Biolistic transformation of rice: now efficient and routine for japonica and indica rices

C.M. Fauquet, S. Zhang, L. Chen, P. Marmey, A. de Kochko, and R.N. Beachy

Rice transformation using microbombardment has been used for 4 yr, but it is only since 1994 that we can consider the process routine for both japonica and indica rices. For japonica transformation, the rate of efficiency of transformation now averages 25%. Our protocol does not generate escapes for hygromycin resistance and the duration of our protocol has been shortened to 8 wk from shooting time to obtaining plantlets. Transformation is accomplished by mixing plasmids containing the hygromycin resistance gene and the gene(s) of interest (GOI). Cointegration of two genes averages 70% and greatly depends on the DNA ratio in the plasmid mixture. Several types of explant tissues such as immature embryos, embryogenic calli, and embryogenic suspensions have been tested and transgenic plants were produced. Embryogenic calli are preferred but, in some cases, embryogenic suspensions can be advantageous. Indica transformation using embryogenic suspensions and the biolistic method is performed regularly with varieties that are difficult to regenerate such as IR72, IR64, and BG90-2 but with a lower efficiency ranging from 1 to 5%. Transgenic plants have been carried through to seven generations, which proves stability in the inheritance of the integrated genes and shows that normal segregation is maintained over generations. Many different GOIs have now been inserted, including the *Xa21* bacterial blight resistance gene, demonstrating that large pieces of DNA coding for large proteins can be successfully integrated and expressed in rice and can produce the expected phenotype.

For some time now, rice has been transformed using protoplast transformation mediated by polyethylene glycol (Toriyama et al 1988; Zhang et al 1988; Zhang and Wu 1988; Shimamoto et al 1989; Datta et al 1990, 1992; Hayashimoto et al 1990; Peng et al 1990, 1992; Terada and Shimamoto 1990; Hayakawa et al 1992; Fujimoto et al 1993;

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Rathore et al 1993). Most of the genotypes have been japonica and the fertility of the transgenic plants has always been a problem. Rice transformation has also been successfully achieved with microbombardment of immature embryos or embryogenic calli (Christou et al 1991, Cao et al 1992, Li et al 1993, Sivamani et al 1996), but it is only recently that biolistic transformation of indica rice has been shown to be effective and capable of producing fertile group I indica plants (Zhang et al 1996). More recently, other methods such as *Agrobacterium*-mediated transformation (Hiei et al 1994) and electroporation of intact seed embryo cells (Xu and Li 1994) have been published.

While fertile japonica transgenic plants can be obtained using other methods, the biolistic system has become the method of choice because it alleviates the need of preparing protoplasts, reduces the time needed to regenerate transgenic plants, and results in transgenic plants with higher fertility. *Agrobacterium*-mediated transformation of rice may provide another alternative technique to biolistic transformation if it is proven to be genotype-independent and routinely efficient.

At the International Laboratory for Tropical Agricultural Biotechnology (ILTAB), successful japonica rice transformations have been carried out since 1991, but it was only in 1994 that we really had an efficient system in place where the production of transgenic independent lines per experiment averaged 25%. It is only now that we can really do experiments properly designed to check a number of variables that are important when transforming rice. In this paper, we discuss results of a number of tissue culture experiments aimed at making the protocol more precise, shorter, and simpler. We also provide information on DNA variables tested so far in our experiments.

Indica rice transformations have always been much more difficult to achieve. We first used the immature embryo system (Rancé et al 1994, Tian et al 1994) and then switched to embryogenic suspensions (Zhang 1996, Zhang et al 1996). Using the latter method, we have had variable success.

Finally, through collaboration with the University of California, Davis, we managed to transfer the bacterial blight resistance gene *Xa21* first to japonica rice (Song et al 1995, Ronald et al 1996) and recently to indica rice (S. Zhang et al, unpubl. data). It is now possible to transfer and express a phenotype resulting from a single gene to any rice genotype. DNA mapping, gene cloning, and genetic engineering are indeed complementary tools that should enable breeders to develop some interesting transgenic lines.

