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6.2

Cassava viruses and genetic engineering

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Abstract

Cassava is affected by a number of viruses, of which the African cassava mosaic virus (ACMV) is the most damaging in Africa, and the cassava common mosaic virus (CCMV) in South America. In 1986, a new application of genetic engineering, coat protein (CP) mediated resistance, was demonstrated as an efficient way of controlling plant virus diseases. A joint programme entitled the International Cassava-Trans Project (ICTP) and being implemented jointly by the Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM) and Washington University aims to apply this technique to cassava in order to reduce the impact of virus infection on cassava production. Because these viruses also infect *Nicotiana benthamiana*, this plant is used as a model for establishing the best molecular strategy for driving resistance against these two viruses. Gene constructions, including those containing ACMV and CCMV CP coding sequences, have been made and transgenic *N. benthamiana* lines obtained. These lines are under investigation for CP expression and resistance to the corresponding virus; preliminary results of these studies are presented. In order to test the gene constructs, transient assays, with a marker gene, have been established to demonstrate the ability of cassava cells to express these constructs. A procedure for the regeneration of cassava plants from somatic embryos has been optimized and the first results of transformation with the particle gun and *Agrobacterium tumefaciens* are presented.

The objective of the International Cassava-Trans Project (ICTP) is to produce by genetic engineering cassava plants resistant to two cassava viruses: the African cassava mosaic virus (ACMV) and the cassava common mosaic virus (CCMV). These viral diseases were chosen because of their economic importance in Africa and South America, respectively (Fauquet and Beachy 1989). The basis of the technique chosen is the integration of genes

encoding viral proteins in the plant genome, in order to reduce viral replication and thus to limit the impact of infection on plant production. The technique used here is the coat protein (CP) strategy, which consists of integrating the viral CP gene into the plant genome. This strategy was first applied to express a gene encoding tobacco mosaic virus (TMV) CP in tobacco; the transgenic tobacco plants obtained were resistant to TMV (Powell et al. 1986). Since 1986 the CP strategy has been successfully applied to control a number of different viruses in several plants (Beachy et al. 1990).

Among the different examples of resistance produced by genetic engineering, an important criterion in the majority of cases is a stable and high level of expression of the inserted gene. The level and pattern of gene expression is greatly dependent upon the chimaeric gene construct used, on the site of insertion of the foreign gene in the plant genome, and on the number of inserted genes. The two last factors are not under the control of the investigator.

On the other hand, it is possible to study the influence of each part of the chimaeric gene on the expression of the gene: the transcriptional promoter, the leader sequence of the messenger RNA, the coding region, the untranslated region, and the termination sequence. It is, therefore, possible to evaluate the role of each part of the construct by generating constructs that differ from each other in each region of the gene. Large numbers of transgenic plants are regenerated following transformation with different constructs and the plants are checked for the presence of the foreign gene, mRNA expression, and CP accumulation prior to challenging the progeny of the transformant with the virus.

In the ICTP, the most significant difficulty is cassava regeneration and transformation. Consequently, indirect methods are necessary to optimize the gene constructs, and to study their expression. In our case, this can be done with *Nicotiana benthamiana* because it is a host for both cassava viruses and it can be readily transformed and regenerated. Furthermore, we can carry out transient assays with the particle gun. The *N. benthamiana* model can tell us if the chosen strategy is efficient for controlling these viruses, and the transient assays can tell us which gene construct is functional and which promoter has the highest level of expression in cassava cells.

Logistics of the International Cassava-Trans Project

The ICPT is a result of cooperation between the Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM) and Washington University, St Louis, USA. It aims to coordinate the institutes and agencies interested in the application of biotechnology to cassava, especially the development of viral resistance in cassava. ICPT researchers are interacting with European and American laboratories working on different aspects that relate to the project, including virology, molecular biology, and regeneration of cassava.

The project is closely related to projects of the international institutes of the Consultative Group on International Agricultural Research (CGIAR) which have a mandate for cassava improvement, namely Centro Internacional de Agricultura Tropical (CIAT) and International Institute of Tropical Agriculture (IITA). The project is also related to national institutes or universities of several developing countries; for example, a young Ivorian has joined the ICPT team for 2 years. Recently, the United States Agency for International Development (USAID) and the Monsanto Company have provided support for a post-

doctoral African scientist, and it is anticipated that this researcher will collaborate with ICPT team in the future. The project receives funds and technical support from a num of different agencies, including ORSTOM, the Rockefeller Foundation, Deuts Geselleschaft für Technische Zusammenarbeit (GTZ), USAID, Monsanto Company, the Technical Centre for Agricultural and Rural Cooperation, the Netherlands (C (Fauquet and Beachy 1989).

