# RICE BIOTECHNOLOGY QUARTERLY

VOLUME 1, 1989

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# Rice Biotechnology Quarterly

At the 1989 Annual Meeting of the Rockefeller Foundation Program on Rice Biotechnology at Columbia, Missouri, it was suggested in one of the workshops that a publication be developed devoted to recent advances in biotechnology, especially as they apply to rice.

The publication would include articles about research in the fields of tissue, cell, anther and protoplast culture, wide hybridization, RFLP mapping, gene sequencing and manipulation, in situ hybridization, gene transfer, transposable elements, mutagenesis, gene isolation, regulation, and analysis, and many other areas.

The idea was to produce a vehicle that would allow fast dissemination of useful information to those researchers all over the world who are working with rice: a way for the scientist to keep up with what was going on in the laboratories of his colleagues, a way to share new ideas and methodologies, a way to bring all of us closer together so that communication becomes easier.

Gary Toenniessen, Director of the RF Program on Rice Biotechnology, suggested that this new publication be published quarterly and be made available not only to those participants of the Annual Meetings, but also to anyone interested in biotechnology and rice.

This first issue contains information from research published during the last several months of 1988 and the first four months of 1989. Future issues will feature recently published research and contributions directly from the labs of RF Annual Meeting participants along with dates of up-coming conferences, symposia, and other sorts of information that might be meaningful to scientists working in the area of rice biotechnology.

We are indebted to the Rockefeller Foundation for their generous support.

We cordially invite manuscripts, abstracts, new findings, short lab reports, or other communications from researchers who use the tools and techniques of molecular biology to investigate the many facets of the rice plant. Please send submissions:

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<u>Rice Biotechnology Quarterly</u>

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## Asymmetric Protoplast Fusion Produces Rice Cybrids

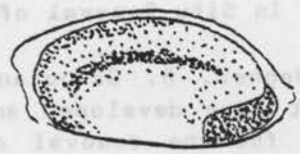
Cytoplasmic male sterility in rice may be determined by the mitochondrial genome as it is in other plants. The transfer of maternally inherited traits (including the mitochondrial genome) by protoplast fusion in monocots has been reported only once, between rice and barnyard grass. By using X-irradiation (inactivates the nucleus) and iodo-acetamide (IOA) (inactivates the cytoplasm), H. Akagi, M. Sakamoto, T. Negishi, and T. Fujimura, Mol. Gen. Genet. 215:501-506 (1989), were able to construct cybrid plants between cytoplasmic male sterile and fertile rice.

The cytoplasmic male sterile rice stain used in this work was MTC-9A. A mutant line, Norin 8, which lacks the aryl acylamidase I gene (which breaks down propanil herbicide) was used as a recessive marker.

Protoplasts were isolated, treated with either X-irradiation or IOA, and placed in a fusion chamber for electrofusion. The cell clusters were cultured for 3 weeks and transfered to callus growth medium. Upon reaching 5 mm in diameter, they were transferred to plant regeneration medium and eventually grown in a greenhouse.

MtDNA from regenerated plants exhibited PstI and Bg/II restriction patterns with fragments specific to both parents. Cytogenetic analysis showed 26 plants with the normal diploid number (2n=24) whereas 12 plants were tetraploid (2n=48). These may represent fusion between 1 CMS cytoplast and 2 N8 protoplasts. Only 2 of 560 regenerated plants set seed at levels similar to the N8 controls. About 80% were completely sterile.

Such cybrids may provide crucial information that will allow analysis of mtDNA from recombinants and provide an understanding of the causes of male sterility in rice.

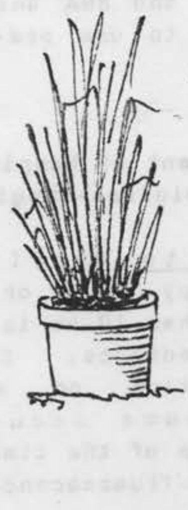


## Fertile Rice from Transformed Protoplasts

K. Shimamoto, R. Terada, T. Izawa, and H. Fujimoto have reported in Nature 338:274-276 (1989) that they have produced fertile transgenic rice plants by transferring both the <u>hph</u> gene (bacterial gene encoding hygromycin B resistence) and the beta-glucuronidase (GUS) gene to protoplasts by electroporation.

Southern blot analysis and Southern hybridization confirmed the presence of the <u>hph</u> gene on the chromosomes of the stable transformants. GUS activity was also demonstrated in the seed and leaf extracts.

This work shows that it is possible to co-transform using both a selectable marker and a non-selectable gene. Such a technique appears as a simple alternative method for generating transgenic plants.



## In Situ Removal of RNA

P. Wagner, B. Oskouian, and G.C. Stewart have developed an effective method for the removal of RNA from plasmid samples when screening by electrophoresis. They report in BioTechniques 7(4):338-339 (1989) that by simply adding RNase directly to the agrose gel during its preparation, RNA is effectively removed making DNA visualization much clearer and the bands sharper.

RNase is added, prior to pouring, at a concentration of 10 ul/ml. It appears that RNA removal is a function of time and migration of tracking dye of less than 2.5 cm resulted in the removal of almost all the RNA.

## Dry Iceless Nucleic Acid Precipitation

For laboratories with short supplies of dry ice but access to a -70C to -80C freezer, C. Bird and G. Wu suggest the following in BioTechology 7(4):337-338 (1989). Fill an empty micropipet box (rack removed) to a depth of 4 cm with 80% isopropanol and place in the freezer til equlibrated. The solution forms a slush into which the microtubes can be inserted. Return the box to the freezer for precipition. This technique has been successful on both DNA and RNA and it eliminates the need to use pre-chilled (-20C) ethanol.

