Paper 27

[Accueil] [Remonter] [Intro 1] [Paper 2] [Paper 3] [Paper 4] [Paper 5] [Paper 6] [Paper 7]
[Paper 8] [Paper 9] [Paper 10] [Paper 11] [Paper 12] [Paper 13] [Paper 14] [Paper 15] [Paper 16]
[Paper 17] [Paper 18] [Paper 19] [Paper 20] [Paper 21] [Paper 22] [Paper 23] [Paper 24]
[Paper 25] [Paper 26] [Paper 27] [Paper 28] [Paper 29] [Paper 30] [Paper 31] [Paper 32]
[Paper 33] [Paper 34] [Paper 35] [Paper 36] [Paper 37] [Paper 38] [Paper 39] [Paper 40]
[Paper 41] [Paper 42]

Genetic transformation of Italian rice cultivars mediated by Agrobacterium tumefaciens and the mature embryo-derived callus system.

Authors :

BALCONI C.(
PERUGINI I.
GIANINETTI A.
RUSSO S.
Istituto Sperimentale per la Cerealicoltura - ITALY
Section of Vercelli, S.S. per Torino km 2.5.
Tel : +39 (0)161-391134
Fax:+39 (0)161-294206

LUPOTTO E
REALI A.
PASSERA S.
Istituto Sperimentale per la Cerealicoltura - ITALY
Section of Bergamo, Via Stezzano, 24.
Tel +39 (0)35-313132
Fax +39 (0)35-316054

Abstract

The research has been focused on the identification and establishment of routine procedures for in vitro culture, plant regeneration and genetic transformation of important Italian rice cultivars.

Experiments have been carried out to test mature embryo-derived callus formation and successive plant regeneration in 30 Italian rice genotypes. The data obtained indicated that the varieties differ in their ability to induce callus and to regenerate plants; several lines showed high embryogenic callus induction frequency.

Experiments have been performed also to test the interaction between the callus systems and 4 different Agrobacterium strains of different chromosomal backgrounds.

The aim of the work is to adapt to Italian genotypes the Agrobacterium methodology developed for tropical japonica and indica type rice varieties. Experiments have been done to optimize the conditions of tissue culture, co-cultivation and plant regeneration since the varieties selected and adapted to Italian environments are genetically very different from the tropical japonica and indica varieties, largely studied. GUS expression in mature embryo-derived calli after Agrobacterium-mediated transformation, indicated that at least 2 Agrobacterium strains are very efficient on several rice genotypes; the other 2 strains resulted less virulent in general, and in some cases no attack was observed, indicating the existence of a specific interaction between Agrobacterium strain x rice genotype.

These indications are very important for the identification and establishment of efficient routine procedures for in vitro culture, plant regeneration and genetic transformation of Italian rice cultivars.

Keywords

- Rice (Oryza sativa L., Japonica type), in vitro culture, transformation, Agrobacterium tumefaciens
- Italy.

Introduction

Low yields of economically important cereal crops, such as rice, caused by environmental stresses and plant diseases, are one of the major problems that biotechnology is expected to address in part by means of plant transformation with engineered resistance genes (Swaminathan, 1982; Khush, 1984; Toenniessen, 1990).

The success of Agrobacterium-mediated transformation could provide a simplified procedure for transformation of monocotyledonous crops. Transformation of dicotyledonous plants by Agrobacterium-mediated gene transfer is well established and has produced stable transgenic plants expressing a number of foreign genes (Zambryski, 1992; Sheng and Citovsky, 1996).

This has not been the case for monocotyledonous plants in general; the ability of Agrobacterium to transform monocotyledonous has been the subject of a serious debate for some time, since these plants are not natural hosts (Smith and Hood, 1995). The controversy now appears to be resolved, since recent studies have clearly demonstrated the stable integration of foreign DNA in rice and in maize in a process mediated by Agrobacterium tumefaciens (Hiei et al., 1994). This process includes the transfer of pieces of DNA with defined ends with minimal rearrangements, the transfer of relatively large segments of DNA, the integration of small copy number of genes into plant chromosomes, and high quality and fertility of transgenic plants, as well as the Mendelian transmission of the DNA to the progeny (Hiei et al., 1996).

The success of any biotechnological application is always strictly dependent upon the possibility to establish, transform and efficiently regenerate an in vitro cell system. Also for rice, the most responsive cereal species for tissue culture, the successful establishment of in vitro culture is dependent on the genotype used, types and ages of tissues inoculated, kind of vectors, strains of Agrobacterium and tissue culture conditions.