Biolistic transformation of japonica rice

ILTAB's transformation protocol was established in 1991 and modified and improved in 1994 for our model japonica variety, TP309. The protocol is a model for optimizing transformation methods for other japonica varieties and, possibly, for a few indica varieties. Using embryogenic suspension and/or embryogenic calli as the target tissue (Sivamani et al 1996), the new protocol is faster and more efficient in selection. One major improvement is the use of osmotic pressure to ensure that wounded cells have a better chance of recovery. Another major improvement is the early visual identification of the transgenic calli, which has tremendously boosted transformation efficiency.

Nature of the explants used for bombardment

Early biolistic transformations used immature embryos, but it is very difficult to obtain immature embryos suitable for transformation on a regular basis. We investigated using other tissues such as embryogenic calli and embryogenic suspensions. Figure 1 shows a diagrammatic representation of the protocol used at ILTAB to produce such embryogenic material for T309 and other japonica genotypes.

Nature of the targeted tissue

We compared the results of transformation efficiency between immature embryos, embryogenic calli, and embryogenic suspensions as target tissues, using T309 in all experiments (Figure 2). Embryogenic calli are much more efficient in producing transgenic plants compared with immature embryos (25 vs 3%). Another set of experiments demonstrated that embryogenic suspensions and embryogenic calli are equally effective in producing transgenic calli (41±3 vs 45±9%).

Tissue size and age

When using embryogenic suspensions for transformation, the size and age of the explants used for bombardment are critical. The suspension should be replicated at least 7 d before—but no more than 14 d prior to—bombardment and the size of the explants should not be smaller than 2 mm for optimal transformation.

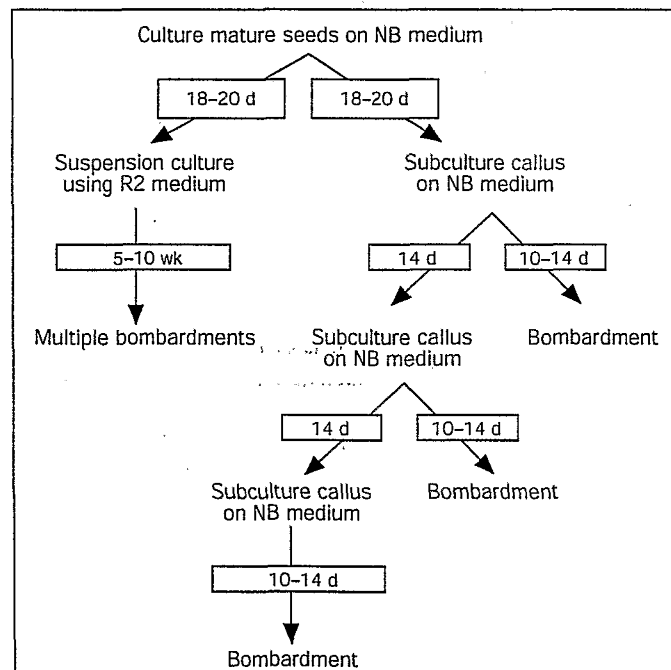

Fig. 1. Preparation of the subcultured embryogenic explants for bombardment.

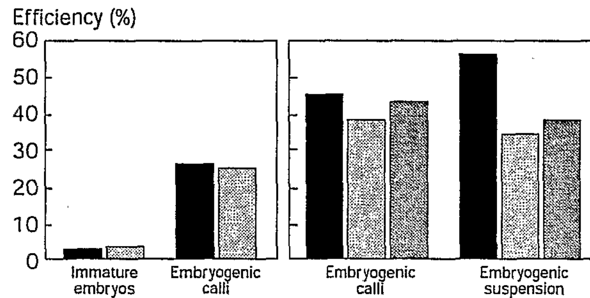

Fig. 2. Comparison of target immature embryos, embryogenic calli, and embryogenic suspension as target tissues used to produce transgenic plants. The patterns designate different experiments (three replicates for each experiment).

