Electronic Journal of Biotechnology ISSN: 0717-3458 Vol.11 No.5, Special Issue, 2008

DOI: 10.2225/vol11-issue5-fulltext-2 SHORT COMMUNICATION

Introduction of antifungal genes in sunflower via *Agrobacterium*

Laura Mabel Radonic
Instituto de Biotecnología
INTA Castelar
De los Reseros y Nicolás Repetto s/n (1712), Castelar
Buenos Aires, Argentina
Tel: 54 11 4621 1278/1447
Fax: 54 11 4621 0199
E-mail: lradonic@cnia.inta.gov.ar

Julián Marcos Zimmermann[#]
Instituto de Biotecnología
INTA Castelar
De los Reseros y Nicolás Repetto s/n (1712), Castelar
Buenos Aires, Argentina
Tel: 54 3878 494492
Fax: 54 3878 493190
E-mail: jzimmerman@cnia.inta.gov.ar

Diego Zavallo
Instituto de Biotecnología
INTA Castelar
De los Reseros y Nicolás Repetto s/n (1712), Castelar
Buenos Aires, Argentina
Tel: 54 11 4621 1278/1447
Fax: 54 11 4621 0199
E-mail: dzavallo@cnia.inta.gov.ar

Nilda López
Instituto de Biotecnología
INTA Castelar
De los Reseros y Nicolás Repetto s/n (1712), Castelar
Buenos Aires, Argentina
Tel: 54 11 4621 1278/1447
Fax: 54 11 4621 0199
E-mail: nelopez@cnia.inta.gov.ar

Marisa López Bilbao*
Instituto de Biotecnología
INTA Castelar
De los Reseros y Nicolás Repetto s/n (1712), Castelar
Buenos Aires, Argentina
Tel: 54 11 4621 1278/1447
Fax: 54 11 4621 0199
E-mail: mlopezbilbao@cnia.inta.gov.ar

Financial support: National Agency of Scientific and Technological Promotion (ANPCyT), PICTO Nº 08-13164 and 08-12925, Argentina.

Keywords: antifungal genes, double expression cassettes, polyprotein cassettes, sunflower, transgenic plants.

[#]**Present address:** Chacra Experimental Agrícola, PO box nº 8 (A4531WAA), Colonia Santa Rosa, Salta, Argentina. E-mail: jzimmermann@chacraexperimental.org.

Abbreviations: CTAB: Cetyltrimethylammonium bromide
DNA: deoxyribonucleic acid
Km: kanamycin
PCR: polymerase chain reaction
Rip: ribosome inhibitor protein
TEV: tobacco etch virus
TMV: tobacco mosaic virus

*Corresponding author

There is evidence that overexpression of transgenes codifying antifungal proteins may confer protection to pathogen attack, and that this protection is increased due to the synergic effect of the expression of two or more genes. On the other hand it is well known that sunflower is a recalcitrant specie, highly difficult to be genetically transformed. In this context, the final aim of this project was to obtain sunflower plants expressing at least two antifungal genes, avoiding sequential transformation. The antifungal genes used encode for two enzymes that degrade the fungal wall (glucanase and chitinase), an osmotin and a ribosome inhibitor protein. Two types of transformation vectors were used: a more traditional system with a double cassette and a novel system producing a unique polyprotein with antifungal proteins released in equimolecular quantities. The polyprotein vector system generated hyperhydric shoots with necrotic areas and abnormal growth at the end of the tissue culture procedure, making impossible the use of this interesting vector in sunflower. Transformation assays carried out with the pHGC39 vector (including glucanase and chitinase genes) vector produced 0.83% efficiency, corresponding to 13 rooted shoots in kanamycin (Km) from a total of 1568 agroinfected shoots. T0 rooted shoots resulted positive by PCR analysis and were transferred to greenhouse to obtain their offspring. In addition, we corroborate the transformation protocol using Km as selective marker, previously described (Radonic et al. 2006) with a reporter gene, but in this opportunity with antifungal genes.

Significant limitations to sunflower (Helianthus sp.) culture are fungal diseases, like those caused by *Verticillium dahliae* and *Sclerotinia sclerotiorum*, because they not only affect yield but also affect the quality of the products. Conventional genetic improvement has not been efficient to confer resistance, caused by the limited sources of effective natural resistance to main pathogens and also to the polygenic character of the resistance to pathogens like Sclerotinia.