## Enhancement of Mapping Single-Copy DNA by Digital-Imaging Microscopy

_In situ_ hybridization of single-copy genes or DNA sequences shorter than 10 kb is often a matter of percentages, that is, the hybridization on any particular chromosome occurs a certain percentage of the time. By using intensified-fluorescence digital-imaging microscopy, E. Viegas-PequignotB. Dutrillaux, H. Magdelenat, and M. Coppey-Moisan report in Proc. Natl. Acad. Sci. USA 86:582-586 (1989) that such statistical analysis of spot distribution is unnecessary.

Slides of **human lymphocytes** were produced and stored at -20C until use. Two probes (6 kb and 2.3 kb) were labeled with bio-11-dUTP. Slides were treated with RNase and the chromosomes were denatured with 70% formamide in 2XSSC at 70C for 2 or 3 min. After rinsing and dehydration, the slides were flooded with the hybridization mixture and covered with a plastic film for incubation overnight at 42C. The slides were again rinsed and stained with propidium iodide and mounted with p-phenylenediamine.

The digital-imaging microscope enhanced the fluorescence signal of the probes to such an extent that unambiguous visualization of the spots could be observed. These probes hybridized to chromosomes 18p and 22q. In every metaphase plate observed, no spots were found on both chromatids of a chromosome other than 18 and 22.

These workers claim that best results are obtained when probes are denatured separately from the chromosomes and that the percentage of cells with labeled chromatids increases with the concentration of the probe.

## Immunoassays of Humicola Fungus in Rice

Much post-harvest deterioration of rice is due to the presence of fungi which reduce nutritional value, change the color, flavor, and texture, and even pose a health hazard through exposure to mycotoxins. Early detection by classical procedures is difficult so F.M. Dewey, M.M. Macdonald, and S.I. Phillips developed several monoclonal antibody assays that recognize Humicola lanuginosa in plant tissues, Jr. Gen. Microbiology 135(2):361-374 (1989).

The immunogen was obtained by washing fungal cultures with phosphate-buffered saline. This was injected into Balb/C mice. Their spleens were removed 4 d after the last booster injection and the splenocytes fused with myeloma cell line SP2/0-Ag14 using PEG.

For the DOT-BLOT immunoassay, polyvinylidine difluoride membranes proved superior to nitrocellulose. Individual grains were soaked overnight at 4C in PBS in Eppendorf tubes to allow fungal antigens to diffuse into solution. Droplets of the diffusate were placed onto the membrane and air-dried. After re-wetting with methanol, the membranes were blocked for 30 min, washed, and exposed to the primary antibody for 2 h. They were then incubated with goat anti-mouse IgG-IgM gold conjugate, washed, silver enhanced, and washed again.

For the DIP-STICK immunoassay, 2X25 mm strips of the membrane were soaked overnight with the grains in PBS, air-dried, and processed as above.

For the SQUASH-BLOT immunoassay, rice grains from the DIP-STICK assay were pressed onto the surface of the membrane and processed as above.

The IgM antibody EC6 worked best with the DIP-STICK assay and proved to be simple to perform and suitable for field use.

## Localization of Proteins and Enzymes by Tissue Printing

G.I. Cassab and J.E. Varner, Cell Biology Intern. Reports 13(1):147-152 (1989), have developed a tissue printing technique to screen for the presence of extensin, a hydroxy proline-rich glycoprotein present in the cell walls of a variety of plants and algae. The simple process consists of slicing through the tissue of interest with a razor blade and pressing the freshly cut surface onto a piece of nitrocellulose paper for 15-20 sec which had been pre-soaked in 0.2 M $CaCl_2$.

The paper is dried and treated with extensin primary antibody for detection of AP-conjugated second antibody with 5-bromo-4-chloroindoxylphosphate and nitroblue tetrazolium.

Tissue prints of soybean seeds show that the distribution of extension resembles that shown by the traditional immunocytological localization of proteins. Tissue printing has also been used to follow peroxidase activity in ethylene treated etiolated pea epicotyls. It appears to be a simple technique suitable for studies of proteins, enzymes, and anatomy of imprinted tissues.

**Detection of Legumin Gene DNA Sequences in Pea**

P.R. Simpson, M.A Newman, and D.R. Davies, Chromosoma (Berl) 96:454-458 (1988) have shown protoplast preps of plant chromosomes to be superior to the standard squash technique and streptavidin-enzyme conjugates to be more sensitive than the antibody-fluorochrome system in detecting low copy number sequences in **pea** during in situ hybridization.

Root tips of cv Greenshaft were incubated in 0.7mM bromonaphthalene at room temperature for 2 h, rinsed, and fixed in methanol/acetic acid (3:1) overnight at 4C. Protoplasts were produced by rinsing twice in cold citrate buffer at pH 4.6 and digested in 1 ml of 1% cellulysin, 1% macerozyme in citrate buffer at 37C for 1 h. After washing twice in citrate buffer, twice in 1 ml cold fixative and resuspended in 400 ul of fresh fixative, small drops (20 ul) of the protoplast suspension were applied to the slides and air dried.

Slides were then rinsed in 2XSSC for 20 min, digested in preboiled RNase A in 2XSSC at 37C for 1 h, rinsed in 2XSSC for 5 min, and dehydrated in an alcohol series. The air-dried slides were stored at 4C.

Several denaturation regimes were tried but the best proved to be with the hybridization mixture consisting of 50% formamide, 10 mM Pipes, pH 8.0, 1 ;mM EDTA, 0.3 M NaCl, 10% dextran sulphate, 25 ng probe DNA, and 500 ug/ml denatured slamon sperm DNA. Ten ul of this mixture was added to each slide and sealed with a coverslip. After incubation for 18 h at 37C, the coverslips were removed, slides were rinsed twice for 30 min in 50% formamide, 30 min 2XSSC at 37C, and 15 min 2XSSC at room temperature.