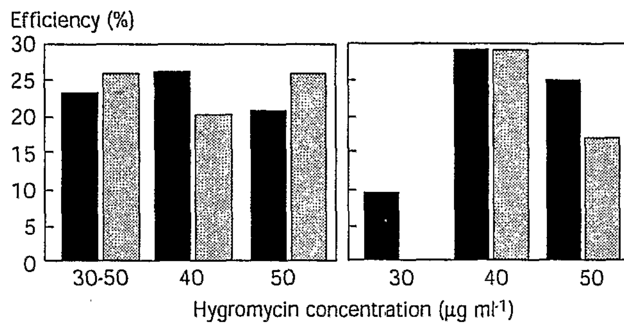

Fig. 3. Comparison of different selection treatments used to produce transgenic rice plants. Each experiment is an average of four or five replicates.

Osmotic treatment

Gold particle bombardment is detrimental to cells. However, it has been demonstrated that wounded cells can be protected by keeping them under high osmotic pressure before and after bombardment. Vain et al (1993) demonstrated this with maize and we confirmed it with rice. It is possible to obtain eight times more transgenic plants (average of three experiments) when using osmotic pressure treatment. We have also found that an optimum osmotic pressure can be reached to optimize rice transformation (data not presented).

Selection with one hygromycin treatment is preferred

Since false-positive plants in transformation work have always been a problem, we investigated various protocols that differed in the amount of selectable marker (30, 40, and 50 mg hygromycin B L^{-1}) and in the number of selection times. Figure 3 shows that a moderate selection pressure is preferable (40 mg hygromycin B L^{-1}) and that one selection is effective.

Need for experienced technicians

An important change at ILTAB since 1992 is the exclusive use of well-trained, experienced technicians to select visually the transgenic calli as early as 21 d after bombardment. Our technicians are trained to observe color, glossiness, and callus structure—all important factors when selecting transgenic calli. Figure 4 provides a diagrammatic representation of the ILTAB protocol for japonica rice transformation, which includes the improvements discussed above.

Transformation efficiency

Transformation efficiency is determined by calculating the number of transgenic lines obtained per explant bombarded. If several plants are regenerated from one explant, they are considered to be siblings and counted as one. In a few experiments, we reached a 50% transformation efficiency but the average is closer to 25%.

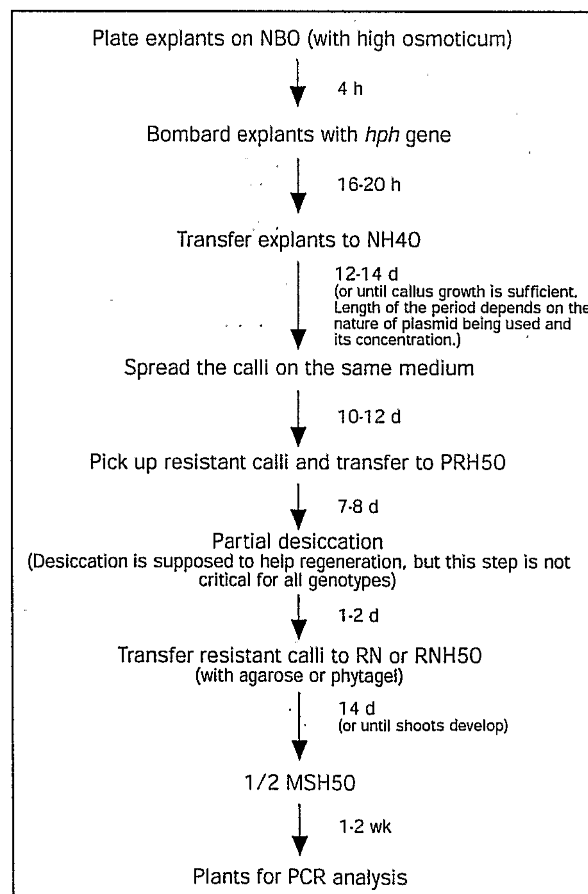

Fig. 4. ILTAB protocol for japonica rice transformation.