There is evidence that the overexpression of transgenes codifying antifungal proteins may confer protection to pathogen attack, and this protection is increased due to the synergic effect of the expression of two or more genes (Punja, 2001). The expression of genes encoding enzymes capable of degrading the major constituents of fungal cell walls (chitin and β-1.3-glucan) have been used as a strategy, for example in tomato, that showed a useful level of resistance to a *Fusarium* wilt disease and in tobacco where the presence of two genes increases the resistance to *Rhizoctonia solani* both examples reviewed in Melchers and Stuiver (2000).

Despite several publications in sunflower genetic transformation (Hewezi et al. 2002; Weber et al. 2003), there was no efficient or reproducible protocol. In a previous publication (Radonic et al. 2006) we determined that the selection by *in vitro* rooting in a Km culture medium is a successful method as no escapes were obtained. Nevertheless some difficulties remain in sunflower transformation as the *in vitro* regeneration of plantlets (T0) is *via* organogenesis, producing chimeras that not necessarily will give an offspring (T1) that carries and/or express the transgene. Therefore, the application of sequential transformation steps to introduce several genes in sunflower is extremely difficult.

In this context, the final aim of this project is to obtain sunflower plants expressing at least two antifungal genes. For the incorporation of these genes, we used two types of transformation vectors, a traditional system with a double cassette and a novel system based on the ability of the NIa protease from the Tobacco Etch Virus (TEV) for autocleavage and to recognize specific cleavage sequences which produce an unique polyprotein producing equimolecular antifungal protein quantities. The antifungal

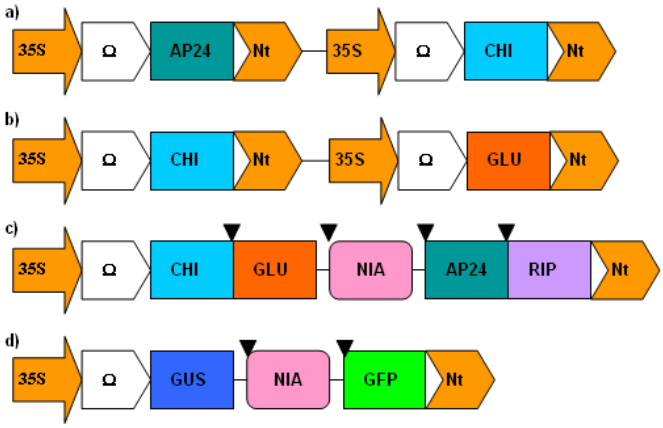

Figure 1. Constructions used for the transformation with antifungal genes. a) and b) Double expression cassettes containing AP24 and chitinase (CHI) genes or CHI and glucanase (GLU) genes respectively. c) Polyprotein construction containing genes of interest (CHI, GLU, NIA protease, AP24, and the Ribosome Inhibitor Protein (RIP). d) Polyprotein construction containing GUS and GFP reporter genes and NIA protease gene.

Figure 2. Transgenic shoots with the construction containing chitinase and glucanase genes (**Figure 1b**).

genes used codify for two enzymes that degrade the fungal wall (glucanase and chitinase), an osmotin and a ribosome inhibitor protein.

MATERIALS AND METHODS

Seeds from the public genotype HA89 were used as plant material. *Agrobacterium tumefaciens* EHA105 and 101 strains (Hood et al. 1993) were used. Transformation and tissue culture were performed as described previously in Radonic et al. (2006) with slight modifications in the selection method: 1 mg/l and 10 mg/l kanamycin were added in the first and second regeneration media, respectively.

The antifungal genes used were *gln2* (glucanase) from *N. tabacum*, *ch5B* (chitinase) from *P. vulgaris*, *ap24* (osmotin) from *N. tabacum* and *rip* (ribosome inhibitor protein). These genes were incorporated using two different types of vector:

1) Double expression cassette vectors: These vectors were kindly provided by L. Hernández (CIGB; Cuba) where genes are in pairs, each one with the *CaMV35S* promoter, the W enhancer from TMV and the nopaline sintase (*Nt*) terminator. These vectors are pHCA35, containing *ch5B* and *ap24* genes (Figure 1a) and pHGC39, containing *ch5B* and *gln2* genes (Figure 1b).