The DNA probe in the hybridization mixture above was labelled with bio-11-dUTP or bio-7-dATP by adding 3 ul of hexamers at 90 units/ml to 28 ul water containing 1-2 ug uncut DNA, boiling for 3 min and chilling on ice. The polymerization reaction contained 20 uM dATP, dCTP, dGTP, 20 uM bio-11-dUTP (or bio-7-dATP), 80 mM Hepes, pH6.6, 5-7 units of Klenow fragment, 20 ug BSA, 0.5 mM $MgCl_2$, 0.05 M Tris-HCl, pH 8.0 which was incubated at room temperature for 16-18 h. Ethanol precipitation with 0.3 M sodium acetate was used to remove unincorporated nucleotides from the probe DNA. DNA was resuspended in 40 ul 1XTE and used in the hybridization mixture above.

Several methods were used to detect biotinylated hybrids but the most effective was with streptavidin-alkaline phosphatase conjugate (SAP). Slides were rinsed in TMN for 15 min and incubated in 50 ul 5% w/v non-fat dry milk in TMN for 10 min, rinsed in TMN, and incubated in 50 ul 1:500-1:1000 dilution SAP in TMN containing 3% BSA for 1 h. The slides are again rinsed in TMN, rinsed in ph 9.5 buffer, and incubated in the dark in 150 ul of the substrate solution: 3.3 ul 5-bromo-4-chloro-3-indolyl-phosphate (50 mg/ml in 100% dimethyl formamide), 4.4 ul nitroblue tetrazolium (75 mg/ml in 75% dimethyl formamide) per ml of pH 9.5 buffer for 1-4 hr.

The reaction was stopped in 1XTE buffer for 1 min and the slides rinsed 3 times in distilled water before staining with Giemsa and mounting in Canada balsam.

This method is sensitive enough to detect a 13.5 kb legumin gene clone located next to the telomere of one chromosome. Work by others have detected the 17 kb Ti sequence in Crepis, and 10.8 and 1.9 kb sequences in maize pachytene chromosomes using $^3$H-labelled probes.

## Three-color Fluorescence *in situ* Hybridization

In Cytometry 10:20-27 (1989), P.M. Nederlof, D. Robinson, A. Avuknesha, J. Wiegnant, A.H.N. Hopman, H.J. Tanke, and A.K. Raap describe a method for visualizing three nucleic acid sequences simultaneously using three different haptenized probes combined with three fluorochromes. Although previous workers have used double hybridizations, this is the first time for triple hybridizations. The success of this technique was due to the use of a newly developed blue immunofluorochrome, aminomethyl coumarin acetic acid (AMCA). Metaphase preparations were made from human lymphocytes and ovarian tumor material. Slides were air dried, washed in PBS, dehydrated with ethanol and stored at 4C.

Probes for four different chromosomes were modified with N-acetoxy-AAF, biotinated with Bio-dUTP, or mercurated. The hybridization mixture contained 50% or 60% formamide, 2XSSC, 1nM KCN, salmon sperm DNA, and the probes. Denaturation took place under a sealed coverslip for 2-4 min at 80C. The slides were incubated for 16 h at 37C for hybridization. After washing twice (20 min each) in formamide, 2XSSC, and 1mM KCN, the slides were rinsed for 20 min in the same solution without the formamide.

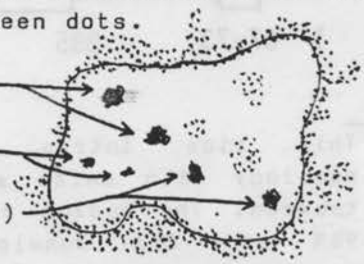
To detect the mercurated probes, the slides were treated with mercury binding sulfhydryl-tri-nitrophenyl ligand in 3XSSC, 1mM EDTA, and 0.05% Tween 20 for 1 h at room termperature at pH 5. To prevent cross reactions from occurring between the secondary antibodies, three steps were necessary using a mixture containing the appropriate antisera in PBS with 2% normal goat serum and 0.05% Tween 20. After incubation for 45 min at 37C, the slides were washed with PBS (made with 0.05% Tween 20) for 5 min. The antisera used for step one: monoclonal mouse anti-AAF IgGI (diluted 1:500) combined with sheep anti-TNP Ig (diluted 1:500). The second step used TRITC-conjugated avidin (diluted 1:1000) combined with FITC-conjugated rabbit anti-sheep Ig (diluted 1:150). The third step contained AMCA-conjugated sheep anti-mouse IgG (diluted 1:150) combined with N-hydroxysuccinimide-AMCA. Lastly, the slides were dehydrated by an ethanol series and mounted in PBS-glycerol (1:9) containing 1,,4-di-azobicyclo-(2,2,2)-octane (2.3% w/v).

The excitation and emission spectra are well separated and the fluorochromes appear as three distinct colors with little overlap. For example, chromosome 15 was hybridized by the mercurated probe, chromosome 1 by the AAF labeled probe, and chromosome 18 by the biotinated probe. In triple-exposed color photomicrographs, three colors are observed: the centromere regions of chromosome 1 appear as two blue dots, chromosome 18 is labeled with red spots, and chromosome 15 is labeled with green dots.

blue (#1)

red (#18)

green (#15)



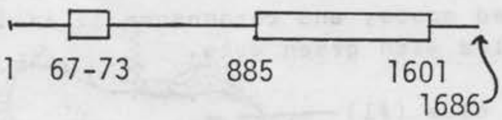
The interphase nuclei also exhibit labeling. Such simultaneous labeling by multi-colored probes may provide more information than would be observed by separate hybridizations.

An obvious application of multiple *in situ* hybridization would be in those cytogenetic studies where rapid analysis of material precludes detailed karyotypic analysis. In such cases, the use of "color-coded" probes for common chromosomes involved in aberrations would greatly facilitate the analyses.

