TRANSIENT EXPRESSION OF *UID*A GENE IN GRAPEVINE PROTOPLASTS AFTER PEG-MEDIATED TRANSFORMATION

EXPRESSION TRANSITOIRE DU GÈNE *UID*A DANS LES PROTOPLASTES DE VIGNE APRÈS TRANSFORMATION AU PEG

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Abstract: Leaf protoplasts, isolated from *Vitis vinifera* «Sakasly» and «Muscat d'Alexandrie» and protoplasts from embryogenic tissue of *Vitis* sp. «Seyval blanc» were incubated in the presence of PEG in a transformation solution containing the plasmid pBI426 which carries the β-glucuronidase (*gus*) and the neomycin phosphotransferase II (*npt*II) genes. The treated protoplasts were cultivated in CPW13 medium without kanamycin selection. 48 h after the PEG transformation, transient expression of gus gene was detected histochemically and fluorimetrically in the protoplast cultures.

Key words: *Vitis vinifera*, β-glucuronidase, PEG transformation, transient expression.

Mots clés: *Vitis vinifera*, β-glucuronidase, transformation au PEG, expression transitoire.

INTRODUCTION

Protoplasts may play an important role in genetic improvement of grapevine, e.g. via somatic hybridization and direct gene transfer. However, reports on genetic transformation of grape protoplasts are still lacking. Transformation of protoplasts from other plant species could be demonstrated using electroporation or PEG mediated transfer (AHMED *et al.*, 1997; DAMM and WILLMITZER, 1991; SALA *et al.*, 1989). The latter technique is rapid and does not require any special expertise or equipment. The objective of this study is to describe, for the first time, the success of transient expression of *gus* gene in grapevine protoplasts transformed with polyethylene glycol.

MATERIAL AND METHODS

I - PLANT MATERIAL AND PROTOPLAST ISOLATION

1) Leaves

Leaves for protoplast isolation derived from vitroplants obtained through apical meristem cultivation from two economically important Tunisian local grape varieties Sakasly and Muscat (*Vitis vinifera*). Protoplasts were isolated from 4-5 week old vitroplants as decribed by REUSTLE and ALLEWELDT (1990).

2) Embryogenic material

Protoplasts were isolated from clusters of somatic embryos of « Seyval blanc », obtained through leaf disk cultivation (HARST, 1995), according to the method described by REUSTLE *et al.* (1995).

II - PROTOPLAST TRANSFORMATION

Purified protoplasts were resuspended to a density of $4x10^6$ cell/ml in the transformation buffer (0.4 M mannitol, 0.1 M MES, $CaCl_2$ or $MgCl_2$ at 0.02 M, 0.075 M or 0.15 M, pH 5.6). Circular plasmid pBI426[1] (7.5Kb) carrying the neomycin phosphotransferase II (*npt*II) and β-glucuronidase (*gus*) as selection and reporter genes were used for the transformation experiments. The calf thymus DNA carrier (GIBCO-BRL) was used to enhance the transfer. The polyethylene glycol ($PEG_{3.350}$) mediated DNA transfer was performed as follows: Aliquots of 250 µl of protoplast suspension (at $4x10^6$ cell/ml) were mixed with 10 to 40 µl of carrier DNA (1 µg/µl in distilled water) and 10 to 40 µl of pBI426 plasmid DNA (1 µg/µl in distilled water). To this mixture, 250 µl of 40 p. cent $PEG_{3.350}$ (filter sterilized and dissolved in transformation buffer) were

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added and the tube was then inverted carefully for several times. After 20 min incubation at room temperature, the transformation mixture was progressively diluted with W5 washing solution every ten minutes until a final volume of 2 ml. The diluted mixtures were centrifuged at 80 g for 4 min and the resulting pellets of protoplasts were resuspended in 0.6 M mannitol solution. The treated protoplasts were cultivated, in the dark at 24 °C, with and without immobilization in sodium alginate gels (REUSTLE *et al.*, 1995) in CPW13 medium (FREARSON *et al.*, 1973 modified).

III - TRANSIENT B-GLUCURONIDASE EXPRESSION ANALYSIS

After 48 h, protoplasts from liquid cultures were centrifuged for 5 seconds at 12,000 rpm and the pellets resuspended in 300 µl of *gus* extraction buffer (50 mM sodium-phosphate buffer; 10 mM Na-EDTA; 10 mM mercaptoethanol; 0.1 p. cent triton X-100). The mixture was vortexed two times, frozen, thawed and finally centrifuged at 1,200 rpm for 5 min. The supernatants were carefully poured off and stored in the freezer for measuring enzyme activity. Protein content of each sample was estimated using the Biorad protein assay Kit (BRADFORD, 1976). For testing the *gus* activity, 50 µl of each extract was mixed with 150 µl of 2 mM ß-glucuronide dissolved in 50 mM of sodium phosphate buffer (on ice) and then incubated at room temperature. The reaction was stopped by transferring 50 µl of the reaction mixture into 1.9 ml of stop buffer (0.2 M $NaCO_3$) at several incubation times (0 to 30 min). Enzyme activity was assessed by fluorometric estimation of 4-MU per µg of protein per minute (VersaFluor, Biorad Fluorometer).