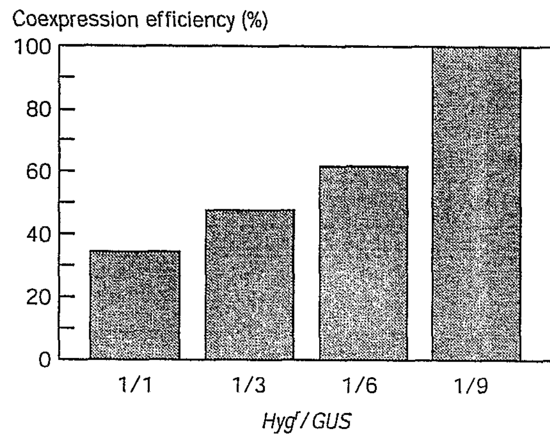

Fig. 5. Percentage of co-expression efficiency for hygromycin resistance and *GUS* expression for various ratios of the DNAs coding for *Hyg*^{r} and *GUS*.

Cointegration efficiency

Because we select with one gene while working to insert another gene, the efficiency of the integration of both genes (cointegration) is a major concern in rice transformation when using the biolistic method—especially since we use mixtures of plasmids rather than plasmids carrying both genes. The percentage of cointegration varies between 5 and 100%. Our experience shows that DNA quality and the nature of the gene are also very important in achieving positive results.

To improve our results, we have studied the molar ratio of the DNA of the gene of interest (GOI) and the DNA of the selectable marker, relative to the efficiency of cointegration and, in some cases, of coexpression. Figure 5 provides preliminary results showing that this ratio is important and that an increase in the molar ratio of the plasmid coding for the GOI increases the coexpression ratio. In this experiment, the two genes were *hph* and *iudA* and 100% coexpression was obtained when using a ratio of 1/9 for *Hyg*^{r}*/GUS* genes.

Fertility and germination

Recently, more than 80% of the transgenic rice plants developed at ILTAB were at least partially fertile (ranging from highly fertile to highly sterile, based on IRRI criteria) and only 18% were completely sterile. In cases where R_0 transgenic plants were partially fertile, fertility was generally restored in the next generation, indicating that partial sterility, in most cases, is not an inherited trait.

Germination rates of seeds from most transgenic rice lines were usually higher than 90%, comparable with the seeds from nontransgenic plants. Lower germination rates were also observed in a few cases, ranging approximately from 10 to 60%.

It is our opinion that transformation itself does not generally induce sterility, but it is the passage through tissue culture and, above all, the conditions under which the transgenic plants are growing that are critical in obtaining high fertility.

Inheritance mode, copy number, and stability of the transgenes

For a majority of the transgenic rice plants assayed so far, the transgenes were inherited as dominant genes in a Mendelian way. However, non-Mendelian inheritance and 1:1 ratio (at some time over several generations) are observed.

Generally, the copy number of the transgenes integrated into the rice genome ranges from one to several when the plants are transformed biolistically. In one study, we used Southern analysis to investigate 41 transformation events and found that 6 (14.5%) had one transgene copy, 31 (80.5%) had from 2 to 10, and only 2 (5%) had more than 10 copies. Using biological assays (*hyg*^{r}), we have detected expression of the *hph* gene in transgenic plants with various copy numbers. The expression of the GOI in some transgenic plants with single- or multiple-copy numbers of the relevant genes was also detected using Northern or Western hybridization. Usually, only a random portion of the vector DNA is integrated into the rice genome, and this portion is different from one transgenic plant to another as revealed in many Southern hybridization analyses. In many cases, when the Southern pattern shows multiple copies, all copies have been inherited together, indicating they were inserted at the same genetic locus.

So far, the *hph* gene has been stably inherited up to the R_7 generation, and the *GUS* gene up to the R_5 generation, except for one offspring line in which *GUS* expression was mostly suppressed. Studies involving other genes are under way.