### Chloroplast b6 Gene Nucleotide Sequence

J.C. Cote, N.H. Wu, and R. Wu report in Plant Molecular Biology 11:873-874 (1988) that they have determined the nucleotide sequence of the **rice** chloroplast apocytochrome b6 (petB) gene using the dideoxynucleotide chain termination method.

Chloroplast DNA from cv. Labelle was digested with HindIII, run on agarose, and transferred to Nytran. The probe consisted of a radioactive fragment (309 bp) containing a section of the spinach chloroplast apocytochrome b6 gene. It hybridized to a single 2.5 kb DNA band which was electroeluted and ligated to HindIII-digested pBR322 and transformed into HB101 competent cells. After colony hybridization, one clone was sequenced and found to contain an 813 bp class II-like intron between nucleotides 73 and 885.




This rice intron shares 86% homology with maize and 63% with tobacco. The coding region shares 98% and 99% homology at the predicted amino acid level with maize and tobacco respectively.

### Genetic Analysis of Rice Regeneration

J. Peng and T. Hodges, In Vitro Cellular & Developmental Biology 25(1):91-94 (1989), used reciprocal crosses of four genotypes (IR8, 36, 54, and 64) in a diallel study in an attempt to understand the genetic mechanism of regeneration from immature embryo-calli.

Plants were grown in the greenhouse for 40 days and then transferred to a short-day growth chamber to stimulate initiation of flowering. Crosses were hand-made in all possible combinations of the four parents. At 7-10 days post-anthesis, immature seeds were collected from the parental varieties and at 6-9 days after pollination for the F1 hybrids. Immature seeds were sterilized, the embryos excized, and plated in callus induction medium composed of N6 basal salts, 3% sucrose, 2 mg/l 2,4-D, 0.5 mg/l 6-BA, 50 mg/l tryptophan, 0.8% agar, and adjusted to pH 5.7 before autoclaving. After three weeks in the dark at 26C, the calli were transferred to light and a regeneration medium containing the same components as the induction medium but omitting the 2,4-D and only 0.2 mg/l 6BA.

The frequency of callus induction of the four parents varied from 90% in IR8, to 100% in IR54 and IR64. However, the regeneration frequency of the four parents was: 0% (IR36), 46% (IR8), 47% (IR64), and 86% (IR54). The hybrids that showed the highest mean plant regeneration frequency were IR54/IR36 and its reciprocal.

The highly significant variences of the general combining ability and specific combining ability indicates that the nuclear genes involved in plant regeneration exhibit both dominant and additive effects. A similar situation has been noted in wheat regenerated from anther derived calli.

These results suggest that the genetc background of elite varieties with poor regeneration response could be incorporated into genotypes with high regeneration abilities. They recommend that genetic effects and environmental conditions both be taken into account to improve and increase plant regeneration.

## Salt-Tolerant Derivatives Limit Accumulation of Ions

D.P. Schachtman, A.J. Bloom, and J. Dvorak in Plant, Cell and Environment 12:47-55 (1989) report on a study that examined changes in salt-stress responses due to the addition of the E genome (Lophopyrum) to the A, B, and D genomes of hexaploid bread **wheat**. They compared parents and derivatives of two crosses: wheat cv. Chinese Spring X L. elongatum (a close relative of wheat which shows a high degree of salt tolerance) and PI178704 (fairly salt tolerant wheat accession) X L. ponticum.

Seeds of the four lines were surface sterilized and germinated on moist filter paper. Chromosome analyses were performed on the root tips and the plants were transferred to two hydroponic tanks. Each tank contained half-strength aerated Hoagland solution. The pH was adjusted to 5.5 by sulfuric acid or potassium hydroxide. Salt stress was imposed by increasing sodium chloride concentration in one tank. A final concentration of Na+ was maintained at 0.2 M. Another experiment was set up with a stronger concentration of NaCl because the previous concentration did not challenge the PI178704 plants.

Plants were harvested and divided into components; roots, shoots, oldest leaves, and newest leaves. The tissue was dried and analyzed for ion content.

The wheat lines with chromosomes from the salt tolerant Lophopyrum showed lower concentrations of Na+ and Cl- and accumulated more dry weight under salt stress than the wheat parents. This may indicate a causal association between low shoot Na+ and Cl- accumulation and salt tolerance. These results are similar to those found in other halophytes.

## Low Temperatures and Tonoplast-Plasma Membrane ATPases

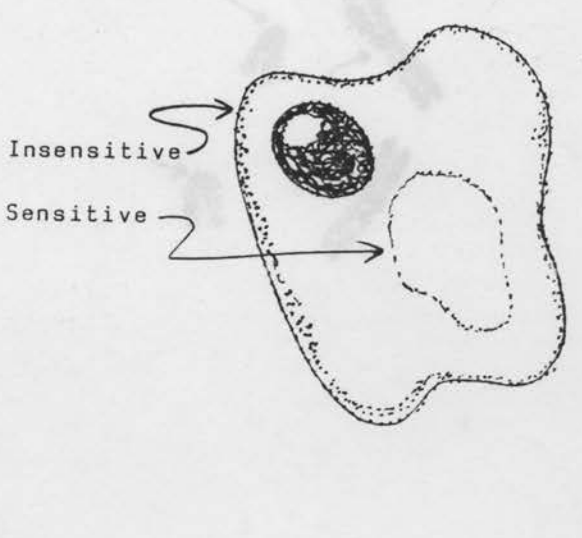
K. Kasamo reports in Plant Cell Physiology 29(7):1085-1094 (1988) on the response of the ATPases of the tonoplast and plasma membrane in chilling-sensitive and chilling-insensitive **rice** culture cells to low temperatures.