The aim of our research is the identification and establishment of routine procedures for in vitro culture, plant regeneration and genetic transformation of important Italian rice cultivars.

Materials and methods

Rice cultivars

The 30 Italian rice genotypes (japonica type), differing for the "grain type" tested are described in Table 1.

Table 1 - Italian rice genotypes (japonica type) tested.

GRAIN TYPE - Short: lenght up to 5.5 mm
- Medium: lenght 5.51 to 6.6 mm
- Long(*,**): lenght 6.61 to 7.5 mm

* Long A : lenght/width ratio : 2 < > 3;** Long B : lenght/width ratio : > = 3

GRAIN TYPE	GENOTYPE
Short	Balilla Elio Selenio Sequial
Medium	Lido Roncarolo Rosa Marchetti Vialone Nano
Long A (LA)*	Anseatico Arborio Ariete Baldo Carnaroli Dorella Drago Gigante Vercelli Koral Lampo Loto Roma S. Andrea Strella Turbo
Long B (LB)**	Graldo Idra Star

Varietes bred by Istituto Sperimentale Cerealicoltura

Short	LA 1
Long B (LB)*	LA 11
Long A (LA)*	LA 12
Long A (LA)*	LA 13
Long A (LA)*	LA 17

Explants, culture media, plant regeneration

Mature seeds were dehusked, sterilized with 70% ethanol for few minutes, and then vacuum infiltrated with 1.5% sodium hypochlorite for 25 min. The seeds were washed 5 times with sterilized distilled water and incubated at 4°C. After 24 hrs the same procedure of sterilization was repeated.

The embryo was dissected out of the kernel and used as explant for callus induction : about 20 explants were placed in Petri dishes (45 mm) containing N6 basal medium (Chu, 1978) supplemented with 1 g/l casamino acids, 30 g/l sucrose, 2 mg/l 2,4-Dichlorophenoxy acetic acid (2,4-D), 9 g/l agar, pH 5.8 and incubated for 4 weeks in dark at 26°C. Actively growing pieces of calli (3-4 mm diameter) were used for transformation experiments.

For regeneration, the induced calli were placed into Petri dishes containing N6 regeneration medium (half-strength N6 major salts, N6 minor salts), 1 g/l casamino acids, 20 g/l sucrose, 0.2 mg/l naphtaleneacetic (NAA), 1 mg/l kinetin, 9 g/l agar, pH 5.8, and aminoacids (AA) as described by Toriyama and Hinata (1985).

The calli were incubated in a growth chamber at 26°C, with 16/8 hrs of light/dark. The plantlets obtained were transplanted into Magenta boxes containing MS basal medium (Murashige and Skoog, 1962) supplemented with 20 g/l sucrose and 9 g/l agar, ph 6.0, lacking growth regulators.

The plantlets with well developed root systems were transferred into pots containing soil and were aclimatized to the green-house conditions.

Transformation

For the Agrobacterium-mediated transformation, a general procedure according to Hiei et al., 1996, with minor modifications was used. The bacteria were grown in AB medium + selection in 2ml aliquots for 3 days. One ml of solution containing bacteria was spun at 14000 rpm and resuspend in 1ml of LS (Linsmaier and Skoog, 1965) infection medium supplemented with 100 uM acetosyringone (AS).

The callus tissues were immersed in the bacterial suspension for 15 min, and subsequently transferred, without rinsing, on the co-cultivation medium (LS + 1.5 mg/l 2,4 D + 100 um AS) and incubated at 25°C in darkness for 3 days.

After the co-cultivation the materials were rinsed in sterile water with 100 mg/l timentin and placed on regeneration medium (as described above) supplemented with 100 mg/l timentin. Samples of calli were analyzed by histochemical assay to detect GUS expression.

Bacterial strains

Agrobacterium tumefaciens strains of different chromosomal backgrounds, used for the transformation are described in Table 2. The Agrobacterium strains contain the marker cassette -INTGUS- utilized for histochemical GUS analysis of the events of transformation. All constructs were kindly provided by Ming Tsair CHAN, Institute of Molecular Biology, Academia Sinica, Nankang Taipei, 11529, Republic of China, in the framework of a cooperative project.