The histochemical *gus* assay (JEFFERSSON *et al.*, 1987) was performed on the protoplasts immobilized in sodium alginate layers. The incubation lasted 2 hours only.

RESULTS AND DISCUSSION

PEG mediated transfer of DNA into protoplasts was reported to be influenced by several factors, such as the DNA concentration, incubation time, the DNA carrier and the plasmid (AHMED *et al.*, 1997; NEGRUTIU *et al.*, 1987; SALA *et al.*, 1989). By variation of some of these factors, a protocol for grapevine protoplast genetic transformation could be established.

I - CONCENTRATION OF DNA

The use of 0.04 µg/µl of carrier DNA and 0.1 µg/µl of circular plasmid DNA gave the best rates of GUS expressing protoplasts (figure 1). KARESH *et al.* (1991) showed the significant effect of the carrier DNA on the transformation frequency. However, they did not find an increase of either absolute transformation frequency or copy number of integrated sequences with various amounts of linearised DNA (5-30 µg/7x10^5 protoplasts). In this case, amounts of 0.02 to 0.1 µg of circular DNA per 10^6 protoplasts were used. In fact, NEGRUTIU *et al.* (1987) demonstrated that the circular plasmid was more efficient for protoplast transformation.

II - TRANSFORMATION BUFFER

For both varieties Sakasly and Muscat, fluorimetric *gus* assays showed best transient expression rates with 0.15 mM $CaCl_2$ or $MgCl_2$, without any preference. However, Mg cations were reported to be more efficient than Ca cations in the transformation of protoplasts from *Nicotiana tabacum* and *N. plumbiginifolia* (NEGRUTIU *et al.*, 1987), and from *Triticum aestivum* (AHMED *et al.*, 1997). Uptake of DNA by *Arabidopsis thaliana* protoplasts was also possible by Ca^{2+} and PEG combination (DAMM and WILLMITZER, 1991). These heterogeneous results showed that the efficiency of transformation via the combination of PEG and salt may depend on the spe-

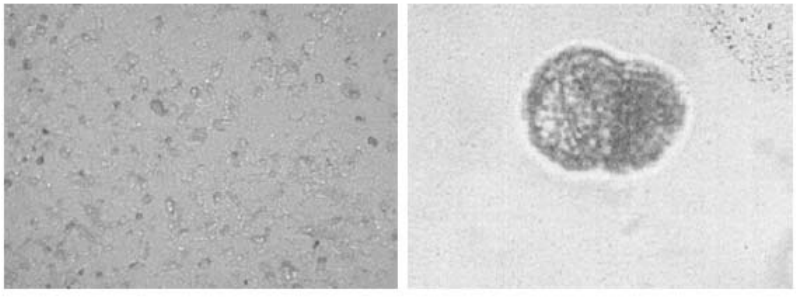

Fig. 1 - *gus* expression in grapevine protoplasts.
Left: transient *gus* expression in grapevine (*Vitis* sp. Sakasly) leaf protoplasts three days after transformation.
Right: first cell division of an *gus* expressing grapevine protoplast originating from embryogenic tissue of Seyval blanc.

Figure 1 - Expression *gus* dans des protoplastes de vigne.
Gauche: expression transitoire du gène *gus* dans les protoplastes de feuille de la variété Sakasly trois jours après la transformation.
Droite: première division d'un protoplaste *gus* positif à partir de tissus embryogène de la variété.

cies. This could be related to the sensitivity of the plasmic membrane for the DNA uptake. With grapevine material, a strong dependence of the transient *gus* expression efficiency on the protoplast source was found. Indeed, protoplasts isolated from leaves of young, vigorous vitroplants revealed to be more competent for PEG-mediated transformation than those deriving from embryogenic tissue (probably due to an incomplete cell wall digestion that prevents the DNA transfer).

II - PROTOPLAST CULTURE

Number of *gus* expressing leaf protoplasts decreased with the time of cultivation. Four weeks after culture initiation, only few protoplasts still showed *gus* expression. Furthermore, we could not get an efficient *gus* positive leaf protoplast division. Continued cultivation of the transformed protoplasts finally resulted in the death of all protoplasts. In contrast, even with the low initial number of GUS positive protoplasts derived from embryogenic tissue we found blue cells being in the stage of first cell division, in the second to third week of cultivation (figure 1).

CONCLUSION

Results of this study show that we could develop a system for transient grapevine leaf protoplast transformation via PEG treatment. Regeneration of grapevine protoplasts is, however, low as well as the transformation competence of protoplasts from embryogenic tissue. Due to these difficulties we are still not able to use the protoplast system as a tool for developing transgenic grapevines. With the described transformation protocol, grapevine protoplasts may be used as a test system for gene expression.

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