Transformation of Sunflower : an Overview. Different Approaches, their Difficulties, How to Overcome Them, and First Results.

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Abstract

Sunflower has long resisted efforts directed to genetic transformation. The encountered problems must be seen, for a large part, in connection with the difficulties posed by plant regeneration from *in vitro* cultured explants. We have conducted an extensive study of different gene transfer methods and their combination with a variety of regeneration systems. Most combinations were unsuccessful, and we shall present some of the reasons for this. This research program has resulted in establishing a pratically useful technique for sunflower transformation. The efficiency of this method depends, among other factors, on an exact timing of its different steps. We shall describe the chain of events occuring in the explants, as their understanding will render the method more transparent and more easy to transfer.

Since a pratically usefull transformation system has not been publicly available until recently, not much information exists characterising transgenic sunflower plants. Results shall be presented concerning diverse genetic constructs, ranging from model constructs to those with biological implications. We have introduced heterologous genes into sunflower coding for enzymes implicated in different steps of the biochemical pathway leading to fatty acids, and some corresponding promoters have been evaluated for their suitability.

Key-words

Transformation - shoot regeneration - *Agrobacterium* - polyethylene glycol - electroporation - particle gun

Introduction

In vitro culture and genetic transformation have become routine approaches to plant improvement and to plant development studies in many Monocotyledonous and Dicotyledonous crops. Despite the fact that the production of transgenic sunflower has been described (Everett *et al.* 1987, Schrammeijer *et al.* 1990, Bidney *et al.* 1992, Espinasse-Gellner 1992, Knittel *et al.* 1994, Malone-Schoeberg *et al.* 1994), a reliable, efficient and universally applicable protocol has not yet been published. Some key factors involved in the process of regenerating fertile plants from transgenic cells are difficult to master in this crop. Which factors precisely are at the origin of this recalcitrancy?

Successful genetic transformation of a plant species involves several phases, *i.e.* the introduction of the foreign gene into the cells, the selection of transgenic cells, and the regeneration of a fertile plant from them.

Gene transfer methods can be performed through several methods either directly or indirectly. *Agrobacterium tumefaciens* is the most commonly used of the indirect methods but it is strongly limited by the nature of the interactions between the plant cells and the bacteria: it requires the plant to be a host and the tissue to be properly wounded to receive the bacteria. Direct gene transfer methods are numerous, ranging from electroporation to particle bombarment.

Substantial progress in sunflower tissue cultures has been made over the last years. A number of regeneration protocols have been described which allow regeneration of plants from explants such as hypocotyls, cotyledons, immature embryos, as well as from protoplasts. Most systems occurre without a long callus phase and prolonged culture in the non-differenciated state appears to be incompatible with plant regeneration.

Production of a transgenic plant requires subtile but efficient combination of a reliable regeneration method with a gene transfer technique appropriate for the cell type used in the regeneration method. We have evaluated different combinations of transformation and regeneration. We have identified the most limiting factors and those that might be responsible for the low transformation efficiency in sunflower. This work led to a transformation protocol now routinely in use in our laboratory.

Results :

In this work, similar genetic constructs were introduced by different methods, in order to compare them, in terms of production of transformed cells and, eventually, plants.

Direct gene transfer to protoplasts

Protoplasts of sunflower can be isolated in high numbers from several tissues, *i.e.* leaves, cotyledons, and hypocotyls. Their culture is easy till the callus stage. Plant regeneration, however, only occured in few cases (Burrus *et al.* 1991, Fischer *et al.* 1992, Krasnyansky and Menczel 1993), and it is restricted to specific but unrelated genotypes, identified through different screening schemes. In the case of genotype 47302bcd, shoot induction occured in a small sub-population of calli, thus severelly reducing the overall efficiency of regeneration to a low but constant level (0.05% of the originally plated protoplasts in our experimental conditions). Optimization was tempted but it was surprising that variations of key media components had little effects on the regeneration efficiency of the genotype.

In order to test direct gene transfer efficiency in sunflower hypocotyl protoplasts, we introduced the pCG35S plasmid containing the uidA gene under control of the 35S promoter and the Nos terminator (Lepetit *et al.* 1991). The presence of a 2-fold excess of carrier DNA increased the enzyme activity by a factor of 2, either in electroporation or PEG experiments. PEG-mediated DNA transfer was more efficient than electroporation, due to severe protoplast damages caused by the electrical field.

