant system, an alternative to reduce the expense consists in directing protein synthesis to seed endosperm [115], from where proteins may be easily extracted. Further studies showed that the recombinant protein (gB) behaves like a plant storage protein and is localised almost exclusively in protein storage vesicles, when expressed in tobacco seeds [115].

In the purpose to facilitate the purification of biologically active hirudin (an anticoagulant found to be an inhibitor of thrombin) in tobacco, an Arabidopsis oleosin promoter combined with a plant oleosin "carrier" was used [116]. The fusion protein was then targeted to the oil body membrane. This system was developed to simplify the initial step of purification and to limit the proteolysis [116]. After a correctly targeting to the oil body membrane, the recombinant protein was separated from the majority of other seed proteins by flotation centrifugation [116].

In plant-cell–suspension cultures, recombinant proteins expressed are either found in the culture supernatant or retained within the cells. This localisation depends on two factors: the presence of targeting/leader peptides in the recombinant protein, and permeability for macromolecules allowed by plant cell wall [111]. Targeting signals can be used to direct the protein for secretion or to intracellular organelles [111]. In this way, genetically

modified *Nicotiana tabacum* cells, grown in suspension culture, produced and secreted into the medium, a biologically active human interleukin-2 and interleukin-4 (IL-2 and IL-4). These two proteins were detected at concentrations of 0.10 and 0.18 µg/ml in the medium, respectively for IL-2 and IL-4 [117,118].

Plant suspension culture has be used to produce and secrete into the medium a variety of biologically active mammalian proteins that are clinical and diagnostic relevance [119]. Either human prepro-sequence or the extracellular tobacco protein PR-S were used to secrete the human serum albumin (HSA) in transgenic potato leaf tissues and in tobacco suspensions [118,119].

Moreover, the epitope tagging of expressed proteins is a versatile tool for the detection and purification of proteins [120]. In the purpose to purify the *E. coli* MutS, MutH and MutL (proteins mediating methyl-directed-mismtatch) proteins, genes were cloned into an expression vector, which allows fusion to the His6 affinity tag. These His6-proteins were then purified by variations of batch binding to Ni(2+)-chelation affinity resin. The yield of purified His6-proteins from these procedures was 0,4-0,6 mg from 40 mL of induced culture [121].

Transient expression represents a method for verifying functionality, integrity and stability of gene product before large scale developing stable transformed plants [104].

But, transient expression could be also used to determine the subcellular localisation of proteins. In this way, plasma membrane localisation of the ACBP2 (Cytosyl acyl-CoA-binding proteins) and both nucleus and plasma membrane localisation of AtEBP (*Arabidopsis thaliana* ethylene-responsive element-binding protein), were demonstrated using GFP autofluorescent protein fusions, in transient expression by agroinfiltration of tobacco leaves [122]. Transient expression by agroinfiltration is a powerfull tool for promoter studies. In this way, a preferential expression in vascular tissues of stems and leaves conferred by the promoter of a rice glycine-rich protein gene was highlighted [123].

Morever, transient expression could be an interesting system for functional analysis of different promoters or for identification of genes *via* functional complementation. For example, it can be exploited in sense-antisense systems, normal and mutated genes, or in studies related to disease resistance genes [106]. These studies demonstrated that interaction between host plant and pathogen is not disrupted by infiltration with *Agrobacterium tumefaciens* [106]. This suggests that the procedure can be used for studying plant/pathogen interactions.

II.3. Control of integrity of products

Transient expression can be used to verify protein activity before proceeding to transgenic plants.

The biochemical analyses of the on-step IMAC-purified protein (Immobilised metal ion affinity chromatography) showed tobacco cells expressed and correctly processed the T84.66/GS8 diabody, and preliminary data from mass spectrometry suggested that post-translational modifications did not occur. This study has also revealed only the presence of functional diabody purified from agro-infiltration tobacco leaves.

