RICE TRANSFORMATION USING THE BIOLISTIC APPROACH

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INTRODUCTION

The basic concept of the biolistic approach of plant transformation is to deliver the genetic material using gold or tungsten particles highly accelerated so they will penetrate through the cell wall and the foreign DNA coated on their surface will then be freed and in some rare cases will recombine with the chromosomal DNA of the host. This approach allows to use a wide variety of tissues as a target for the bombardment, embryogenic calli, cell suspension, mature embryos derived calli or immature embryos (IE)...

The choice of the tissue depends on the competence for transformation, the transformation efficiency (some tissue are more difficult to penetrate than others or, on the contrary, too fragile and they might be destroyed by the bombardment), and on the competence for regenerating a fertile plant from the shot sample.

TARGET TISSUE

Concerning rice, after testing all the mentioned tissues, the best results were obtained when immature embryos (IE) were used with japonica varieties. Some other techniques like protoplasts transformation may give a better yield of transformed plants but this technique is difficult to set up for indica rice, it needs adaptation with each different genotype as the genetic variability is quite important among this sub-species of *Oryza saliva*. Furthermore, protoplast transformation leads very often to sterile plants and integration of multi-copies of the transgene(s) is too frequent.

Calli derived from mature embryos should be a very interesting material because of its continuous and nearly unlimited availability, unfortunately, as for the protoplasts, it is difficult to obtain regenerated plants and to date the transformation efficiency is lower than with IE, but improvements are under way and it might become in the future an alternative for rice transformation.

Embryogenic cell suspension give good results but not better than immature embryos. On a regular base, for varieties that do not present difficulty for regeneration, there is no reason to use them as it is necessary to maintain a fresh suspension of embryogenic cells, which is time consuming and requires a high expertise in tissue culture. Nevertheless, when the yield of regenerated plants is low, or null, with IE, it might be advantageous to use this kind of tissue.

An other alternative to IE for rice transformation consists in using subcultured calli obtained from mature embryos. During the subculture process it is possible to select easy to regenerate calli and increase dramatically the regeneration efficiency. At each transfer (once a week), a callus is divided, one half is placed on a growing medium, the other half is put on a regeneration medium, only the calli that regenerate are conserved for the next transfer. This tissue allows also to have a dense target for the particles. At last, this procedure is independent of the seed setting and might be applied all year long. Its disadvantage is that after 6 transfers, the calli have to be renewed as they rise too much abnormal plants and the sterility of the regenerated plants is high.

SELECTION OF THE TRANSFORMED PLANTS

At the same time than the gene of interest (GOI) it is necessary to introduce in the plant to transform a selectable marker which allows the selection of the plants expressing it. For this purpose it is preferable to use a gene which expression will allow the growth of a plant submitted to a selective pressure.

The most widely used markers are resistance genes against herbicide or antibiotics. Herbicide resistance genes present the risk to be spread to weed once the transformed plant is in the field. The antibiotic resistance gene may also be considered as undesirable in a cultivated plant. For this purpose, in order to get rid of the resistance gene once the transgenic plant produced, the simplest solution is to proceed to cotransformation. The selectable marker is located on a different vector than the GOI. If mostly of the time, whatever the transformation procedure is used, the two introduced DNA are integrated at linked sites, some transgenic plants have them located far enough to allow recombination between them and produce segregating offspring containing only the GOI.

We choose to use antibiotic resistance. After testing kanamycin and G41 8 we decided to change to hygromycin B as the first two antibiotics gave nearly 100% of sterile plants. This situation seem to coincide with others laboratories involved in rice transformation. The selection by hygromycin is very efficient and the regenerated plants have an overall good fertility.

The *hph* gene from *Escherichia coli*, which encodes a hygromycin phospho transferase, is incorporated downstream the 35S promoter of the cauliflower mosaic virus (CaMV) on a separate plasmid which is coated on the gold particles with a molar ratio of 1:2 in respect to the plasmid containing the GOI

The selection might start a day after the shooting or a short delay of recovery (3 to 4 days) on a callus induction medium might be left before starting the selection.

PREPARATION OF THE IMMATURE EMBRYOS AND SHOOTING PROCEDURE

Ten to twelve days after fecundation, the IE are collected, separated from the seeds, sterilized (45 min. in bleach or 45 sec. if antibiotics are used) and placed, scutelum up, on growing medium in a Petri dish. In case of short sterilization in bleach, the NB medium is supplemented with cefotaxime (100mg/L) and carbenicillin (400mg/L).

An osmotic treatment might be applied too by transferring the embryos 4 hours prior shooting on a medium (NB with 2,4D) containing sorbitol or mannitol (0.4 M). 1 6 hours after shooting the embryos are removed from the hypertonic medium and transferred on a normal callus induction medium. For the shooting about 40 embryos are placed in the center of a Petri dish.

