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***Commercial production of antibodies in cereals:
setting up the system***

*P. Galeffi, G. Petrighi Polidori, C. Rasi, A. Latini, G. Cavicchioni, E.
Palmieri, M. Sperandei and C. Cantale*

BIOTEC-GEN, ENEA CR Casaccia –, Via Anguillarese 301, 00060 Rome, Italy

running title: Plant factory and cereals

Abstract

Plant molecular farming is receiving more and more interest and is going to be considered an attractive system for producing “valuable recombinant pharmaceutical molecules” by plant-factory in industrial/agricultural scale. In particular, expression in cereals allows for rapid scale-up of production to accommodate large-volume users. Moreover, seed is an excellent storage vehicle for proteins maintaining long-term stability.

Among valuable recombinant proteins single chain variable fragment (scFv) represent a particularly promising class for biomedical applications. We have obtained an engineered antibody, scFv800E6, specific for the Herb 2 oncogene (strongly related to the human breast and ovary cancers) and its production in plant has been studied both in stable and transient systems. We are now interested to set up its production in cereals, namely in *Triticum aestivum*. Generally, cereal transformation has been carried out using biolistic procedure and just a few examples are reported concerning the use of *Agrobacterium tumefaciens* as vector for transferring the exogenous gene. In order to find the optimal starting conditions for accomplishing the cereal transformation by *Agrobacterium*, we explored the metabolic activities of different bacterial strains and the regenerative abilities of various *Triticum aestivum* and Triticale varieties. Preliminary results are presented.

Keywords: Single chain antibodies, plant-factory, *Agrobacterium tumefaciens*, cereal transformation

INTRODUCTION

Biopharmaceutical production is expected to rise considerably in the next future and concerns are growing about the deficit in manufacturing capacity. Plants ability to express xenogenic proteins has been largely investigated as a emerging producing system. Plants represent an exciting chance, rich of economic and qualitative benefits, able to join the known agriculture practice and simplicity to a safer system, in comparison with the conventional mammalian cell culture.

However, some challenges still face the production of therapeutic proteins in transgenic plants. These challenges include the level of product that is accumulated per unit biomass and the protein recovery and purification. Both of them contribute to the yield, defined as the final amount of purified recombinant protein recovered, a critical factor as regards marketing because of its impact on overall costs.

Estimates of the downstream processing costs of plant biopharmaceutical products are difficult because they are not yet produced on a large scale, but experience from plant commercial production of enzymes, such as avidin, can be used as a guide, even if therapeutic uses require more extensive purification to comply with regulatory requirements.

Evangelista et al., in 1998, showed that protein extraction and purification accounted for about 90% of the production costs and that the expression level was the most important factor in reducing unit production costs, an expression level as low as 0.015% being still profitable.

Various different elements influence the recombinant protein accumulation levels in transgenic plants. In general, it is assumed that crops that have a higher protein content would result in a higher accumulation of recombinant proteins, and consequently are more cost-effective, requiring less crop to be grown and harvested and less amount of biomass per unit weight of recombinant protein to be treated in extraction/purification processes -Kusnadi et al., 1997.

A large number of crops have been experimented with bioreactors. From the crop-handling viewpoint, one of the more attractive possibilities is the seed of the plant. As known, seeds and tubers represent the natural storage organs of the plant where the proteins are accumulated for long periods of time without degradation -Stoger et al., 2000. Furthermore, seed accumulation of xenogenic proteins allows an easy storage after harvesting simplifying its delivery toward the downstream processing facility, that can be also very far from the field. It has been demonstrated that transgenic cereal seeds are suitable for storage of potentially therapeutic macromolecules -Stoger et al., 2000. Our aim is to develop a commercial scale production system for a well characterized engineered antibody, scFv800E6, that is a good candidate for clinical applications. A protocol for cereal transformation has been set up using

Agrobacterium-mediated transformation. Different bacterial strains and cereal varieties have been tested to identify the optimal pair and preliminary transformation results are described.

MATERIALS AND METHODS

Agrobacterium strains

The following *Agrobacterium tumefaciens* strains were used: LBA4404 (containing pAL4404 plasmid), GV3101 (containing C58C1 plasmid), EHA101(At502) (containing pEHA101 plasmid), EHA105(At542) (containing pTiBo542 plasmid), C58Z707(At503) (containing pC58 plasmid), and At368(SE3111) (containing pTiB6S3 plasmid).

Cereal varieties

Three *Triticum aestivum* varieties have been tested: Bobwhite, Nadro and Lona and two Triticale varieties, Liron and Pollmer.

Plasmid

pBG-BIN-scFv α CTV -Galeffi et al., 2002- was used for engineering the *Agrobacterium* strain. Electroporation of the *Agrobacterium* competent cells was carried out according to Shen and Forde (1989).

Analyses of the metabolic capacities of the bacteria strains

The bacteria were grown on the opportune selective medium (LB plus specific antibiotics).

The Biolog MicrostationTM and the automatic identification system 4.0TM (Biolog Inc. California, USA) –Bochner, 1989- were used to analyse the metabolic pattern of the *Agrobacterium* strains, besides confirming that they belonged to the *Agrobacterium tumefaciens* species.

Bacterial cultures were freshly grown (overnight, 28°C) in BUGMTM (Biolog Universal Growth Medium) medium for 24 h. According to the Biolog procedure, a final concentration of 10⁸ cells ml⁻¹ was incubated at 28°C in GN-Biolog-microplates pre-filled with 95 different carbon sources.

Positive reactions in microplates were detected after 4, 24, 48 and 72 h by the oxidation of the tetrazolium dye (forming a purple colour read at a wavelength of 590 nm). The test was carried out in triplicate for each strain.