In therapeutical application, the use of plant system production for glycosylated proteins is still limited because of differences between plant and animal glycosylation machinery. For example, a higher number of Guy's 13 glycoforms in plant than in mammalian expression system has been reported [124]. But despite high structural diversity of the plantibody (antibody product in plant) N-glycans, glycosylation appears to be sufficient for the production of a soluble and biologically active IgG in the plant system and is not a limitation to the use of plantibody Guy's 13 for topical immunotherapy [124]. However, these plants N-glycans are immunogenic. One strategy that could be used to produce recombinant glycoproteins with non-immunogenic glycans is to produce these glycoproteins in plants devoid of one or several enzymes, present in the Golgi apparatus, improved in the N-glycans maturation [114]. Arabidopsis *cgl* mutants, deficient in N-acetylglucosaminyltransferase I (*GnTI*) activity, enzyme initiating the formation of complex N-linked glycans on secretory glycoproteins, were generated. Studies on these mutants demonstrated an effective reduction of *GnTI* activity which can be achieved in mature tissues by means of *GnTI*-mediated gene silencing [125]. These researches opened the way for the production of therapeutic glycoproteins in transgenic plant species carrying minimal compatible *N*-glycans of uniform *N*-acetylglucosamine structure [125]. This methodology has already been reported for mammalian cell lines [126]. Humanisation of the N-glycosylation in transgenic plants contributed to expand the use of plant system for the glycoproteins production [114].

Conclusion

Many biotechnological applications require high-level expression of proteins. Thus, in the past decade, plant-based expression systems have emerged as a serious competitive force with the aim of large-scale production of recombinant valuable proteins. To verify functionality, integrity and stability of gene product proteins, we developed a flexible and versatile system, for a rapid and large production in rice and tobacco. The strategy developed, and first results obtained, using an RYMV-amplicon system combined with RYMV-silencing suppressor, show that this system seem to be a promising tool for the production of recombinant proteins (Figure 6). This system is a good alternative for the production of proteins, like anti-*leishmania* vaccine, having an incorrect folding in bacteria system. Moreover, high-level