Callus was initiated from the roots of seedlings growing on agar and maintained on N6C medium with agar. From 3 months to 2 years later, suspension cultures were started in liquid N6C medium on a reciprocating shaker at 25C.

After exposure to chilling temperatures (5-10C for 2-10 days), callus and suspension cells were homogenized and treated to produce tonoplast-rich and membrane-rich pellets. By solubilizing these membranes, the ATPases could be recovered.

The chilling insensitive rice varieties included Yuukara, Nipponbare, and Hayayuki. The chilling sensitive varieties included Wasenishiki, Had sadri, Boro, and Boro II.

The results show that the tonoplast ATPase is more sensitive to low temperature than the plasma membrane ATPase. This difference is probably due to interactions between components of the membranes, the proteins and the phospholipids.



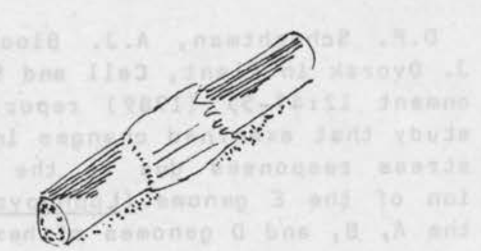
## Double *in situ* Hybridization

In Experimental Cell Research 181:126-140 (1989), P. Emmerich, P. Loos, A. Jauch, A.J.N. Hopman, J. Weigant, M.J. Higgins, B.N. White, M.V. Der Ploeg, C. Cremer, and T. Cremer describe their work dealing with double *in situ* hybridization using biotinylated and mercurated DNA probes which were analyzed by the production of digital images.

Slides were prepared of human lymphocytes and cells from amniotic fluids and fibroblasts. Two probes were used: one contained a tandemly organized sequence isolated from the human satellite DNA fraction II/III and the second contained a 1.8 kb Sau3A fragment. Each was nick-translated with biotin-11-dUTP or mercurated with mercury acetate.

For double hybridizations, both probes were used at about 1ug/ml in a hybridization mixture containing 65% formamide and 2XSSC.

Camera lucida drawings were digitized and stored in a VAX 11-780 computer to produce 2D images. Appropriate analyses of these images indicated that, at least in this material and with chromosomes 1 and 15, there is no distinct association of homologous chromosomes in somatic cells. This technique enabled the researchers to simultaneously determine the positions of heterochromatin of two different chromosomes during interphase.



## Chromosome Doubling in Haploid Rice

C.K. Wong reported on a new approach to doubling the chromosome number of haploid rice plants in Theoretical and Applied Genetics 77:149-151 (1989). Haploids produced by anther culture were mass produced by excising nodal segments and planting in vermiculite watered with a simple inorganic nutrient solution. At or just after anthesis, nodes (about 3 cm on either side of the node) of the haploid plants were harvested into plastic bags and incubated 12-18 h at 30C.

The nodal segments were then treated with either 0.3% or 0.4% colchicine containing 2% DMSO at 30C for 6-10 h. After rinsing for 12-18 h, the cut ends were coated with 50% Benlate powder (DuPont) and planted in vermiculite moistened with nutrient solution.

Although the treated plants were slower to sprout (10-20 days later) and appeared stunted, 42% survived the doubling treatment. And 12% of the plants that went on to produce seeds were shown to be diploid or diploid-like.

Additional experiments indicated that by growing the treated segments at a lower temperature of 26C, the survivial rate could be increased as high as 85%. Also the use of IBA at 50-100 ppm would increase rooting frequency.

## Exponential Amplification of Hybridization Probes

In Bio/technology 6:1197-1202 (1988), P.M. Lizardi, C.E. Guerra, H. Lomeli, I. Tussie-Luna, and F.R. Kramer reported on the synthesis of of recombinant RNA molecules that function both as probes and as templates of exponential amplification by QB replicase. This enzyme initiates the synthesis of a large number of RNA strands, greater than a million-fold increase as a result of the exponential reaction mechanism. That is, single-stranded RNAs are copied into complementary single stranded products which in turn serve as templates in the next round of synthesis. As long as there is an excess of the enzyme QB replicase, the number of RNAs increases exponentially.

Working with a repetitive DNA sequence from **Plasmodium falciparum**, these workers constructed an RNA molecule that would serve as a probe and template. Four different RNAs were produced: MDV-1, MDV-fal-un, MVD-fal-st, and MVD-poly.

Using MVD-fal-un at decreasing amounts with QB replicase, it was shown that by starting the reaction mxture with as few as 1000 molecules of recombinant RNA, in just 30 min there was a one billion fold increase in the amount of RNA. Similar results were obtained with the other two probes.

In addition, each of the newly constructed RNAs bound specifically to denatured plasmids containing target sequences of P. falciparum.

This method may provide an alternerative to the exponential amplification of targets by the PCR technique (DNA polymerase chain reaction). Such amplification should permit detection at extremetly low levels of target, increase speed of detection, and provide for simultaneous detection by using a mixture of different probes.

## Transgenic Rice After Direct Gene Transfer

K. Toriyama, Y. Arimoto, J. Uchimiya, and K. Hinata in Bio/Technology 6:1072-1074 (1988) report they have regenerated whole plants from protoplasts which had been electroporated with a plasmid gene coding for aminoglycoside phosphotransferase II (APH(3')II).

Fragments produced by BamHI and BglII on pHlKl which contained APH(3')II and CaMVT were inserted into the promoter terminator cassette of CaMV35SP/NosT of pUC12.

Protoplasts were isolated from anther-derived cell suspensions of cv. Yamahoushi, electroporated, and transferred to 2.5 ml B5 medium supplemented with 2 mg/l 2,4-D and 5% mannitol. After two weeks, this medium was supplemented with 1 ml B5 medium (without ammonium sulphate, 2 mg/l 2,4-D, and 3% glucose). After three weeks, the medium was replaced with B5 medium (without ammonium sulphate, 2 ug/ml G418 sulphate. One month later, microcalli were transferred to B5 medium (without ammonium sulphate, 20ug/ml G418 sulphate and 1% agarose). Finally, two weeks later, growing calli were transferred to N6 medium containing 0.2 mg/l indol-3-acetic acid, 1 mg/l kinetin, and 1% agarose to stimulate regeneration.