Japonica transformation at ILTAB

Over the last 18 mo, one experienced technician and two assistants produced 2,135 independent transgenic lines with 187 different constructs. These numbers affirm that japonica transformation is now routine at ILTAB. We can now address scientific and technical questions that need to be answered before transferring any GOI to a genome of interest, including indica rice.

Recent improvements in the biolistic method used for japonicas will have an impact on all rice transformation research. It is now possible to look for the expression of an inserted gene and to study a large number of genes and promoters in rice. These developments will permit rice biotechnology to progress rapidly over the next few years.

Major advantages in the ILTAB protocol are summarized below.

- Preparation of the target tissue is independent of the environment. Instead of immature embryos, we now use subcultured embryogenic calli (Sivamani et al 1996) obtained from mature seeds, as well as embryogenic suspensions (Zhang et al 1996).
- The transformation protocol is rapid. Generally, it does not take more than 8-9 wk from bombardment to appearance of transgenic shoots/plantlets.

- There are no escapes in terms of hygromycin resistance.
- Transformation efficiency is consistent and uniform. An experienced technician can achieve efficiency ranging from 8 to 54% with an average of 25%, i.e., 25% of the bombarded explants will produce an independent transgenic line.
- The cointegration efficiency has improved to an average of 76%. In some cases, 100% cointegration has been obtained.
- Most of the transgenic plants are fertile or at least partially fertile. Fertility problems are associated more with greenhouse conditions than with the transformation protocol itself.

Much can still be done to improve the transformation protocol. For example, there is too much variability in transformation efficiency and we need to investigate DNA concentration and quality. And the nature of the sequences to be inserted is an important issue since we see a lot of variation from among the plasmids.

Biolistic transformation of indica rice

Four laboratories (Peng et al 1990, 1992; Christou et al 1991, 1992; Datta et al 1992; Xu and Li 1994) have reported accomplishing transformations using group 1 (Glaszmann 1987) indica rice, but with limited success in obtaining fertile transgenic plants. Fertile lines were regenerated only through bombarding immature embryos (Christou et al 1991, 1992) or electroporating embryogenic cells (Xu and Li 1994). Unfortunately, these techniques are genotype-dependent and cannot be extrapolated to a wide range of indica varieties. In addition, both the immature embryo and electroporation techniques are time-consuming and labor-intensive. Since immature embryos are environment-dependent, it has been difficult to maintain a continuous supply of suitable explants at ILTAB.

ILTAB protocol for indica transformation

The ILTAB protocol calls for using regenerable embryogenic suspensions as the target tissue for bombardment. Over the last 2 yr, we have obtained fertile transgenic plants from elite varieties IR24, IR64, and IR72 and advanced breeding line IR57311-95-2-3 and from indica varieties popular in Vietnam (Nang Huong Cho Dao), India (Basmati, Co 45), Malaysia (MR80, MR81), Thailand (Khao Dawk Mali), and West Africa (BG90-2).

Although indica transformation efficiency is quite low compared with that of japonica transformation, the steps described in the following sections are simple and repeatable and can be used to introduce GOIs into indica germplasm. This protocol has produced 163 green plants, representing 49 independent transgenic lines, expressing marker genes and GOIs of IR24, IR64, IR72, IR57311-95-2-3. Most of the plants produced seeds.

Callus induction and cell suspension. Small, compact, and globular embryogenic calli can be induced in equal quantities using either mature seeds or immature embryos (Zhang 1995). ILTAB prefers using mature seeds to avoid the extra work of maintaining a constant supply of immature embryos. The most difficult step—and a limiting

factor—in establishing embryogenic suspensions is cell browning and death in the liquid media at the suspension initiation stage. To avoid cell death, small, compact, and loosely attached globular embryogenic calli are carefully selected and cultured in R2 medium with 2 mg 2,4-D L^{-1} and 20 g sucrose L^{-1}. If the callus begins to turn brown, the R2 medium is changed to 2 mg 2,4-D L^{-1} and 20 g maltose L^{-1} and the cultures are subcultured at short intervals (1-5 d) for 2-3 wk. Following this protocol, group 1 indica rice suspensions can be established in 6-8 wk. These suspensions can be used as target tissue for transformation for up to 3.5 mo; after this time, plants can be regenerated, but with a low fertility. Depending on experimental needs, large quantities of this embryogenic suspension can be easily produced.