Table 2 - Agrobacterium STRAINS

Ref	*Agrobacterium* strain	chimeric genes of interest	selection for *Agrobacterium* (ug/ml)	selection for plant cells
2	C58c1(pINTGUS)	p36S-INTGUS-nos3' pnos-HPT-nos3'	100 Sp 50 Rif 100 Cb 300 Sm	Hygromycin 30-50 mg/l
4	Agt 121 (pGUS)	p35S-INTGUS-nos3' pnos-NPTII-nos3'	100 Kan	Kanamycin 100-200 mg/l
6	EHA101(pMTCA23GUS)	p35S-INTGUS-nos3' pnos-NPTII-nos3'	100 Sp	Kanamycin 100-200 mg/l
9	EHA105(pMT1)	p35S-INTGUS-nos3' pnos-NPTII-nos3'	100 Kan	Kanamycin 100 mg/l

Abbreviations:

nos3'= 3' terminal signal of nopaline synthase pnos = nopaline synthase promoter
INT= Adh1 intron 1, from maize
GUS= B-glucuronidase
HPT= hygromycin phosphotansferase
NPTII= neomycin phosphotransferase
p35S= 35S CaMV promoter t35S= 3' signal of 35S gene

Antibiotics:

Sm= streptomycin; Sp= spectinomycin; Rif= riphampicin; Cb= carbenicillin; Kan= kanamycin

Results and discussion

Methods for genetic transformation and production of transgenic rice plants are generally based on protoplasts transformation derived from embryogenic suspensions (Lynch et al., 1991) and bombardment of immature embryos (Christou et al., 1992). Immature embryos are limited to short periods of growth cycle of the plant, thus limiting the ease of manipulation. The use of mature embryos as explant offers a suitable source of continuously available material for tissue culture. A tissue culture system with efficient degree of

somatic embryo formation is a prerequisite for genetic manipulation studies for engineering rice cultivars.

The results of our research allowed to set up a method suitable for obtaining embryogenic callus, somatic embryos and plant regeneration in Italian rice cultivars using mature embryos.

The data obtained indicate that the genotypes tested differ in their ability to induce callus; in particular :

- the Callus Induction Frequency (CIF%) showed a range between 32 and 93%, with a mean value of 66%. In detail, the best responsive genotypes were : Selenio (Short), 93%; Rosa Marchetti (Medium), 78%; Baldo (LA), 79%; Idra (LB), 80%.
- several genotypes showed high Callus Regeneration Frequency (CRF%); particularly : Selenio (Short), 50%; Roncarolo (Medium), 65%; Baldo (LA), 75%; Star (LB), 69%.

On the other hand, the callus derived from some genotypes, showed a tendency to the necrosis and those genotypes were discarded. From other varieties, regenerated plants have been analyzed for fertility as well as for several agronomical traits of interest. Experiments are in progress to analyze the regeneration capacity of other genotypes.

Experiments have been performed also to test the interaction between the callus system and 4 different Agrobacterium strains of different chromosomal backgrounds (as described in « Material and methods » The aim of the work was to optimize the conditions of tissue culture, co-cultivation and plant regeneration since the effect of the transformation via Agrobacterium is mostly reflected in a drastic loss of regenerative capability of the callus tissue.

GUS expression in mature-derived calli after Agrobacterium-mediated transformation, indicated that at least 2 Agrobacterium strains are very efficient on several rice genotypes (Table 2: strain 2, C58c1 and 6, EHA101). The other 2 strains resulted less virulent in general, and in some cases no attack was observed, indicating the existence of a specific interaction between Agrobacterium strain x rice genotype.

The histochemical analysis of transversal sections of the mature-seed embryos induced to callus formation, after the infection with Agrobacterium, indicates that the GUS expression is restricted to the scutellum-proliferating cells, from which the embryogenic callus with somatic embryos derives.

In order to test more in detail at which stage of the scutellum-derived callus is susceptible to attack Agrobacterium, an experiment for the evaluation of "time infectability" was performed. Mature-embryos of 10 rice genotypes, were infected with 4 Agrobacterium strains, either directly explanted or after 1 and 5 days of in vitro culture. Also at these early stages of callus induction, the Agrobacterium strains 2 (C58c1) and 6 (EHA101) resulted very efficient on several rice genotypes and no difference of attack was detected .

Our research indicates that the 30 Italian rice genotypes tested show variability in the response to the in vitro culture, plant regeneration and genetic transformation. Some of the genotypes are efficiently cultured in vitro and establish a regenerable callus system from mature seed embryos. The target tissue displays infectability with Agrobacterium tumefaciens, thus allowing to apply directly the system of transformation.

These indications are very important for the identification and establishment of efficient routine procedures for in vitro culture, plant regeneration and genetic transformation of Italian rice cultivars.

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