Stable integration of the transgene into sunflower protoplasts has been shown (Moyne *et al.* 1989). In our conditions, when cultured, the protoplasts surviving the transformation treatment proceeded through the first divisions like untreated protoplasts. Regeneration of transgenic plants should be possible by this approach. However, this has never been demonstrated because of the very low efficiency of both transformation and regeneration : 0.05% for protoplast culture and 0.01-0.1% for stable integration events (Moyne *et al.* 1989). Furthermore, the calli from genotype 47302bcd loose their regeneration ability very quickly in culture, thus preventing the possibility of selecting for transgenic colonies.

We are now working to increase the efficiency of plant regeneration to a level which allows the recovery of rare events by studying the cytological characteristics of competent cells and by establishing genetic fingerprints correlated to the regeneration potential.

Particle bombardment of cotyledons and immature embryos

Particle bombardment has proven to be highly efficient for the production of transgenic plants from a number of species which cannot be transformed otherwise, or which are not amenable to protoplast isolation, or to injure tissues in order to prepare them for *Agrobacterium* infection. For transgenic plant recovery, it is important that particles reach the meristems or the cells competent for regeneration. The cell type in which microprojectiles are delivered is determined by physical and technical shooting parameters, whereas the cell type competent for regeneration is under genetical or physiological control.

Therefore, we studied both physical (particle types, acceleration conditions, target distance) and physiological (nature of the explants, age of the explants, pre-bombardment treatments, culture conditions) parameters with the pCG 35S plasmid (Lepetit *et al.* 1991). Two different gun designs were also tested: the

gunpowder driven device (Zumbrunn *et al.* 1989) and the helium particle inflow gun (Finer *et al.* 1992). Because, for everyday use, the gunpowder driven model was less convenient and required more particles than the second model for comparable efficiencies, we used the helium device for routine experiments, but most of the results described have been verified with both guns.

Proximal halves of cotyledons of young seedlings put on BAP containing medium form multiple shoots near the petiole area without any callus phase (Knittel *et al.* 1991, Chraibi *et al.* 1992). Plant regeneration occurres for several genotypes, and it is highest in cotyledons isolated from young seedlings, below 7 days.

In all experimental conditions tested, particle bombardment of cotyledons was inefficient: almost all the tungsten particles were stopped by the thick cuticle, a few gold particles reached the epidermis, almost none the palisadic parenchyma, independantly the shooting conditions. Transient expression was low (on average 1 to 8 GUS positive spots in maximum 27% of the explants) and always restricted to the epidermis. No improvement could be achieved. Furthermore, most epidermis cells harboring a tungsten particle were dead, as shown by microscopical observation. The most likely explanation for the low efficiency is that particle delivery without excessive cell damage is difficult in sunflower. No stable transformants were obtained and this approach was then abandoned.

Immature zygotic embryos are a suitable material for regeneration (Finer 1987, Espinasse *et al.* 1989, Jeannin *et al.* 1991).

We used a variety of regeneration protocols, different stages of embryo development and several bombardment conditions.

Best transient expression and multiple shoot production were obtained with the smallest embryos (1.5 mm large or less) precultured for 3 days on a MS medium with NAA and BAP. As for mature cotyledons, dead epidermis cells containing one or more microprojectiles could be observed. Most blue spots were located several cell layers deep, between the epidermis and the fourth mesophyll layer. Particle bombardment did not modify further organogenesis into multiple shoots and GUS expression could still be detected 4 weeks after bombardment. But, even under selective pressure, the expression level constantly decreased compared to the level detected 3 days after bombardment and most spots had not developed any larger. It could indicate that no active cell division had occured in these cells or that no stable integration had occurred. To date, no transgenic plant could be recovered.

Cotyledons of 2-3 mm immature embryos and halves of larger embryos gave high transient expression but were not efficient for plant regeneration.

Since GUS expression levels were not sufficient to obtain transformed sunflower, we tested another uidA gene construct with the stress-inducible promoter of the str 246 C gene and the nos terminator (pPH105) (Godiard *et al.* 1991). Immature embryos were bombarded, analysed 2 and 4 weeks after bombardment and the expression compared to the pCG35S (table 1). Higher expression was detected with plasmid pPH105 than with plasmid pCG35S: tissue culture and bombardment are stressful conditions for the explants, thus yielding larger amounts of the GUS protein. This difference was even more important over long periods of culture.