Callus regeneration from cereal embryos

Plants were grown in greenhouse for seed productions and embryos were extracted in sterile conditions. Immature embryos were extracted from seeds after 14-18 days from flowering in sterile conditions. Embryos were grown at 26 °C, in dark room for about two months in MSE3 medium (Sigma-Aldrich, USA) containing 2 mg/l 2,4-D (2,4-Dichlorofenoxyacetic acid) in order to obtain callus regeneration.

Calli transformation

Regenerated calli were co-cultivated with *Agrobacterium* strain at 25 °C for three days in dark room, adding 150 mg/l timentin and 150 mg/l cefotaxime. After a week, the survived calli were grown in presence of 100 mg/l kanamicin, 100 mg/l cefotaxime and 100 mg/l timentin for at least 2 months in a climatic chamber at 25 °C, using a 16-hour photoperiod, changing weekly the medium.

PCR analysis

DNA extracted from transformed calli was analysed by PCR analyses carried out using specific oligonucleotides accounting for a known fragment of the scFv800E6 transgene.

RESULTS AND DISCUSSION

Metabolic analyses of *Agrobacterium* strains

Even if the ability of *Agrobacterium* strains to transform the dicotyledones is known, some difficulties have been reported for the monocotyledon transformation. Although the various strains of *Agrobacterium* have the ability to introduce the exogenous DNA in the genome of a plant cell, both the *Agrobacterium* strain and the plant influence transformation efficiency -van Wordragen et al.,

1991. We are interested in determining the optimal working conditions with a view to mass production of transgenic cereal plants able to generate functional scFvs. Thus, an evaluation of the metabolic activity of the various strains as an indirect measure of their ability in plant transformation has been carried out using Biolog MicrostationTM automatic system. Results confirmed that all the five strains belong to *Rhizobium radiobacter* genus, the updated classification of the *Agrobacterium strains* -Young et al., 2001. Five different *Agrobacterium* strains have been tested, some of them are very usual for using in plant transformation other less. Different metabolic behaviours were observed among the strains just for fourteen substrates, while for the other ones they show a relatively similar behaviour. In Fig. 1 the behaviour of the five strains using these fourteen substrates is shown. The LBA4404 strain was able to use a wider variety of carbon sources than the other ones and these results suggest a wider adaptability for this strain. The availability of data at four different growth times allow to evaluate the kinetic of the substrate oxidation and also in this case LBA4404 shows the same trend (data not shown).

Evaluation of regeneration ability

Wheat transformation remain a relatively difficult task and many attempts have been carried out using various techniques to overcome some specific issues inherent that inhibit transformation of this recalcitrant plant -Akutzu et al., 2004. Triticale is an artificial hybrid cereal obtained by the cross between two different cereal species, *Triticum* and rye. It is used mainly as feed and for this reason can represent a good biofactory, avoiding the concern of inadvertent mixing of bioengineering plant material with that intended for food. It has been demonstrated that higher transformation and regeneration frequencies in wheat have been obtained using immature embryos for growing calli. -Weeks et al 1993; Cheng et al., 1997. The regenerative ability of the tested cereal varieties resulted largely variable, as shown in Table I. In particular, about 39% of *Triticum aestivum* var. Bobwhite regenerated in alive calli showing the higher friability, vitality and a ochre-colour indicating a callus of class II -Koichi et al., 2002; Przetakiewicz et al., 2003. Triticale var. Liron is comparable to wheat performances but globally Triticale develops a lower number of calli.

Cereal transformation

Triticum aestivum var. Bobwhite and the engineered LBA4404 *Agrobacterium* strain have been used to carry out a preliminary transformation experiment. After transformation, five selected and survived calli have been analysed by PCR. Fig. 2 shows that all samples present a band corresponding to the expected fragment of the transgene, proving the insertion of the xenogenous DNA coming from transformation into the plant genome.

This preliminary result can be considered a promising starting point towards the optimization of our protocols in view of the use of cereals as biofactory for biopharmaceutical products.

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Fig. 1 The metabolic abilities of the *Agrobacterium* strains with reference to the fourteen selected carbon sources

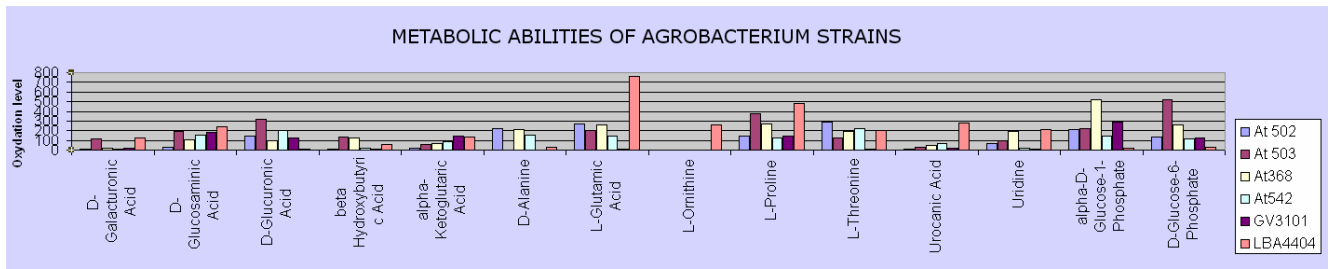


Table I Summary of the regeneration abilities of the tested varieties

	<i>Triticum aestivum</i>			Triticale	
	BOBWHITE	LONA	NADRO	LIRON	POLLMER
% protein content	na	15.5	15.5	12.5	10
Number of embryos	98	97	94	87	114
Number of calli	38	13	4	6	3
% regenerant calli	39	13	4.3	6.3	2.6
Callus type	II	II	II	II	II
Vitality	+++	++	+	++	++
Ochre-Colour	+++	++	+	++	++
Friability	+++	++	+	++	++