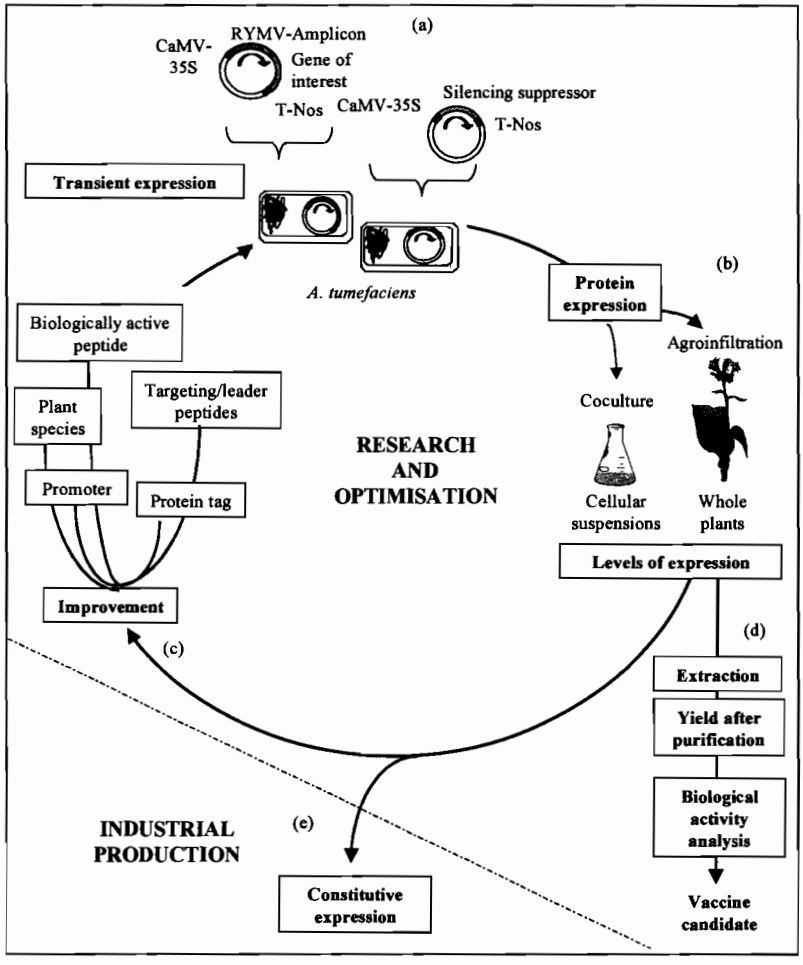

Figure 6. Optimisation of transitory system in order to use the powerful system for constitutive expression of recombinant proteins. (a) Vectors containing gene of interest or silencing suppressors are inserted into *Agrobacterium tumefaciens*. (b) Recombinant proteins are transiently expressed in whole plants with agroinfiltration assays or in cellular suspensions with coculture assays. (c) System of expression could be improved: using other plant species, using other promoters to target the protein, using a tag to facilitate the purification step and using the only biologically active peptide. (d) Products are extracted and analysed to test the structure and the activity of proteins. (e) As soon as the system was optimised to produce a biologically active protein, the production of recombinant proteins could be developed with constitutive system.

expression of recombinant proteins obtained by transitory system is sufficient for structure and activity analysis. The transient expression developed is a powerful tool to choose best expression system to improve yield of production or to facilitate the purification step. Next, production of correct folding and biologically active protein will permit development of a constitutive expression system for the production of the anti-*leishmania* vaccine. This transient system will also be promised to produce another interest protein.

References

1. Hacker, D.L., and Sivakumarann, K. 1997, Virology, 234, 317.
2. Ngon a Yassi, M.N., Ritzenthaler, C., Brugidou, C., Fauquet, C., and Beachy, R.N. 1994, J. Gen. Virol., 75, 249.
3. Qu, C., Liljas, L., Opalka, N., Brugidou, C., Yeager, M., Beachy, R.N., and Fauquet, C.M. 2000, Structure, 80, 1095.
4. Delseny, M., Salses, J., Cooke, R., Sallaud, C., Regad, F., Lagoda, P., Guiderdoni, E., Ventelon, M., Brugidou, C., and Ghesquière, A. 2001, Plant Physiol. Biochem., 39, 323.
5. Delseny, M. 2003, Curr. Opin.Plant Biol., 6, 101.
6. Causse, M.A., Fulton, T.M., Cho, Y.G., Ahn, S.N., Chunwongse, J., Wu, K., Xiao, J., Yu, Z., Ronald, P.C., and Harrington, S.E. 1994, Genetics, 138, 1251.
7. McCouch, S. 2000, Proc. Natl. Acad. Sci., 95, 1983.
8. Tyagi, A.K., and Mohanty, A. 2000, Plant Sci., 158, 1.
9. Christou, P. 1997, Plant Mol. Biol., 35, 197.
10. Goff, S.A., Ricke, D., Lan, T-H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P., and Varma, H. 2002, Science, 296, 92.
11. Yu, J., Hu, S., Wang, J., Wong, G.K.-S., Li, S., Liu, B., Deng, Y., Dai, L., Zhou, Y., and Zhang, X. 2002, Science, 296, 79.
12. Sasaki, T., and Burr, B. 2000, *Curr.* Opin. Plant Biol., 3, 138.
13. Yuan, Q., Ouyang, S., Liu, J., Suh, B., Cheung, F., Sultana, R., Lee, D., Quackenbush, J., and Buell, C.R. 2003, 31, 229.
14. Miyao, A., Tanaka, K., Murata, K., Sawaki, H., Takeda, S., Abe, K., Shinozuka, Y., Onosato, K., and Hirochika, H. 2003, Plant Cell, 15, 1771.
15. Sallaud, C., Meynard, D., van Boxtel, J., Gay, C., Bès, M., Brizard, J.P., Larmande, P., Ortega, D., Raynal, M., and Portefaix, M. 2003, Theor. Appl. Genet., 106, 1396.
16. Sallaud, C., Gay, C., Larmande, P., Bès, M., Piffanelli, P., Piégu, B., Droc, G., Regad, F., Bourgeois, E., and Meynard, D. 2004, Plant J., 39, 450.
17. Miki, D., and Shimamoto, K. 2004, Plant Cell Physiol., 45, 490.
18. Verdaguer, B., De Kochko, A., Fux, C.I., Beachy, R.N., and Fauquet, C. 1998, Plant Mol. Biol., 37, 1055.
19. Verdaguer, B., De Kochko, A. and Beachy, R.N. 1996, Plant Mol. Biol., 31, 1129.
20. Gleba, Y., Marillonnet, S., and Klimyuk, V. 2004, Curr. Opin. Plant Biol., 7, 182.
21. Scholthof, H.B., Scholthof, K-B.G., and Jackson, A.O. 1996, Ann. Rev. Phytopathol., 34, 299.
22. Ahlquist, P., French, R., and Bujarski, J.J. 1987, Adv. Virus Res., 32, 215.

23. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. 1994, Science, 263, 802.
24. Chiu, W-L., Niwa ,Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J. 1996, Curr. Biol., 6, 325.
25. Haseloff , J., and Amos, B. 1995, Trends Genet., 11, 328.
26. Sheen, J., Hwang, S., Niwa, Y., Kobayashi, H., and Galbraith, D.W. 1995, Plant J., 8, 777.
27. Baulcombe, D.C., Chapman, S., and Cruz, S. 1995, Plant J., 7, 1045.
28. Yusibov, V., Shivprasad, S., Turpen, T.H., Dawson, W., and Koprowski, H. 1999, Curr. Top. Microbiol. Immunol., 240, 81.
29. Pogue, G.P. 2002, Ann. Rev. Phytopathol., 40, 45.
30. Callaway, A., Giesman-Cookmeyer, D., Gillock, E.T., Sit, T.L., and Lommel, S.A. 2001, 39, 419.
31. Hwang, D., Roberts, I.M., and Wilson, T.M.A. 1994, Proc. Nat. Acad. Sci., 91, 9067.
32. Leffel, S.M., Mabon, S.A., and Stewart, C.N. 1997, BioTechniques, 23, 912.
33. Takken, F.L.W., Luderer, R., Gabriels, S.H.E.J., Westerink, N., Lu, R., de Wit, P.J.G.M., and Joosten, M.H.A.J. 2000, Plant J., 24, 275.
34. Lu, R., Martin-Hernandez, A.M., Peart, J.R., Malcuit, I., and Baulcombe, D.C. Methods, 30, 296.
35. Baulcombe, D.C. 1999, Curr. Opin. Plant Biol., 2, 109.
36. Ruiz, M.T., Voinnet, O., and Baulcombe, D.C. 1998, Plant Cell, 10, 937.
37. Robertson, D. 2004, Ann. Rev. Plant Biol., 55, 495.
38. Burton, R.A., Gibeaut, D.M., Bacic, A., Findlay, K., Roberts, K., Hamilton, A., Baulcombe, D.C.,and Fincher, G.B. 2000, Plant Cell, 12, 691.
39. Johnson, J. 1997, Ann. Rev. Phytopathol., 35, 67.
40. Sugiyama, Y., Hamamoto, H., Takemoto, S., Watanabe, Y., and Okada, Y. 1995, FEBS Lett., 359, 247.
41. Wu, L., Jiang, L., Zhou, Z., Fan, J., Zhang, Q., Zhu, H., Han, Q., and Xu, Z. 2003, Vaccine, 21, 4390.
42. Scholthof, H.B. 1999, J. Virol., 73, 7823.
43. Ratcliff, F., Martin-Hernandez, A.M., and Baulcombe, D.C. 2001, Plant J., 25, 237.
44. Wu, L., Fan, J., Jiang, L., Wang, H., Song, R., Zhang, Q., Zhu, H., Li, N., Liu, Z., and Xu, Z. 2003, J. Virol. Meth., 111, 101.
45. Thomas, C.L., Jones L., Baulcombe, D.C., and Maule, A.J. 2001, Plant J., 25, 417.
46. Voinnet, O., Pinto, Y.M., and Baulcombe, D.C. 1999, Proc. Natl. Acad. Sci., 96, 14147.
47. Ratcliff, F.G., MacFarlane, S.A., and Baulcombe, D.C. 1999, Plant Cell, 11, 1207.
48. Holzberg, S., Brosio, P., Gross, C., and Pogue, G.P. 2002, Plant J., 30, 315.
49. Attere, A.F., and Fatokun, C.A. 1983, Plant Dis., 67, 420.
50. Abo, M.E., Sy, A.A., Alegbjo, M.D. 1998, J. Sust. Agri., 11, 85.
51. Bakker, W. 1974, Characterization and ecological aspects of Rice yellow mottle virus in Kenya. [Doctorat], Wageningen.
52. Fargette, D., Pinel, A., Abubakar, Z., Traore, O., Brugidou, C., Fatogoma, S., Hebrard, E., Choisy, M., Sere, Y., and Fauquet, C. 2004, J. Virol., 78, 3252.
53. Fargette, D., Pinel, A., Halimi, H., Brugidou, C., Fauquet, C., and Van Regenmortel, M. 2002, Arch. Virol., 147, 5863.

