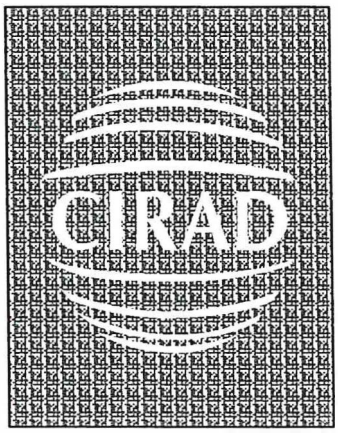
Cirad Ca
UPR Systèmes cotonniers en petit paysannat



Evogene Mission Report

2 - 9 april 2005

C. Pannetier

Report on the Evogene Mission from 2 to 9 April 2005

The mission was performed within the framework of Project 1 of the Evogene-Cirad framework contract that currently consists of two projects:
- Project 1: "Training and communication of know-how on cotton genetic engineering by CIRAD to Evogene by way of technology transfer and training in genetic transformation of cotton"
- Project 2: "Evaluation of natural germplasm provided by CIRAD to Evogene".

I should like to thank the following persons for their welcome, their availability and the quality of discussions: Rafi Meissner, Scientific Director; Laura Beckerman, Head of the Genetic Transformation Project; Haigai Karchi, Head of the Abiotic Stress Tolerance Project; Ofer Haviv, Director of Evogene; Julien Meissonnier, Business Development; together with all the members of Evogene with whom I had scientific or 'cultural and tourism' contacts.

1. The ABST project

Fresh results have been obtained by Evogene within the framework of the ABST project. In particular, the first F1 lines given by the crossing of a resistant T3 line of Micro-Tom with a commercial variety were tested on 180mM NaCl. The illustrations that I saw showed that the 'transgenic' F1 displayed distinctly increased root development, vegetative development and fruit production than the control. The results were obtained with Gene 36 under the control of Evogene promoter 6669. This construction has been introduced in cotton since February 2005. Until then, the p35S-GusIntron construction was practically the only one used to calibrate the production of transgenic cotton under Evogene laboratory conditions using the CIRAD protocol provided to Naama Zelinger (the former head of the transformation laboratory) during her training in Versailles in April 2004.

2. Progress of the setting up of regeneration of transformed cotton plants

2.1. Using material produced during the training course in Versailles.

The material brought from Versailles (transformed explants, transformed callus and control callus) gave the results expected (transformed calli from explants, embryogenic tissues from transformed or controls calli). Embryo production has remained small.
The observations that I was able to make appear to show that the culture room conditions were not optimum, at least during the initial stages of the project (low temperature, no humidity control, etc.). Furthermore, embryogenic tissue was not handled properly (no selection of interesting tissue at each subculture). During the mission, three plantlets were successfully transferred to the greenhouse. Grafting trials were also conducted. When the scion has grown sufficiently (1 or 2 young leaves), the graft takes successfully. I also explained again to Laura Beckerman what the best culture conditions are for obtaining plantlets: strong gas exchanges, light provided by GroLux fluorencent tubes, the possibility of using vermiculite as a culture substrate in tubes and watering with liquid culture medium.

2.2. Setting up the procedure at Evogene

Difficulties were encountered in obtaining transformed callus from hypocotyl fragments: dense greencallus not particularly suitable for obtaining embryogenic tissue (compact green callus that I was not able to see). It seems that these difficulties result mainly from the quality of the seeds used to ontain under aseptic conditions the that form the initial material, and also the conditions for the obtaining of these seedlings (too high intensity of light). Rafi Meissner had decided to work on cotyledon explants and apply the culture conditions recommended by an Indian team that had supplied them with grains said to possess high embryogenic potential (Dr Pental's laboratory). However, the quantitative results obtained from hypocotyl explants seem satisfactory (Table 1).

2.2.1. The problem of seed germination

Seed germination is very poor and the embryo must be excised to obtain a plantlet *in vitro*. Furthermore, germination is conducted at 25°C and in normal light (more than 40microEinsteincm-1). The medium used is not that recommended by CIRAD.

I brought a few seeds back to Versailles where they displayed 100% germination after a normal length of time and under our disinfection and germination conditions. The protocol proposed by CIRAD should therefore be applied with precision with regard to disinfection and germination medium.

Culture temperature does not play an essential role in the germination stage. In contrast, it is important for the seedlings to develop under low light conditions (see protocol) to obtain good quality calli.

2.2.2. Obtaining transformed callus

I was not able to judge the quality of the calli produced from hypocotyl as all the cultures since September 2004 have been performed with cotyledon fragments from seedlings grown in light.

The number of calli obtained from cotyledon explants is satisfactory but their appearance did not seem very favourable as it was soft and grey. Few calli were beige and thick, like those formed from hypocotyl explants (see illustrations of the CIRAD protocol).

Recommendations were given for the selection and subculturing of this callus.

2.2.3. Obtaining embryogenic strains

A large number of embryogenic lines have been obtained but I was unable to draw up a complete balance of the experiments in terms of the percentage of explants giving embryogenic strains as the results had not yet all been entered in the database. Data provided on the experiments performed with hypocotyl explants and the strain Agro C58:pGC2260 pBinGusIntron give the results shown in Table 1.