DNA was digested with HindIII and EcoRI, electrophoresed, blotted onto a nitrocellulose filter, and hybridized with the DNA probe (Amersham, multi-translated).

Five plants were regenerated which were not albino and which did not bleach in the presence of 50 ug/ml kanamycin sulphate. APH(3')II activity was detected in leaf extracts. Similar results have been shown in maize protoplasts.

## RFLPs Associated with Water Use Efficiency

Working with a large number of F3 and backcross families obtained by crossing the drought-sensitive **tomato** cv. UC82B and UC204B, with the wild drought-tolerant species <u>Lycopersicon pennellii</u>, B. Martin, J. Nienhuis, G. King and A. Schaefer reported in Science 243:1725-1728 (1989), that they had found three RFLPs strongly associated with water use efficiency. Evaluation for this component of drought-resistance has been unreliable in field grown plants. RFLPs have already been associated with insect resistance and soluble solids in tomato.

Plants were grown in the field and thinned to 20 plants spaced 30 cm apart within rows. The soil was kept moist during seedling growth. About two months later, irrigations were reduced to evaluate the performance of plants under drought. Leaves were sampled and combusted under vacuum and the carbon dioxide formed was released into a mass spectrometer to determine the ${}^{13}C/{}^{14}C$ ratio. This ratio had earlier been shown to be an excellent predicter of water use efficiency in plants including wheat, peanut, and barley.

Eight families with a high ratio and 11 with a low ratio were selected for RFLP analysis. Seeds were harvested from these plants, grown for 2 months, and their leaves lyophilized and ground for DNA extraction. The DNA was digested with BglII or HindIII, subjected to electrophoresis, and blotted onto nylon membranes. Seventeen probes were hybridized to the blotted DNAs which covers 12 of the 19 known linkage groups in tomato. Analysis of the data indicated significance for all three RFLP markers.

Each of these markers could be associated with several or more genes but such work should open new ways of studying water use efficiency and other difficult traits.

## Transient Expression After Particle Bombardment

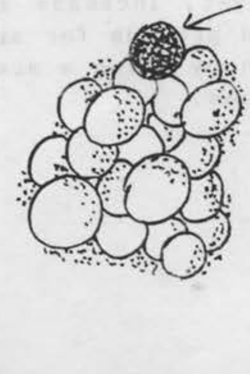
In Plant Molecular Biology 11:433-439 (1988) Y.C. Wang, T.M. Klein, M. Fromm, J. Cao, J.C. Sanford, and R. Wu describe a technique to transfer foreign genes directly into **rice**, wheat and soybean cells. Callus of rice dv. Taipei 309 was produced from immature embryos and developed into a cell suspension which was cultured in medium containing 3% sucrose, 2 mg/l 2,4-D, modified White's vitamin and an AA formula (uses amino acids as the nitrogen source).

All cell suspensions were placed on a rotary shaker at 120 rpm, in the dark, and at 26C. Twice a week, the rice cell suspensions were subcultured. The plasmids used were $pAI_1GusN$ and $pCaMVI_1CN$. Recombinants were grown in <u>E. coli</u>, lysed with alkali, phenol extracted, and purified by CsCl/ethidium bromide density centrifugation.

Before bombardment with the DNA coated particles, the cell suspension was applied to 3 layers of filter paper and placed in a plastic Petri dish. After bombardment, the cells were incubated for 48 h in the dark at 26C.

Intact cells of rice (and wheat) produced detectable levels of GUS activity. Rice cells (and wheat, maize, and soybean) that had been bombarded with particles coated with plasmid DNA containing the CAT gene also showed expression.

This method can avoid the difficulties encountered during the regeneration of plants from protoplasts. The frequency of foreign gene transfer is estimated as 1/1000. The next step is to obtain a stable incorporation of the foreign genes.



## Efficient Transformation of Potato

An efficient method of obtaining transgenic **potato** plants has been reported by R. Tavazza, M. Tavazza, R.J. Ordas, G. Ancora, and E. Benvenuto in Plant Science 59:175-181 (1988).

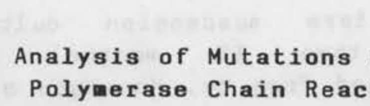
Leaf discs were inoculated with Agrobacterium tumefaciens LBA4404 containing a binary vector, pGA492, in which the tumor inducing genes had been replaced by Neomycin phosphotransferase, a selectable marker.

By using a feeder layer technique and selecting transformants for kanamycin resistance, these workers were able to isolate transgenic plants within just 4 weeks. Several factors influence transformation frquencies: 1) use plants younger than 3 weeks, 2) keep termperatures of growth below 19C.

Since the adventitious shoots grew rapidly with little callus formation, it is expected that such plants should exhibit greater genetic stability. Other workers have also reported minimum variation in plants produced by this technique.

## Analysis of Mutations Using Polymerase Chain Reaction

In the technical newsletter Clontech Cruiser 1(1):1-6 (1989), R. Frye, P. Cogswell, and E. Liu describe the methods they currently use to detect ras mutations in **human** tissues. There are three ras genes that code for three similar 21 kd proteins located in the cell membrane and serve in a regulatory capacity. All three display intrinsic GTPase activity, converting GTP to GDP. The ras gene was shown in 1982 to be responsible for induced transformation of mouse cells to tumor cells.