54. Brugidou, C., Holt, C., Yassi, M.N., Beachy, R.N., and Fauquet, C. 1995, Virology, 206, 108.
55. Fargette, D., Pinel, A., Abubakar, Z., Traore, O., Brugidou, C., Fatogoma, S., Hebrard, E., Choisy, M., Sere, Y. and Fauquet, C. 2004, J. Virol., 78, 3252.
56. Kouassi, N., Brugidou, C., Chen, L., Ngon, A. Yassi, M., Beachy, R.N., and Fauquet, C.M. 1997, Intern. Rice Res. Notes, 14.
57. Bonneau, C. 1998, Etude cytologique et moléculaire du mouvement du virus de la panachure jaune du riz (RYMV), Orsay, Université Paris XI.
58. Mallory, A.C., Parks, G., Endres, M.W., Baulcombe, D.C., Bowman, L.H., Pruss, G.J., and Vance, V.B. 2002, Nature Biotechnol., 20, 622.
59. Susi, P., Hohkuri, M., Wahlroos, T., and Kilby, N.J. 2004, Plant Mol. Biol., 54, 157.
60. Cogoni, C., and Macino, G. 2000, Curr. Opin. Genet. Develop., 10, 638.
61. Aravin, A.A., Klenov, M.S., Vagin, V.V., Rozovskii, Y.M., and Gvozdev, V.A. 2002, Mol. Biol., 36, 180.
62. Carthew, R.W. 2001, Curr. Opin. Cell Biol., 13, 244.
63. Hammond, S.M., Caudy, A.A., and Hannon, G.J. 2001, Nature Rev. Genet., 2, 110.
64. Wang, M.-B., and Waterhouse, P.M. 2000, Plant Mol. Biol., 43, 67.
65. Han, Y., Griffiths, A., Li, H., and Grierson, D. 2004, FEBS Lett., 563, 123.
66. Hamilton, A., Voinnet, O., Chappell, L., and Baulcombe, D. 2002, EMBO J., 21, 4671.
67. Elbashir, S.M., Lendeckel, W., and Tuschl, T. 2001, Genes Develop., 15, 188.
68. Hammond, S.M., Bernstein, E., Beach, and D., Hannon, G.J. 2000, Nature, 404, 293.
69. Matzke, M.A., Matzke, A.J.M., Pruss, G.J., and Vance, V.B. 2001, Curr. Opin. Genet. Develop., 11, 221.
70. Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C., and Voinnet, O. 2003, EMBO J., 22, 4523.
71. Waterhouse, P.M., Wang, M.-B.,and Finnegan, E.J. 2001, Trends Plant Sci., 6, 297.
72. Mlotshwa, S., Voinnet, O., Mette, M.F., Matzke, M., Vaucheret, H., Ding, S.W., Pruss, G., and Vance, V.B. 2002, Plant Cell, 14, S289.
73. Lecellier, C.-H., and Voinnet, O. 2003, Immunol. Rev., 198, 285.
74. Ding, S.-W., Li, H., Lu, R., Li, F., and Li, W.-X. 2004, Virus Res., 102, 109.
75. Kasschau, K.D., and Carrington, J.C. 1998, Cell, 95, 461.
76. Voinnet, O. 2001, Trends Genet., 17, 449.
77. Moissiard, G., and Voinnet, O. 2004, Mol. Plant Pathol., 5, 71.
78. Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Mallory, A.C., Smith, T.H., and Vance, V.B. 1998, *Proceedings of the National Academy of Sciences* 1998, **95**:13079-13084.
79. Brigneti, G., Voinnet, O., Li, W.-X., Ji, L.-H., Ding, S.-W., and Baulcombe, D.C. 1998, EMBO J., 17, 6739.
80. Roth, B.M., Pruss, G.J., and Vance, V.B. 2004, Virus Res., 102, 97.
81. Lu, R., Folimonov, A., Shintaku, M., Li, W.-X., Falk, B.W., Dawson, W.O., and Ding, S.-W. 2004, Proc. Natl. Acad. Sci., 101, 15742.
82. Voinnet, O., Lederer, C., and Baulcombe, D.C. 2000, Cell, 103, 157.
83. Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. 2003, Plant J., 33, 949.

