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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

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

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

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.

References

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