The polymerase chain reaction (PCR) can be used to amplify DNA segments in regions of the three ras genes where mutations occur. The PRC-amplified DNA segments are then analyzed by sequencing or probe hybridization. Amplification takes several hours and can be done in an automated thermal cycler, thanks to the availability of heat stable Taq DNA polymerase.

To amplify the genomic target DNA, 0.5 ug is mixed with amplification primers, Taq polymerase, PCR buffer, 10X dNTP stock solution, and water. Mineral oil is layered over the solution to prevent evaporation. The first cycle of amplification includes denaturation at 94C for 3 min, annealing at 50C for 30 secs, and primer extension for another 30 secs. All forty subsequent cycles were run for 30 secs at each temperature. These researchers have found the concentration of Mg++ to be critical: less than 1.5 mM gives almost no amplification and more than 5 mM causes increased random priming.

After PCR amplification, the mineral oil is removed by extraction with cloroform. About 80 ul of the amplified sample is labeled and hybridized onto filters. Using these techniques, these researchers have been able to analyze the DNA from small numbers of cells, as few as 30 cells.

They found that, depending on the GC content, the temperature of denaturation and annealing may need to be adjusted. The higher the GC content, the higher the temperature needed to reduce nonspecific binding. Also, the wash conditions should be monitored very carefully. They recommend measuring the temperature of the wash container, not the water bath, and keeping the water bath covered.

### Ultrastructural Resolution with In Situ Hybridization

G.I. McFadden compares in Cell Biology International Reports 13(1): 3-21 (1989) several in situ methods for localization of RNAs in plant tissues. Of the isotopic probes used on various plant tissues (**pea, barley,** and **tobacco**), the finest resolution was achieved with $^{3}H$. But it was found that non-radioactive probes (biotinylated) produced even finer resolution than isotopic labels, although with reduced sensitivity.

### Differential Expression of mRNA and a Cloned Histone Gene in Rice

There are many problems associated with the biochemical analysis of gene expression in the anthers of **rice**. Some of these problems may be overcome by using cytological methods for the detection of macromolecules according to V. Raghavan in Journal of Cell Science 92:217-229 (1989). He determined the localization of mRNA and a histone gene during microsporogenesis by hybridization of $^{3}H$ poly(U) and cloned $^{3}H$ histone asymmetric RNA probes.

Florets and anthers were fixed in 70% ethanol/acetic acid (3:1) for 24 h, dehydrated, and embedded in glycol methacrylate. Sections were attached to slides and dried overnight at 55C. The slides were rinsed in hybridization buffer, and treated with 80 ul of the hybridization mixture, sealed with a coverslip, and incubated at 47C for 4 h. After incubation the slides were rinsed in cold tap water, hybridization buffer, and RNase digestion buffer. Pancreatic RNase was used to remove unhybridized $^{3}H$ poly(U). The binding sites were detected by dipping the dry slides in diluted Kodak NTB-3 liquid emulsion. After 4 weeks in the dark, the slides were developed and stained with 0.1% eosin Y, dried and mounted.

The histone gene was a 1.3 kb insert from an IR26 genomic library. This sequence shows 80% and 92% homology with the H3 histone genes of sea-urchin and wheat, respectively. After hybridization, the slides were developed and stained in Toluidine Blue, dried, and mounted.

Results show that poly(A)+RNA and the histone message are not localized in the same pattern, histone mRNA is not necessarily found in rapidly dividing cells, and that the lack of binding of the probe to the generative cell may indicate that histones are not involved in the regulation of transcription in anther cells.

### High-Frequency Embryogenesis in Rice

Long term suspension cultures (older than 18 months) were established from cv. Konansou by K. Ozawa and A. Lomanine, Theor Appl Genet 77:205-211 (1989). Subculturing occured every three days with N6 medium supplemented with 2,4-D (1 mg/l), sucrose (30 g/l), casein hydrolysate (300 mg/l), and proline (10 mM). One millimeter cell clusters were transferred to solid N6 medium with kinetin (5 mg/l), NAA (1 mg/l), sucrose (30 g/l), and Gelrite (2 g/l). The frequency of embryogenesis was estimated at 90%. Smaller clusters produced lower rates of embryogenesis, about 50%. This is the first report on the establishment of high-frequency embryogenesis from long-term cell suspensions of rice.

# REFERENCES


Akagi, H., M. Sakamoto, T. Hegishi, and T. Fujimura, 1989. Construction of rice cybrid plants. Mol. Gen. Genet. 215:501-506. (Mitsui Toatsu Chemicals, Plant Biotechnology Department, 500, Nishikubo, Chigasaki City, Kanagawa Pref., 253, Japan)

Bird, R.C., and G. Wu, 1989. An alternative to the dry ice/ethanol bath for the precipitation of nucleic acids. BioTechniques 7(4):337-338.(Department of Pathobiology, College of Veterinary Medicine, Auburn University, AL, 36849, USA)

Cassab, G.I., and J.E. Varner, 1989. Tissue printing on nitrocellulose paper: a new method for immunolocalization of proteins, localization of enzyme activities and anatomical analysis. Cell Biology International Reports 13(1):147-152. (Plant Biology Program, Department of Biology, Washington University, St. Louis, MO, 63130, USA)

Cote, J.C., N.H. Wu, and R. Wu, 1988. Nucleotide sequence of the rice chloroplast apocytochrome b6 gene (petB). Plant Molecular Biology 11:873-874. (Section for Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY, 14853, USA)

Dewey, F.M., M.M. MacDonald, and S.I. Phillips, 1989. Development of monoclonal-antibody-ELISA, -DOT-BLOT and -DIP-STICK immunoassays for <u>Humicola lanuginosa</u> in rice. Jr. Gen. Microbiology 135(2):361-374. (Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RA, United Kingdom)