84. Bonneau, C., Brugidou, C., Chen, L., Beachy, R.N., and Fauquet, C. 1998, Virology, 244, 79.
85. Daniell, H., Streatfield, S.J., and Wycoff, K. 2001, Trends Plant Sci., 6, 219.
86. Twyman, R.M., Stoger, E., Schillberg, S., Christou, P., Fischer, R. 2003, Trends Biotechnol., 21, 570.
87. Desjeux, P. 1996, Clin. Dermatol., 14, 417.
88. Sundar, S. 2001, Trop. Med. Int. Health, 6, 849.
89. Lira, R., Sundar, S., Makharia, A., Kenney, R., Gam, A., Saraiva, E., and Sacks, D. 1999, J. Infect. Dis., 180, 564.
90. Sundar, S., More, D.K., Singh, M.K., Singh, V.P., Sharma, S., Makharia, A., Kumar, P.C., and Murray, H.W. 2000, Clin. Infect. Dis., 31, 1104.
91. Singh, N., Singh, R.T., and Sundar, S. 2003, J. Infect. Dis., 188, 600.
92. Merlen, T., Sereno, D., Brajon, N., Rostand, F., and Lemesre, J.L. 1999, Am. J. Trop. Med. Hyg., 60, 41.
93. Lemesre, J.L. 1993, IRD-Patent FR N° 9305779.
94. Lemesre, J.L., 1994, IRD-Patent PCT/FR N°94/00577.
95. Lemesre, J.L., Holzmuller, P., Cavaleyra, M., Bras Gonçalves, R., Hottin, G., and Papierok, G. Vaccine, in press.
96. Handman, E. 2001, 14, 229.
97. Sjolander, A., Baldwin, T.M., Curtis, J.M., Bengtsson, K.L., and Handman, E. 1998, Vaccine, 16, 2077.
98. Stöger, E., Vaquero, C., Torres, E., Sack, M., Nicholson, L., Drossard J., Williams, S., Keen, D., Perrin, Y., and Christou, P. 2000, Plant Mol. Biol., 42, 583.
99. Horn, M.E., Woodard, S.L., and Howard, J.A. 2004, Plant Cell Rep., 22, 711.
100. Vaquero, C., Sack, M., Schuster, F., Finnern, R., Drossard, J., Schumann, D., Reimann, A., and Fischer, R. 2002, FASEB J., 16, 408.
101. Hood, E.E., Witcher, D.R., Maddock, S., Meyer, T., Baszczynski, C., Bailey, M., Flynn, P., Register, J., Marshall, L., and Bond, D. 1997, Mol. Breeding, 3, 291.
102. Zhong, G.-Y., Peterson, D., Delaney, D.E., Bailey, M., Witcher, D.R., Register III, J.C., Bond, D., Li, C.-P., Marshall, L., and Kulisek, E. 1999, Mol. Breeding, 5, 345.
103. Witcher, D.R., Hood, E.E., Peterson, D., Bailey, M., Bond, D., Kusnadi, A., Evangelista, R., Nikolov, Z., Wooge, C., and Mehigh, R. 1998, Mol. Breeding, 4, 301.
104. Fischer, R., Vaquero-Martin, C., Markus, S., Drossard, J., Emans, N., and Commandeur, U. 1999, Biotech. Appl. Biochem., 30, 113.
105. Krebitz, M., Wagner, B., Ferreira, F., Peterbauer, C., Campillo, N., Witty, M., Kolarich, D., Steinkellner, H., Scheiner, O., and Breiteneder, H. 2003, J. Biology, 329, 721.
106. Kapila, J., De Rycke, R., Van Montagu, M., and Angenon, G. 1997, Plant Sci., 122, 101.
107. Wagner, B., Fuchs, H., Adhami, F., Ma, Y., Scheiner, O., and Breiteneder, H. 2004, Methods, 32, 227.
108. Shivprasad, S., Pogue, G.P., Lewandowski, D.J., Hidalgo, J., Donson, J., Grill, L.K., and Dawson,W.O. 1999, Virology, 255, 312.
109. Ratcliff, F., Harrison, B.D., and Baulcombe, D.C. 1997, Science, 276, 1558.
110. Al-Kaff, N.S., Covey, S.N., Kreike, M.M., Page, A.M., Pinder, R., and Dale, P.J. 1998, Science, 279, 2113.