2) Polyprotein vector: This vector is based on the ability of the NIa protease from the Tobacco Etch Virus (TEV) for autocleavage and to recognize specific cleavage sequences. This vector contains the four antifungal genes which translate as a single polyprotein under the regulation of a single *CaMV35S* promoter and the W enhancer from Tobacco Mosaic Virus (TMV) and the *Nt* terminator. Each gene is preceded by the cleavage recognition sequence for the protease action (Figure 1c). As a control and model of this system a vector containing *gus* and *gfp* reporter genes was used (Figure 1d).

In addition, all these vectors contain the *nptII* (neomycin phosphotransferase) gene regulated by *nos* (nopaline sintase) promoter and *oct* (octopine sintase) terminator.

Genomic DNA extraction was performed using the CTAB method (Saghai-Maroof et al. 1984). PCR was performed for a fragment of *ch5B* gene using Invitrogen Taq polimerase (1 U), Promega Green Go Taq Buffer (1X) and 0.1 µM of each primer (35S f: 5'-CGCACAATCCCACTATCCTT-3' and chi r: 5'-GAGGGCGCTGAGATCAGTAG-3'). Negative controls: mix without the addition of DNA and another with DNA from a non-transformed plant. Positive control: pHGC39 plasmid DNA.

RESULTS AND DISCUSSION

Both polyprotein vectors, containing antifungal or reporter genes, were used to transform a total of 1524 shoots. All these shoots presented hyperhydric aspect, with necrotic areas and abnormal growth at the end of the culture procedure. Besides, and probably associated to this *in vitro* behavior, no shoots rooted in the selective media containing kanamicyn. The *in vitro* response described here was never observed in our lab previously. It must be mentioned that materials (media, seeds, Agrobacteria strains), conditions (growing chambers, laminar flows, vacuum bombs, etc.) and researchers and technicians were the same in all the assays done by our group, both with antifungal vectors described in this work as with other vectors carrying reporter genes or different selective genes. Therefore this hyperhydric aspect and abnormal *in vitro* growth could be related to the presence of the NIa gene. Virus diseases of cultivated sunflower (*Helianthus annuus* L.) or other wild species of the genus *Helianthus* are rare, and nine of the

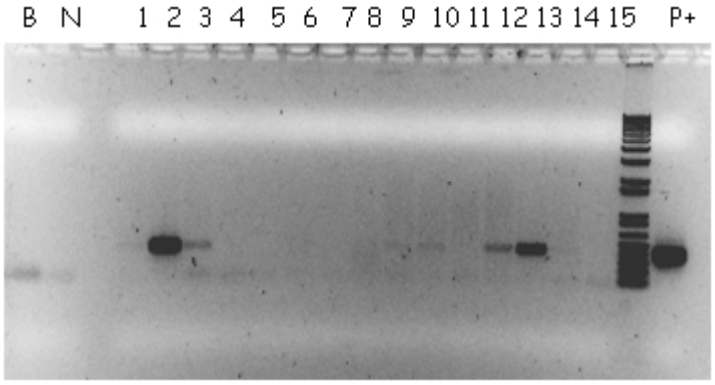

Figure 3. PCR performed for a fragment of chitinase gene. 1, 2, 3, 8, 9, 10, 11, 12 and 13: DNA from *in vitro* rooted shoots. 4, 5, 6, 7, 14 and 15: DNA from non-rooted shoots chosen randomly.
B: mix without the addition of DNA, N: DNA from non-transformed sunflower, P+: positive control pHGC39 plasmid DNA.

viruses reported to infect sunflower naturally or experimentally, belong to the family *Potyviridae* (Gulya et al. 2002). The NIa protease gene used in these constructions is from the TEV the *Potyviridae* most closely related to the Sunflower Mosaic Virus (SuMV) (Gulya et al. 2002). For that reason, sunflower tissues could be detecting the presence of this protease activity not only in stable transformed shoots but also in those shoots with a transient transformation expression. This tissue culture response described here resulted in the impossibility of using this interesting vector system in sunflower.

The double expression cassette vectors used in this work were successfully used in strawberry (Vellicce et al. 2006). When these double expression cassette vectors were used for sunflower transformation, we observed different results at the *in vitro* response level. In the case of the pHGC39 vector, the transformation efficiency obtained was 0.83 %, corresponding to 13 rooted shoots in Km of a total of 1568 agroinfected shoots (Figure 2a). PCR analysis from rooted shoots (T0), using primers *35S f* and *chi r*, produced the expected amplified fragment from the 35S promoter to the chitinase gene sequence (Figure 3). All these rooted shoots were transferred to greenhouse to obtain their offspring (Figure 2b). T1 and T2 are being molecularly characterized and they will allow us to analyze the response to pathogens.

