ANNUAL REPORT
COMPREHENSIVE RESEARCH ON RICE
January 1, 1994 - December 31, 1994

PROJECT TITLE:
Marker-assisted Breeding in California Rice Varieties

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LEVEL OF 1993 FUNDING:
$10,000

OBJECTIVES AND EXPERIMENTS CONDUCTED BY LOCATION TO ACCOMPLISH OBJECTIVES:
The long-term goal of this project is to develop an easy screening technique utilizing DNA-based markers to identify genes for economically important, but difficult to measure traits in California rice cultivars and wild relatives. We accomplished both objectives that were stated in last year's proposal: 1) develop a PCR-based micro-assay that can be done on a small segment of leaf tissue and 2) optimize methods for converting dominant markers into co-dominant markers that both give more information from a cross and are easier to use.

Objective 1: PCR-based micro assay.
Experiments were conducted to optimize conditions for quickly isolating DNA from small leaf segments so that many samples could be processed in a short time. These samples would then be used in polymerase chain reactions (PCR) to generate molecular markers that could be used in rice breeding. The following conditions were varied for DNA extraction: amount of leaf tissue needed, extraction buffer components, buffer

infiltration method, cell lysis time and temperature, resuspension volume. Conditions were varied to produce the PEX DNA isolation protocol that worked reliably with all ages of tissue (6 days - 4 months post-germination) and with material grown in both the greenhouse and field (fig. 1). In addition to its use with rice, the technique was adapted for use with barley, oat, corn and bean (fig. 2). The optimal conditions were published (Williams and Ronald, 1994).

Objective 2: convert dominant markers into co-dominant markers.
Two approaches were successful in converting dominant markers into co-dominant PCR-based markers that gave increased information from a cross. One method involved generating sequence-tagged sites (STSs) from a single band in a PCR pattern such as that shown in fig.1. This approach involved excising the band of interest from a gel, cloning the DNA into a bacterial plasmid and transforming the bacteria, isolating large quantities of the clone, determining the DNA sequence, and designing 24-base long PCR-primers. This process did not always result in conversion from a dominant to a co-dominant marker, but did always generate more reliable markers that were easier to use. The marker shown in fig. 3 is a codominant STS that was generated by this method.

The second approach that was successful in generating co-dominant markers involved changes in the parameters for separating DNA markers on a gel. From colleagues at Cornell University (McCouch and Yanagihara) we received primers for an STS linked to the rice wide compatibility locus of rice. The STS was generated as described above and was genetically linked (about 4cM) to the wide compatibility locus from a javanica variety that could be crossed to both indicas and japonicas without F1 sterility. Transfer of this locus to japonica and indica breeding lines would make it possible to intercross the three varieties and to introgress desirable traits. However, the STS version of the marker was present in all three races of rice and was no longer able to distinguish them. By changing the conditions for separating DNA we were able to identify two alleles, one found in japonicas and the other shared by indicas and javanicas. The STS marker could then be used to follow the introgression of the wide compatibility locus from javanicas into japonicas (fig. 4). The resultant progeny could then be crossed with indicas. The important factors involved in separating the DNA were to use Synergel along with agarose in the gel matrix and to run the gel overnight at 4°C and at a low voltage.

SUMMARY OF 1993 RESEARCH (MAJOR ACCOMPLISHMENTS) BY OBJECTIVE:
By achieving the two objectives, we were able to apply the new techniques to two areas of research. In collaboration with Dr. D. J. Mackill we used the wide compatibility-linked STS to screen F_3 populations resulting from crosses of Lemont (containing the javanica STS allele) and breeding lines M202 and M204 (containing the japonica STS allele). We identified the segregants that contained the javanica STS allele and these

were then crossed to IR50, an indica tester (fig. 5). If the progeny from these crosses are fertile, then we have successfully used the STS to transfer the wide compatibility locus into both japonica and indica rice. In the spring of 1995 we will know the results of these crosses.

The second research project involved the use of our newly developed STSs that are linked to *Xa21*, a locus that confers resistance to bacterial leaf blight disease of rice. We consider this a model system for optimizing techniques that can be used to make codominant STSs that are linked to any gene of interest. The PEX DNA isolation protocol made it possible to process about 2000 samples quickly. These samples were from an F_2 population that was segregating for resistance. We are currently using the STSs to precisely map the position of *Xa21* and to predict which setregants will contain the locus. So far, 2400 F_2 plants have screened with five of our six STSs.