111. Fischer, R., Emans, N., Schuster, F., Hellwig, S., and Drossard, J. 1999, Biotechnol. Appl. Biochem., 30, 109.
112. Doran, P.M. 2000, Curr. Opin. Biotechnol., 11, 199.
113. Matsumoto, S., Ikura, K., Ueda, M., and Sasaki, R. 1995, Plant Mol. Biol., 27, 1163.
114. Faye, L., Landry, N., Lerouge, P., Gomord, V., and Vézina, L.-P. 2001, Médecine/Sciences, 17, 867.
115. Wright, K.E., Prior, F., Sardana, R., Altosaar, I., Dudani, A.K., Ganz, P.R., and Tackaberry, E.S. 2001, Transgenic Research, 10, 177.
116. Parmenter, D.L., Boothe, J.G., van Rooijen, G.J., Yeung, E.C., and Moloney, M.M. 1995, Plant Mol. Biol., 29, 1167.
117. Fischer, R., Drossard, J., Emans, N., Commandeur, U., and Hellwig, S. 1999, Biotechnol. Appl. Biochem., 30, 117.
118. Magnuson, N.S., Linzmaier, P.M., Reeves, R., An, G., HayGlass, K., and Lee, J.M. 1998, Prot. Expres. Purif., 13, 45.
119. Sijmons, P.C., Dekker, B.M., Schrammeijer, B., Verwoerd, T.C., van den Elzen, P.J., and Hoekema, A. 1990, Biotechnol., 8, 217.
120. Darain, F.B.C., and Shim, Y.B. 2004, Biosensors Bioelectronics, 20, 857.
121. Feng, G.W.M. 1995, Biotechniques, 19, 956.
122. Li, H.-Y., and Chye, M.-L. 2004, Plant Mol. Biol., 54, 233.
123. Liu, Z.-Z., Wang, J.-L., Huang, X., Xu ,W.-H., Liu, Z.-M., and Fang, R.X. 2003, Planta, 216, 824.
124. Cabanes-Macheteau, M., Fitchette-Laine, A., Loutelier-Bourhis, C., Lange, C., Vine, N., Ma, J., Lerouge, P., and Faye, L. 1999, Glycobiology, 9, 365.
125. Wenderoth, I., and von Schaewen, A. 2000, Plant Physiol., 123, 1097.
126. Stanley, P. 1989, Mol. Cell. Biol., 9, 377.

Siré C., Piron F., Bangratz Reyser Martine, Lemesre Jean-Loup, Brugidou Christophe (2006)

Rice and virus biotechnology : application for the expression of an anti-*leishmania* vaccine

In : Franche Claudine (ed.). *Molecular biology of tropical plants*

Kerala : Research Signpost, p. 17-39

ISBN 81-308-0138-8