The Tobacco Model with ACMV and CCMV

Detection of the coat proteins of ACMV and CCMV

One critical requirement in the proposed studies is the detection of CPs in transgenic pla The level of expression of CP can be very low (Beachy et al. 1990), and it is essential develop techniques that enable detection of these low levels.

Two methods are currently used, the enzyme-linked immunosorbent assay (ELIS technique and the western blot technique. For the ACMV, both have been used; the ELI technique can detect up to 10μg of virus per well, which corresponds to a level of expressi of 0.01% of total protein content in transgenic plants. The western blot technique can det as low as 1μg of capsid protein (0.002% of total protein content) but with a relatively hi background. For CCMV, the antibodies are much more efficient and the methods readily detect levels of virus and CP of 1 and 0.1μg (0.001 and 0.0002% of total prot content for ELISA and western blot techniques, respectively), without significant ba ground due to the plant proteins.

Constructs with ACMV and CCMV coat proteins

The gene constructs and vector should have two major characteristics: the ability integrate into the plant genome at a high efficiency; and a constitutive expression (that in all cell types throughout the life of the plant) in the transgenic plants, which can interf with the viral infection. The binary vector, which is derived from the Ti plasmid *Agrobacterium tumefaciens*, has been chosen because of its high efficiency of gene tran into the plant genome. Most such vectors can also be used with direct DNA delivery, s as with the particle gun or by direct DNA uptakes into protoplasts.

For eucaryotes, gene expression is the result of complex mechanisms comprising: 1) transcription in the nucleus; maturation of RNA transcripts and transportation to cytoplasm; translation of mRNA to produce a protein; and possible post-translati modification of the protein. Transcription is regulated by sequences called promoters There are many types of promoters, which are expressed under different conditions: currently use p35S, isolated from cauliflower mosaic virus; p35S is referred t constitutive because it is expressed in many types of cells throughout the growth of the pl It has been proven that leader sequences (upstream of the coding region) are implicate the translational phase of gene expression (Kozak 1988). With the aim of increasing level of gene expression, we have modified, by mutagenesis, the leader of the ACM gene in order to obtain the consensus sequence of the plant gene leaders.

Construction of chimaeric genes with the coat protein of ACMV

ACMV is a geminivirus with a genome composed of two molecules of single-stranded DNA of approximatively 2.7 kb (Bock and Harrison 1985). The sequence of the genome was elucidated in 1983 (Stanley and Gay 1983), and the CP open reading frame located on the positive strand of DNA A segment was isolated. Several constructs have been made using the CP coding region: with different promoters (p35S, pE35S, p35S+4xOCS); with two viral leader length sequences; with two different termination sequences; and in a sense and antisense orientation. Lastly, as noted above, a construct was also developed that included an improved translational consensus sequence.

Construction of chimaeric genes with the coat protein of CCMV

CCMV is a potexvirus, and its genome is composed of one molecule of single-stranded RNA of approximately 6.3 kb (Costa and Kitajima 1972). Partial cDNA cloning of the virus has been achieved, and the CP coding region located near the viral 3' end has been sequenced. The CP of CCMV has a molecular weight of 25 kd and comparisons of its amino acid sequence with six other potexviruses showed a 47-62% homology amongst them (Fauquet et al. 1990). In the case of CCMV, we developed constructs with only the enhanced promoter of the cauliflower mosaic virus (pE35S) (Kay et al. 1987), but we varied the viral leader sequences and the viral termination sequence in order to test their effect on the level of expression of the CP gene in transgenic tobacco plants.