Agrobacterium infection of cotyledons and immature embryos

Seedling cotyledons are not easily transformed by *Agrobacterium tumefaciens* under standard culture conditions optimized for regeneration (Knittel *et al.* 1991): few GUS positive spots were observed after 7 days of culture. While searching to improve the transformation efficiency, we showed that interaction of the bacteria with the tissue is strongly influenced by the coculture conditions, mostly the hormones. Best results were obtained with MS or SH medium without hormones, but, in these conditions, no shoot regeneration occurred. Thus, efficient regeneration was incompatible with efficient transformation, and *vice-versa*. A compromise was found, giving acceptable transformation and shoot regeneration. GUS positive chimeric shoots were then recovered but at a very low frequency . The histological study of regeneration indicated that shoot formation occurres mostly in the subepidermal cell layers, whereas transformation occurres almost exclusively in the perivascular tissues. These two regions do not often

overlap, thus giving rise to chimaeric shoots only rarely. The potential to recover transformed shoots was so low that this approach had to be abandoned for routine uses.

This work on cotyledons is a good exemple of the complexity of genetic transformation : cells that are transformed are not necessarily competent for regeneration.

Agrobacterium infection of embryonic axes

The apical meristems of embryonic axes are easily excised from imbibed seeds, as shown by Schrammeijer *et al.* (1990) and Bidney *et al.* (1992). These meristems form shoots naturally, even when wounded or split into two halves. This capacity may be increased by an appropriate hormonal environment which will induce the development of the axillary shoots.

After *Agrobacterium* infection, the developing shoots are often chimaeras, because of the high number of meristematic cells involved in their formation. These shoots, however, may exhibit positive axillary buds, whose development can be induced by further subculture onto selective medium. For this purpose, kanamycin is appropriate: untransformed sectors turn white, while resistant sectors remain green. The kanamycin concentration has to be low, in order not to kill the untransformed sectors who are important for shoot growth. Transgenic sectors are mostly located on stems or on leaves, but, in 5% of the explants, apical meristems of the main shoots were GUS positive. These meristems are expected to produce transgenic offspring. Indeed, Malone-schoneberg *et al.* (1994), Knittel *et al.* (1994), through related approaches, were able to recover transgenic seeds of different genotypes. In our hands (Rousselin *et al.* 1995), transgenic sunflower plants expressing the uidA and kan genes with either single copy and simple GUS expression pattern or higher copy number and complex integration patterns were obtained.

The efficiency of this technique depends on experimental details, such as the genotypes, the physiological state of the explants (preculture, hormonal environment), the coculture conditions, the wounding of the explants (Bidney *et al.* 1992).

Agrobacterium transformation of embryonic axes is labour-intensive but, to date, it is the only technique available which permits to use genetic engineering with sunflower.

Conclusion

All described approaches to sunflower transformation have their own merits and difficulties.

Our experiments with different gene transfer methods have shown that transformation of sunflower is not problematic, but rather efficient. Direct gene transfer into protoplasts and particle gun bombardment of immature embryos are good means for studying transient expression. Stable integration of the introduced DNA is also possible.

It is now clear that difficulties in regenerating transgenic sunflower plants reside in the fact that regeneration is mostly direct and affects only a small proportion of the cells in the explants. These few cells have to be transformed. Regeneration through a callus phase is rare and too inefficient to be exploited for the selection of the few transformed cells. Furthermore, the selection procedure may seriously alter the regeneration. The difficulty to find a gene transfer technique that specifically reaches cells competent for regeneration may explain the failure to produce easily transgenic sunflower.

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Table 1 : Bombardment of immature zygotic embryos by the particle inflow gun : comparison of two plamid constructs

Gus assay after # days	Plasmid	# bombarded embryos	% embryos with blue spots	GUS expressing embryos related to pCG35S (%)
3	pCG35S	79	90.2	-
	pPH105	68	97.0	107.5
14	pCG35S	68	53.6	-
	pPH105	66	78.8	147.0
28	pCG35S	69	24	-
	pPH105	79	45	187.5