Emmerich, P., P. Loos, A. Jauch, A.H.N. Hopman, J. Weigant, M.J. Higgins, B.N. White, M. Van der Ploeg, C. Cremer, and T. Cremer, 1989. Double <u>in situ</u> hybridization in combination with digital image analysis: a new approach to study interphase chromosome topography. Exp. Cell Res. 181:126-140. (Institute of Anthropology and Human Genetics, University of Heidelberg, INF 328, D-6900 Heidelberg, Federal Republic of Germany)

Frye, R., P. Cogswell, and E. Liu, 1989. The analysis of mutations in the <u>ras</u> gene family using the polymerase chain reaction (PCR). Clontech Cruiser 1(1):1-6. (227 Lineberger Cancer Research Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599, USA)

Lizardi, C., C.E. Guerra, H. Lomeli, I. Tussie-Luna, and F.R. Kramer, 1988. Exponential amplification of recombinant-RNA hyridization probes. Biotechnology 6:1197-1202. (Centro de Investigacion sobre Ingenieria Genetica y Biotecnologia, Universidad Nacional Autonoma de Mexico, Apartado Postal 510-3, 62270, Cuernavaca, Morelos, Mexico)

Kasamo, K., 1988. Response of tonoplast and plasma membrane ATPases in chilling-sensitive and -insensitive rice (<u>Oryza sativa</u> L.) culture cells to low temperature. Plant Cell Physiol. 29(7):1085-1094. (Department of Cell Biology, National Institute of Agrobiological Resources, Kannondai, Tsukuba Science City, Ibaraki 305, Japan)

Martin, B., J. Nienhuis, G. King, and A. Schaefer, 1989. Restriction fragment length polymorphisms associated with water use efficiency in tomato. Science 243:1725-1728. (Native Plants, Inc., 417 Wakara Way, Salt Lake City, UT, 84108, USA)

McFadden, G.I., 1989. In situ hybridisation in plants: from macroscopic to ultrastructural resolution. Cell Biology International Reports 13(1):3-21. (Plant Cell Biology Centre, School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia)

Nederlof, P.M., D. Robinson, R. Abuknesha, J. Weigant, A.H.N. Hopman, H.J. Tanke, and A.K, Raap, 1989. Three-color fluorescence in situ detection of multiple nucleic acid sequences. Cytometry 10:20-27. (Department of Cytochemistry and Cytometry, University of Leiden, 2333 AL Leiden, The Netherlands)

Ozawa, K., and A. Komamine, 1989. Establishment of a system of high-frequency embryogenesis from long-term cell suspension cultures of rice (Oryza sative L.). Theor. Appl. Genet. 77:205-211. (Biological Insitute, Tohoku University, Sendai, 980 Japan)

Peng, J., and T.K. Hodges, 1989. Genetic analysis of plant regeneration in rice (Oryza sative L.). In Vitro Cellular & Developmental Biology 25(1):91-94. (Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana, 47907, USA)

Raghavan, V., 1989. mRNAs and a cloned histone gene are differentially expressed during anther and pollen development in rice (Orzya sative L.). Journal of Cell Science 92:217-229. (Department of Botany, The Ohio State University, Columbus, OH, 43210, USA)

Schachtman, D.P., A.J. Bloom, and J. Dvorak, 1989. Salt-tolerant Triticum X Lophopyrum derivatives limit the accumulation of soium and chloride ions under saline-stress. Plant, Cell and Environment 12:47-55. (Department of Vegetable Crops, University of California, Davis, CA, 95616, USA)

Shimamoto, K., R. Terada, T. Izawa, and H. Fujimoto, 1989. Fertile transgenic rice plants regenerated from transformed protoplasts. Nature 338:274-276. (Plantech Research Institute, 1000 Kamoshida, Midori-ku, Yokohama, 227, Japan)

Simpson, P.R., M.A. Newman, and D.R. Davies, 1988. Detection of legumin gene DNA sequences in pea by in situ hybridization. Chromosoma(Berl) 96:454-458. (Department of Applied Genetics, John Innes Institute, Colney Lane, Norwich NR4 7UH, United Kingdom)

Tavazza, R., M. Tavazza, R.J. Ordas, G. Ancora, and E. Benvenuto, 1988. Genetic transformation of potato (Solanum tuberosum): an efficient method to obtain transgenic plants. Plant Science 59:175-181. (ENEA, Dipartimento Agrobiotecnologie, Divisione Ingegneria Genetica C.P. 2400, Roma A.D. Italy)

Toriyama, K., Y. Arimoto, H. Uchimiya, and K. Hinata, 1988. Transgenc rice plants after direct gene transfer into protoplasts. Bio/Technology 6:1072-1074. (Faculty of Agriculture, University of Tohoku, Sendai 980, Japan)

Viegas-Pequignot, E., B. Dutrillauz, H. Magdelena, and M. Coppey-Moison, 1989. Mapping of single-copy DNA sequences on human chromosomes by in situ hybridization with biotinylated probes: enhancement of detection sensitivity by intensified-fluorscence digital-imaging microscopy. (U.R. 620, Structure et Mutagenese Chromosomiques, Section de Biologie, Insitut Curie, 26 rue d'Ulm, 75231, Paris Cedex 05 France)

Wagner, P.M., B. Oskouian, and G.C. Stewart, 1989. In situ removal of RNA during agarose gel electrophoresis. BioTechniques 7(4):338-340. (Department of Microbiology, University of Kansas, Lawrence, KS, 66045, USA)

Wang, Y.C., T.M. Klein, M. Fromm, J. Cao, J.C. Sanford, and R. Wu, 1989. Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. Plant Molecular Biology 11:433-439. (Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY, 14853, USA)

Wong, C.K., 1989. A new approach to chromosome doubling for haploid rice plants. Theor. Appl. Genet. 77:149-151. (Institute of Botany, Academia, Sinica, Nankang, Taipei, Taiwan 11529, Republic of China)

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