Table 1. Example of the production of embryogenic lines from hypocotyl explant

Trial	Transformation date	Number of explants	Number of calli that gave embryogenic strains *
36 TrV11	12/04/2004	37	19
37 TrV12	17/04/2004	180	52
38 Naama1	26/04/2004	130	17
6	06/07/2004	127	44
7	07/07/2004	120	6
15	03/08/2004	86	16
16	10/08/2004	176	20
17	16/07/2004	197	24
18	25/08/2004	84	10
23	07/09/2004	225	7
29	04/10/2004	70	4

* Several strain isolation operations may have been performed on the same callus but it is not possible to know whether they are different transformation events.
Trials performed in Versailles in bold type

It can be concluded that although the obtaining of embryogenic strains from hypocotyl explants is variable from one trial to another, it is satisfactory on average.

The most important observation concerns the appearance of these embryogenic lines : they were very soft and their colour is often markedly brownish grey.
The problem may result from the culture medium used and in particular the quality of the gelling agent (Phytagel from Sigma and not Gelrite from Duchefa) as the cultures were too soft. In addition, the medium contained glucose at 2% and not sucrose at 3%; this forms a significant difference as regards carbon sources but the effect here could be essentially du to difference in osmotic pressure.
A key parameter is that these cultures are subcultured with no selection made of the most favourable zones: pale, friable zones.
Subcultures were performed during the mission and the appearance of the resulting cultures has improved considerably (information sent by e-mail by Laura Beckerman after our return).
I recommended that the pH of the culture medium should be monitored. A tiny difference in pH can cause considerable differences in the texture of the medium, especially when the latter is sterilised in an autoclave at a high temperature (120°C for 25 min).
After the mission, the media are currently dried for a while under a laminar flow hood before use. This practice has also improved the appearance of the cultures.

I brought back different control and transformed embryogenic cultures. After treatment as performed in Versailles (medium, type of subculture), the cultures now have excellent appearance and a satisfactory proliferation rate and embryos are beginning to develop. Photographs have been sent to Laura Bekerman.

The appearance of the Evogene embryogenic cultures leads to supposing that they were not truly transformed (poor development on the selective agent). For this reason, we checked using a Gus test that the potentially transformed cultures expressed the *Gus* gene. All the 9 cultures tested expressed the *Gus* gene. Evogene was informed of the results.

3. In short

- Ensure the quality of the culture media: composition and hardness of solid media. Return as much as possible to the culture media recommended in the protocol document and in particular eliminate growth substances as soon as embryogenic tissue has been isolated.
- Do not use media that are too 'fresh'. Dry them under the lamina flow hood first.
- Put much attention to the subculturing of embryogenic cultures by selecting the parts whose development and appearance are satisfactory.
- Use sucrose for the isolation and culture of embryogenic strains.
- Perform a comparative test of the medium used and 1) using Duchefa gelrite, 2) adding $MgCl_2$ to Sigma Phytagel and 3) using plant agar.
- Try—with caution as there is no moisture control—to allow as much as possible gazeous exchanges in the culture recipients for the development of embryos and plantlets.
- Use vermiculite substrate (soaked regularly with liquid culture medium) to enhance the development of plantlets that seem to display a blockage in either the apical or the root part.
- If plantlet development is blocked, graft the apical part (1 or 2 very young leaves) on a hypocotyl.

- Transfer the plantlets to a greenhouse when root development is sufficient, conserving a certain confinement to be removed gradually after several days.

4. Material supplied

- OCI-Gus seeds for the fibre project calibration of Gus assay)
- 0065 AF1seeds for the production of transformed embryogenic strains of cotton
- *Agrobacterium* strain C58:pGV2260

5. Continuation of collaboration

Ofer Karchi expressed his desire to continue the partnership of Evogene and CIRAD on the project on resistance to abiotic stresses. Project 2 will continue as per the terms defined in the framework agreement.
Funding is currently sought for Project 3 through Eureka projects. Evogene received a negative response (for various reasons) to its request for Israeli funding. However, Julien Meissonnier is to do the necessary to gain the Eureka label with own funding. This will enable CIRAD to continue its approaches to the Ministry of Research for the obtaining of funding.
When this report was written, Julien Meissonnier had met the Israeli body but we have not yet received a reply. For CIRAD, a meeting took place at the Ministry delegated to Research with Mr Richard-Molard (Assistant Director of the Bio-Engineering Department at the Technology directorate) and Mr Vicaire, a member of his staff. The presentation of the CIRAD project was well received and Mr Richard-Molard is providing support on the basis of the quality and importance of the project.
The question of label thus remains on the Israeli side and that of the type of funding on the French side (intervention by the Agence Nationale de la Recherche, ANR?).

The possibility of developing Evogene - Bayer - CIRAD three-cornered collaboration was discussed at the meeting with Ofer Haviv, Director of Evogene.
Marnix Peferoen (BayerCropScience), met by persons from Evogene at the last Beltwide Conferences, is reported to have desired such collaboration. Ofer Haviv would like this organisation to be set up and asked me to discuss the point with Bernard Hau and the managers of the Cotton Research Unit.