Transgenic tobacco plants with ACMV and CCMV coat proteins

For each construct described above, *N. benthamiana* was transformed with *Agrobacterium* using the leaf-disc transformation technique (Horsch et al. 1985) and 20 independent lines of transformed tobacco plants were regenerated. The plasmid used for transformation contained one of the CP genes and also a gene conferring kanamycin resistance (*Npt*II gene). All the master plants regenerated were checked for the expression of the *Npt*II gene by ELISA, as well as for the expression of the CP genes of ACMV and CCMV. Because the *Npt*II gene and the CP gene are positioned very close to each other on the plasmid vector, there is a high probability that they will both be integrated at the same site in the plant genome and consequently the expression of *Npt*II gene can be used as a marker gene for selecting transgenic plants harbouring the CP genes. This is also expressed in the F_1 (and succeeding) generation; most of the plants contain one copy of the genes and are segregated in a Mendelian ratio (of 1:3:1). Assuming that the expression of the *Npt*II gene is strongly correlated to the expression of the viral gene, the plants that are saved for F_2 seed production are selected from among the highest expressors of *Npt*II. In most cases, this technique leads to plants homozygous for the viral CP and the *Npt*II gene.

Expression of coat protein genes of ACMV and CCMV in tobacco

The presence of the CP genes of ACMV and CCMV in the plant genome is verified by extracting the DNA from transgenic plants and hybridizing this DNA with DNA complementary to the coding region of the genes. The number of inserted genes can be evaluated by the number of bands and/or the intensity of the hybridizing bands compared with a known amount of DNA. Most of the transgenic tobacco plants had one gene or at least one site of integration of several genes. The transgenic plants are then checked for level of mRNA expressed from the CP gene with a similar hybridization technique but using poly-adenylated RNA instead of DNA. The ACMV transgenic *N. benthamiana* plants tested thus far have very low amounts of mRNA corresponding to the ACMV CP gene, while the CCMV plants have higher levels of mRNA. The final assay is to determine the amount of CP accumulated in the transgenic plants by western immunoblotting. As predicted from the mRNA analysis, the amount of CP produced by the ACMV gene is very low; the maximum concentration registered so far is 0.01% of the total soluble protein. The amount of CCMV CP in the transgenic tobaccos is extremely high in nearly all the transgenic plants, reaching as much as 4% of the total soluble protein. The ELISA technique has also been used for rapid evaluation of protein levels on a large number of plants.

Resistance of transgenic tobacco plants to ACMV and CCMV

After the production of F_2 transgenic tobacco plants, which are normally homozygous for the CP gene, the transgenic plants were challenged with the viruses to evaluate their level of resistance. In the case of ACMV, a limited number of F_1 plants have been tested, for a limited number of constructs; some of them showed a trend of resistance. This resistance was expressed as a short delay (7 days maximum) in the expression of infection in plants and/or a lower percentage of infected plants (for example, 30% instead of 90%). These encouraging preliminary results need to be confirmed in the F_2 generation to be sure that the CP strategy will control ACMV. A larger number of F_1 lines expressing the CCMV CP gene have been tested, and a few F_2 lines have also been tested. Most of these lines showed some degree of resistance and some were apparently highly resistant, nearly immune. In the case of the best resistant transgenic line, the percentage of infected plants was between 0 and 5% at 50 days after inoculation, using an inoculum concentration of CCMV of 1 µg/mL. This concentration of CCMV normally kills *N. benthamiana* in 30 days. The few plants that are infected developed very mild symptoms compared to the control plants, that is, a mosaic or a few necrotic spots instead of complete necrosis.

Transient Gene Expression Assays with Cassava

Principle and choice of promoters

Transient assays allow a rapid evaluation of gene constructs without the inconvenience of transformation and regeneration. Furthermore, such assays are important when transformation has not been achieved, as is the case with cassava. The principle is very simple: a reporter gene under the control of a chosen promoter is introduced into a cell, there is no integration of the gene into the plant genome, and expression of the gene is measured after a short period of time (24-48 hours). The reporter gene that we chose was the ß-glucuronidase (*Gus*) gene, and it was introduced into epidermal and mesophyll cells of intact leaflets of cassava with the help of a particle gun.

Cassava belongs to the Euphorbiaceae family and until now the expression of a foreign gene has not been studied in a member of this family. The first task was to determine if the available promoters were active in such a plant and, if so, whether it was possible to boost this expression by altering the promoter. Although p35S seems to be a very ubiquitous promoter in general, it needed to be tested in an euphorbiaceous host before being used for cassava transformation. As mentioned above, several promoters or modifications of the p35S promoter were tested, including a regulatory element of the promoter of the octopine synthase gene of *Agrobacterium* that was shown to enhance 200-fold the activity of heterologous promoters (Ellis et al. 1987). The promoter isolated from the ubiquitin gene of *Arabidopsis* species was also tested.