5 µg of total DNA (GOI vector + resistance gene vector) are used to coat the gold particles. Two shots at 1100 psi (pounds per square inch) are performed using the helium gun PDS-1000/He from BIO-RAD. The plate is at 8 cm from the stopping grid. P.Christou reports that using heterogeneous sizes of particles, ranging from 0.5 to 2 µm gives better yield.

SELECTION PROCEDURE

Four days after shooting, the embryo derived calli are transferred to a new NB medium added with 30 mg/L of hygromycin B. A week after the hygromycin concentration is increased to 50 mg/L and the selective pressure is maintained during four weeks allowing only the cells expressing the *hph* gene to grow.

The surviving calli are then transferred on a regeneration medium containing the same concentration of hygromycin B. After 4 to 5 weeks, the regenerated plantlets are transferred to soil for a weaning period in a growth chamber and then to the green house.

In order to avoid siblings, only one plant per embryo-derived callus will be considered.

ANALYSIS OF THE TRANSGENIC PLANTS

Once the regenerated plants are obtained, nothing distinguished the different procedures of transformation. The analysis of the transformed plants, using molecular biology tools, has to be performed following the same steps.

The first step of analysis is in general discriminatory in order to select quickly the most interesting plants. A test of expression of the GOI is certainly the most accurate (ELISA, Protein slot blotting) but in some cases the level of expression of the transgene is not sufficient, or when the antisense strategy is applied, there is no protein synthesized. In those situations, a PCR will indicate the presence of the gene. Amplifying the whole cassette (promoter+gene+terminato r) will give a good estimate of the expression, indeed, the presence of an intact cassette is strongly correlated to the expression of the gene.

The integration of the foreign gene and the copy number are surveyed by DNA blotting (Southern), the expression is confirmed by RNA and protein blottings (Northern and Western).

Of course when a reporter gene is used, (*gus*) testing the enzymatic activity is the first step to perform, but this will not give indications about the integration in the plant genome and the number of integrated copies. Further analysis are still needed.

RESULTS

The transformation efficiency, calculated as the number of independent transgenic plants obtained in relation to the number of shot IE or calli, is variable from one experiment to another. Our range, on 11 experiments, goes from 3% to 44%. Among the regenerated plants, almost 44 % have a panicular fertility of at least 50% (number of grain/number of spikelets insertions), and only 1 8 % are completely sterile

The rate of co-transformation, in our case, is difficult to estimate, we calculate it based on PCR results as the ratio of plants possessing the GOI among the plants expressing the *hph* gene. Calculated on 11 independent experiments this proportion of cotransformation is almost 40%.

EXPRESSION OF THE RICE TUNGRO BACILLIFORM VIRUS COAT PROTEIN GENE:

In order to test the efficiency of the coat protein mediated resistance strategy against the tungro disease which is induced by two viruses: the rice tungro bacilliform virus (RTBV) and the rice tungro spherical virus (RTBV), we identified on the RTBV genome, the sequence encoding the coat protein (CP). It is comprised in a large open reading frame which is processed after translation. Four fragments, of different length but all containing the core sequence corresponding to the CP, were isolated and integrated in different constructs.

Four different promoters were used to control the expression of the RTBV coat protein. Those promoters were: the 35S from the cauliflower mosaic virus, the rice actin 1 gene promoter followed by the first intron, the RTBV promoter itself (phloem specific) and the maize ubiquitin gene promoter with the first intron of that same gene.

Co-transformation experiments were performed as described and the selected plants were analyzed as indicated.

The 35S promoter gave very low level of expression, hardly detectable by protein blotting and/or RNA blotting.

The actin 1 promoter gave better results in term of expression in the RO plants, but the offspring showed a much lower level if not null, Furthermore some recombination seems to occur which may lead to the loss of the introduced sequence, this phenomenon was also observed by T. Hodges (pers. com.).

The RTBV promoter, as it is phloem specific gives a product difficult to quantify. Northern, performed on polyA RNAs, show a band of the expected size, but the protein is not detected due to its low amount in the extract.

Finally, the maize ubiquitin gene promoter, in association with the first intron of that same gene, gave better results. When intact cassette was present as shown by DNA blotting, the expression was sufficient to identify the encoded protein by Western immunoblots.

The inheritance was confirmed among the offspring of 4 different transgenic rice lines. The level of expression was comparable to the one in the RO plants. But a 1:1 segregation was observed in 2 of the 4 lines. This might be explained by the death of the microspores (or pollen grains) expressing the RTBV CP.

In order to be challenged for virus resistance, the transgenic plants produced will be sent to IRRI, (International Rice Research Institute, Los Banos, Philippines), MARDI (Malaysian Agricultural Research and Development Institute, Kuala Lumpur, Malaysia) and the Agricultural College of Fujian (Fuzhou, China, PR).

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