In those transformation assays where pHCA35 double expression vector was used no rooted shoots were obtained of a total of 452 treated shoots. This high dependence of the transformation efficiency on the DNA sequence used is a known effect in other species transformation.

This work corroborates the efficiency of the transformation/selection method in presence of kanamycin where no escapes were obtained, since all shoots that were able to root *in vitro* in presence of the antibiotic carried the transgene.

It must be mentioned that in our laboratory we have found some examples of transgenic instability as some T1 plants which expressed the transgen then lost it at the following generation. This situation was described by McCabe et al. (1999) in lettuce, also a *Compositae* as sunflower, when using the viral promoter *CaMV35S*. In consequence, our next step in sunflower transformation will be to look for better promoters than the *CaMV35S* used in our assays until now.

ACKNOWLEDGMENTS

We wish to thank Mr. A. Montenegro and Ms. V. Peralta for their collaboration at the greenhouse.

REFERENCES

GULYA, T.J.; SHIEL, P.J.; FREEMAN, T.; JORDAN, R.L.; ISAKEIT, T. and BERGER, P.H. Host range and characterization of *Sunflower mosaic virus.* *Phytopathology,* 2002, vol. 92, p. 694-702.

HEWEZI, T.; PERRAULT, A.; ALIBERT, G. and KALLERHOFF, J. Dehydrating immature embryo split apices and rehydrating with *Agrobacterium tumefaciens*: A new method for genetically transforming recalcitrant sunflower. *Plant Molecular Biology Reporter*, December 2002, vol. 20, no. 4, p. 335-345.

HOOD, E.; GELVIN, S.B.; MELCHERS, L.S. and HOEKEMA, A. New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Research*, July 1993, vol. 2, no. 4, p. 208-218.

MCCABE, M.S.; SCHEPERS, F.; VAN DER AREND, A.; MOHAPATRA, U.; DE LAAT, A.M.M.; POWER, J.B. and DAVEY, M.R. Increased stable inheritance of herbicide resistance in transgenic lettuce carrying a petE promoter-bar gene compared with a CaMV 35S-bar gene.

Theoretical and Applied Genetics, 1999, vol. 99, no. 3-4, p. 587-592.

MELCHERS, L.S. and STUIVER, M.H. Novel genes for disease-resistance breeding. *Current Opinion in Plant Biology*, 2000, vol. 3, no. 2, p. 147-152.

PUNJA, Z.K. Genetic engineering of plants to enhance resistance to fungal pathogens-a review of progress and future prospects. *Canadian Journal of Plant Pathology*, 2001, vol. 23, no. 3, p. 216-235.

RADONIC, L.M.; ZIMMERMANN, J.M.; ZAVALLO, D.; LÓPEZ, N. and LÓPEZ BILBAO, M. Rooting in Km selective media as efficient *in vitro* selection method for sunflower genetic transformation. *Electronic Journal of Biotechnology*, April 2006, vol. 9, no. 3, p. 315-319.

SAGHAI-MAROOF, M.A.; SOLIMAN, K.M.; JORGENSEN, R.A. and ALLARD, R.W. Ribosomal DNA Spacer-Length Polymorphisms in Barley: Mendelian Inheritance, Chromosomal Location, and Population Dynamics. *Proceedings of the National Academy of Sciences of the United States of America,* December 1984, vol. 81, no. 24, p. 8014-8018.

VELLICCE, G.R.; DIAZ RICCI, J.C.; HERNÁNDEZ, L. and CASTAGNARO, A. Enhanced resistance to *Botrytis cinerea* mediated by the transgenic expression of the chitinase gene *ch5B* in strawberry. *Transgenic Research*, February 2006, vol. 15, no. 1, p. 57-68.

WEBER, S.; FRIEDT, W.; LANDES, N.; MOLINIER, J.; HIMBER, C.; ROUSSELIN, P.; HAHNE, G. and HORN, R. Improved *Agrobacterium* -mediated transformation of sunflower (*Helianthus annuus* L.): assessment of macerating enzymes and sonication. *Plant Cell Reports*, January 2003, vol. 21, no. 5, p. 475-482.