Optimization of the particle gun

The first particle gun was described in 1987 (Klein et al. 1987). The principle is as follows: DNA is coated onto tungsten particles 1.2 µm in diameter, which are then accelerated so that they can penetrate several layers of cells. Once in the cell, the DNA is freed and introduced genes are expressed. After optimization of our air-powered particle gun, 100-500 cassava cells expressed the *Gus* gene in each experiment (Franche et al. 1990).

Activity of *Gus* gene constructs in cassava leaves

Beta-glucuronidase is an enzyme that reacts with 5-bromo-4-fluoro-3-indolyl glucuronid to produce a blue precipitate, and the intensity of the reaction is correlated with the expression of the gene, and reflects the transcriptional efficiency of the promoter. We have demonstrated that all the promoters tested are active in cassava cells; p35S is slightly more active than the ubiquitin promoter, and pE35S is slightly more active than p35S+4OCS, and four to five times more active than p35S. This activity was evaluated by the intensity of blue spots on the cassava leaves, and by the activity of enzyme extracted from the leaves and assayed spectrophotometrically (Bogusz et al. 1990; Franche et al. 1990).

Regeneration of Cassava

The ICTP's objective of producing virus-resistant transgenic cassava plants can be achieved only if we are able to regenerate transformed cassava plants. In previous studies, many tissues of the cassava plant have been tested for regeneration but apparently only young leaves, meristems, and cotyledons are able to produce embryos that can be later regenerated into plants (Stamp 1984; Stamp and Henshaw 1986). This technique has been confirmed at CIAT with several cultivars which have been cultivated in the field (Szabados et al. 1987). Nevertheless, the technique remains difficult and is restricted to a few South American cultivars. The number of plants regenerated is generally very low, compared with the number of initial explants taken to produce embryos. We have been able to reproduce these results with two cassava cultivars from Colombia, MCol 22 and MCol 1505 (Schöpke et al. 1990). A third cultivar from Colombia, CMC76, produces many embryos, which can be used to produce secondary and tertiary embryos. This culture grows rapidly and is very

convenient for transformation experiments, but the regeneration is unfortunately very difficult. Somatic embryogenesis is restricted to the tissue along the veins of very young leaflets of cassava (2-5 mm long) and there is a tendency for callus cells to overgrow the embryogenic cells. Finally, cassava embryos grow very slowly in culture and at least 8 weeks are needed to complete one experiment.

Transformation of Cassava

Transformation by *Agrobacterium*

Transformation by *Agrobacterium* requires a wound, but if there is a wound the explant has a tendency to produce callus tissue that will not regenerate into a plant. We nevertheless decided to transform callus tissue, in order to prove that we can transform cassava cells and detect the expression of the CPs. To do this, we used the leaf-disc transformation technique commonly used for tobacco transformation (discussed earlier), and selected transgenic cells on 100 µg/mL of kanamycin. The transformed cells were selected through several cycles of growth onto kanamycin and checked for their expression. We integrated the work with *Gus* expression and totally blue calluses have been obtained, showing that the transformation and the selection were effective. The same experiments with the CPs of ACMV and CCMV are currently in progress and the transformation will be confirmed by northern and western blotting analyses.

Transformation by particle gun

Different cassava tissues have been shot with DNA via the particle gun to study the ability of cassava tissues to express the *Gus* reporter gene. Transient expression of this gene has been demonstrated in young leaflets, in leaf embryogenic tissues, and in somatic embryos. The expression of the *Gus* gene has also been observed in different cell types, including the epiderm, the mesophyll, and associated with the phloem cells. These experiments showed the potential of the method and the ability of these different cell types to express a foreign gene and to be transformed. Stable transformation of embryogenic tissue has also been observed 4 weeks after gene introduction, and 2 weeks after selection on kanamycin. Clumps of several hundreds of transformed cells suggest that the transformation occurred and that multiplication of these cells also occurred, but this needs to be proven (Schöpke et al. 1990).