Effect of osmotic pressure on transformation efficiency. As in japonica transformation, it is crucial to maintain cell turgor after wounding with the gold particles. An osmotic treatment (30 g mannitol L^{-1}, 30 g sorbitol L^{-1}) of cell suspension cultures for 4 h before and 16-20 h after bombardment (Vain et al 1993) enhances transformation efficiency as much as fourfold.

Transformation and selection of embryogenic rice cell suspension. We use hygromycin B (hyg B) as the selection agent in our studies. The effect of hyg B on the growth of embryogenic suspensions of each variety is examined prior to initiation of an experiment. Cell growth is significantly reduced on solid medium containing 30 mg hyg B L^{-1}, and growth is greatly inhibited on medium containing 50 mg hyg B L^{-1}. However, if cells are plated from suspension cultures on solid growth medium without hyg B for a few days, the selection on hyg B becomes more difficult, presumably because the rapidly growing cell clusters do not allow for efficient selection on hyg B. Therefore, bombarded cell suspension cultures, kept on high osmotic pressure medium for 16-20 h after bombardment, are then transferred directly to selection medium with 30 mg hyg B L^{-1}.

Most explants gradually turn brown 2-3 wk later and it is easy to identify any white, growing cell clusters. At this stage, however, not all growing clusters are transgenic, so they are carefully removed from dying explant tissue and transferred to fresh selection medium containing 50 mg hyg B L^{-1} for further selection. To increase plant regeneration, after 2 wk of selection on 50 mg hyg B L^{-1}, the hyg^r calli are transferred to preregeneration medium containing 50 mg hyg B L^{-1} before transferring to the regeneration medium.

Regeneration of hygromycin-resistant plants. When the hyg^r calli reach approximately 2-3 mm in diameter, they are transferred to regeneration medium containing 50 mg hyg B L^{-1}. In preliminary experiments, we had observed that the absence of hyg B leads to regeneration of nontransgenic plants, although the calli were previously grown on medium with hyg B for more than 1 mo. Plantlets are then transferred to a rooting medium without hyg B; rooting in the absence of hyg B appears to be important in retaining fertility of the R_0 plants.

Fertility of transgenic rice plants and germination of transgenic seeds

Transgenic group 1 indica plants developed from protoplast transformation protocols have reportedly been sterile (Peng et al 1990, 1992; Datta et al 1992). This may be

Table 1. Fertility of transgenic IR72 lines and germination rates of transgenic seeds.

Transgenic rice line	Fertility[a] (%)	Seeds germinated[b]/ seeds planted
IR72-1	NT	47/50
IR72-2	NT	139/150
IR72-5	45.1+14	47/49
IR72-7	40.1+17	55/56
IR72-10	49.5+18	47/47
IR72 (control)	65.3+7	63/63

[a]Mean of fully set seeds per hundred seeds collected from six random panicles. NT: not tested. [b]Seeds were germinated in magenta boxes containing half-strength MS salts, 30 g sucrose L^{-1} , 0.1 mg NAA L^{-1} and with 0.25% (w/v) phytagel medium.

Table 2. Inheritance of the hyg^r trait and *uidA* gene expression in transgenic IR72.