Conclusion

Although the ICTP has not yet produced cassava plants resistant to ACMV and CCMV, it has nevertheless thrown light on many previously unanswered questions. A very strong resistance to CCMV has been achieved in many different lines of *N. benthamiana*, and lines tolerant to ACMV have been obtained, showing that the CP strategy can be effective for resistance to cassava viruses, at least in tobacco. The weak resistance against ACMV can be related to the low mRNA and CP content in the transgenic plants, and further studies of

mRNA stability will be needed to improve the level of resistance. It is not certain that a result in a tobacco model can be extrapolated to cassava, but we currently have no alternative. As yet, there is no known example where resistance achieved in a tobacco has not been confirmed or improved in the natural host of the virus under consideration. We have also demonstrated that cassava is able to express each of the gene constructs that we have produced and that the promoters used are efficient in different types of cassava cells. This could be a very important fact because ACMV is naturally transmitted by whiteflies, which inject the virus into cells associated with phloem cells, prior to the invasion of other cell types after the first cycles of viral replication. Cassava callus cells can be transformed and selected, and we anticipate that transformed calluses will express the CP genes of ACMV and CCMV. Lastly, we have been able to regenerate cassava plants from somatic embryogenetic tissues in a limited number of cassava cultivars, and we are currently using this route of regeneration to transform cassava by *Agrobacterium* and the particle gun. It appears that all the pieces of the puzzle have been gathered and that the production of genetically engineered virus-resistant cassava plants is now a matter of assembling the puzzle.

Discussion

QUESTIONS: Are the tomato plants growing in the field resistant to some antibiotics? What is the amount, expressed as % of total protein, of the TMV? What kind of promoters were used to express the TMV gene, and is it possible to drive the expression of these genes only when requested and in the right organ?

FAUQUET: The first generations of transgenic plants were resistant to kanamycin, but the next generations will use herbicide-resistant genes. With regard to the second question, the amount of CP expressed in transgenic plants depends on the nature of the virus, the plant, and the generated line. It can range from 0.001% to 4% of the total amount of protein. The correlation between the CP expression and the resistance obtained is not clear. It seems to be linear in the case of tobamoviruses, and not related in the case of potyviruses. In answer to the third question, so far we are using only p35S from CaMV or modified versions of that promoter. But other promoters, tissue specific or inducible, are under study.

HAMILTON: With regard to the 54 kd protein described by Dr Zaitlin, might conserved sequence in their protein be used to transform tobacco for resistance to several other tobamoviruses?

FAUQUET: I don't know if there is a conserved region in the 54 kd protein of TMV which can be used for transforming plants. But these transgenic plants were resistant to a strain which in fact was obtained by mutation from the original strain used, and not at all resistant for the most closely related tobamoviruses.

MURDOCK: Are you confident that coat protein gene-mediated resistance, once deployed over wide areas, will be durable — that is, not break down?

FAUQUET: Yes, for two reasons. First, plant viruses are very stable in time and space and it seems difficult to break resistance, even vertical resistance. Second, experiments in the laboratory proved that cycles of virus infection through transgenic plants have been unable to break this coat protein-mediated resistance. This resistance is, in most cases,

a monogenic resistance, which is easy to transfer to other varieties but may be easi break. Even in that event, it is easy to come back to the original coat protein, modif and produce a new transformant. Furthermore, it has been demonstrated that spectrum of specificity with a virus group is fairly wide; viruses having at least 60' homology with this coat protein are protected, leaving room for a lot of mutation

ROBERTSON: Please do not regard this as a hostile question. Could you explain the pre status of the patents for the coat protein approach and of the genes involved?

FAUQUET: A patent has been deposited in the USA by Roger Beachy from Washing University and Monsanto Company, covering the whole concept of coat prot mediated resistance. If accepted, this patent will cover all the transgenic plants using coat protein strategy. But at least in the case of cassava viruses, we have got a writ agreement from Monsanto allowing us to freely use this patent. Such an agreem might be extended to other examples for the developing countries.

SINGH: Monsanto Company has been working on transgenic varieties for several years. you showed beautiful pictures of transgenic tomato and tobacco varieties show significant differences. Some of these date back to 1987. Have some of these transge varieties been released for general cultivation? If not, can you please enlighten us on procedures and problems involved?

FAUQUET: Only field trials for research purposes have been done and Monsanto forecasting that it will release transgenic plants by 1993-95, provided that rul regulations and patents about transgenic plants are in place.

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