Transgenic line	Seeds planted (no.)	R_1 germinated seedlings (no.)	hyg^r seedlings (%)	GUS-positive seedlings (no.)	χ^2
IR72-1	50	47	35 (74.5)	35	0.007
IR72-2	100	91	76 (83.5)	76	5.280
IR72-5	49	47	34 (72.3)	34	0.177
IR72-7	56	55	39 (70.9)	39	0.491
IR72-10	47	47	35 (74.5)	35	0.007
IR72 (control)	63	63		0	

[a]Transgenic rice seeds (R_1) were planted on 1/2 MS salts medium for 6 d, then transferred to medium containing hyg B (50 mg L^{-1}). χ^2 tests indicate good agreement with segregation ratios of 3:1 except in line IR72-2.

due, in part, to the time involved in establishing the cell suspension cultures. In our experiments, cell suspensions are used for biolistic transformation 6-8 wk after initiation. All of the regenerated transgenic plants tested were fertile. Table 1 shows the fertility level of three independent R_0 transgenic IR72 lines. Their fertility is lower than that of nontransgenic plants, probably due to the effects of the tissue culture protocol. However, normal fertility can be restored in the R_1 plants (data not shown). The seed germination rate in R_0 transgenic rice lines is comparable with that of the nontransgenic plants (Table 1).

Inheritance of *hph* and *uidA* genes

Integration of the *hph* gene into the genome of transgenic rice plants was confirmed by Southern blot hybridization using the hph sequence as probe. All lines tested contained the intact cassette (promoter, *hph* gene, and Nos terminus), as expected. Some lines contained more than one copy. The segregation of the hyg^r trait and *uidA* gene expression among offspring of the transgenic plants were demonstrated by germinating R_1 seeds on medium containing 50 mg hyg B L^{-1} and by GUS assays,

respectively (Table 2). Results showed that the *hph* and *uidA* genes segregated together in tested offspring and most of the lines exhibited a 3:1 segregation ratio among the offspring, indicating Mendelian inheritance from a single genetic locus of functional *hph* and *uidA* genes. Inheritance of *hph* and *uidA* by Southern blot and GUS assay of R_3 transgenic plants was also demonstrated (data not shown).

Production of bacterial blight-resistant rice plants

We collaborated with the University of California, Davis (UCD), to transfer the bacterial blight resistance gene *Xa21* from *Oryza longistaminata* to *O. sativa*. The gene, which had previously been transferred through crossing, has been mapped on chromosome 11 and cosmid-bacterial artificial chromosome (BAC) libraries containing it have been made from line IRBB21. Through cloning and hybridization, UCD researchers managed to isolate eight clones corresponding to the *Xa21* locus. These constructs were integrated, as such, into 306 independent T309 transgenic lines, produced in 4 mo. Most of the R_0 plants were then challenged with *Xanthomonas oryzae*. Eighty percent of the 15 lines corresponding to construct B were found extremely resistant to *X. oryzae* under laboratory conditions. After sequencing the clones, it was found that only the construct possessing the entire *Xa21-B* gene induced the resistance phenotype (Song et al 1995, Ronald et al 1996). For six resistant lines, it was possible to obtain seeds and check the inheritance of the trait, and indeed the resistance phenotype followed a 3:1 ratio as expected for a transgene present at a unique locus.

Since then, we have transformed the indica rice varieties IR64 and IR72, with the same *Xa21-B* construct, and we have managed to produce several independent transgenic lines for both varieties that have been challenged with *X. oryzae* and subsequently shown to be extremely resistant. These plants are now producing seeds, and we should be able to challenge the second generation very soon. After that, seeds will be mass-propagated and bacterial blight resistance tests will be performed on a large scale by bacteriologists. Breeders will then evaluate these transgenics for other traits to determine their characteristics.

These results demonstrate that an attempt to transform indica rice can be made with less than complete molecular information. Even in the case of a very large protein (the Xa21 protein is about 113 kDa), it is possible to express it correctly and above all to obtain the expected phenotype. It will be possible to use BAC subclones directly in transformation experiments and to immediately begin the search for the phenotype—if a very efficient transformation system like that currently used for japonicas becomes available. When the effective clone has been precisely identified, we have demonstrated that it is possible to transfer a useful gene to improve indica varieties.

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Notes

Authors' address: Tropical Agricultural Biotechnology (ILTAB)/L'institut français de recherche scientifique pour le développement en coopération (ORSTOM), The Scripps Research Institute, Division of Plant Biology-MRC7, 10666 N. Torrey Pines Rd, La Jolla, CA 92037, USA.

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