PUBLICATIONS OR REPORTS:
To date, one publication has resulted from this research which was funded by the National Institutes of Health and the Rice Research Advisory Committee (Williams, C. E. and P. C. Ronald, 1994. PCR template-DNA isolated quickly from monocot and dicot leaves without homogenization. *Nucleic Acids Research* 22: 1917 - 1918).The importance of this research is reflected in that we have received over 60 reprint requests. An abstract describing results of the STS-mapping at the *Xa21* locus has been submitted to the international meeting, Plant Genome III that will be held in San Diego in January. Other manuscripts will be submitted upon completion of the projects.

CONCISE GENERAL SUMMARY OF CURRENT YEAR'S RESULTS:
We optimized techniques and generated DNA-markers that can aid in the introgression of important genes into Californis rice cultivars. We developed a quick, small-scale DNA prep that could be used to generate markers linked to genes that are important for rice breeding. We then optimized methods for designing easy to use molecular markers. Using these techniques, we produced a marker for the wide compatibility gene in javanica rice. This gene makes it possible to intercross the three varieties of rice (indica, japonica and javanica) in order to transfer useful traits without barriers caused by sterility of the F_1 hybrid. We used this marker to identify javanica X japonica progeny that should contain the gene and crossed them to an indica tester. In other experiments, we tested our new techniques by generating markers linked to *Xa21*, a gene that confers resistance to bacterial leaf blight disease of rice. These markers were then used to accurately determine the chromosomal location of *Xa21*, and to predict which progeny would contain the gene. In the future, we will apply these techniques to the development of DNA-markers linked to stem rot resistance genes so that they can more easily introgressed during improvement of California rice cultivars.

Figure legends

Figure 1. Comparison of DNA amplified from a large-scale isolation protocol (lanes 1 and 2) vs. the PEX protocol (lanes 3 through 6). The RAPD markers were resolved on an agarose gel after PCR.

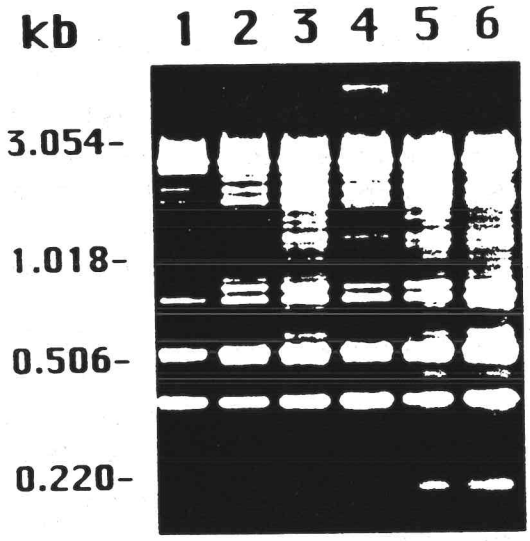


Figure 2. The PEX DNA prep was optimized for use with many plants. RAPD primer AB09 generated amplification products from bean, barley,oat, corn and rice (lanes 1-6, respectively).

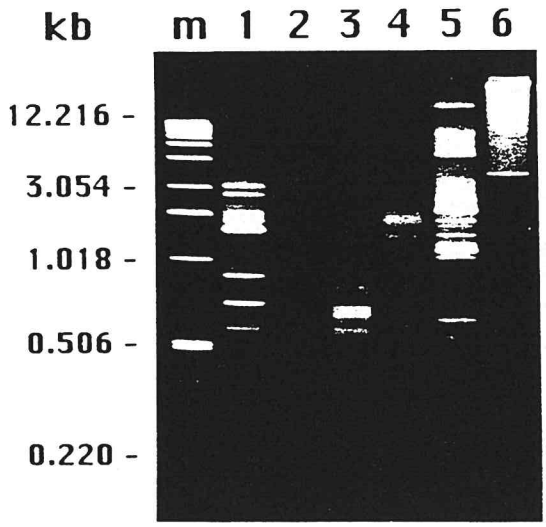


Figure 3. A codominant STS marker can distinguish the two homozygous types (lanes 1 and 2) from eachother as well as from heterozygotes (lanes 3-5).

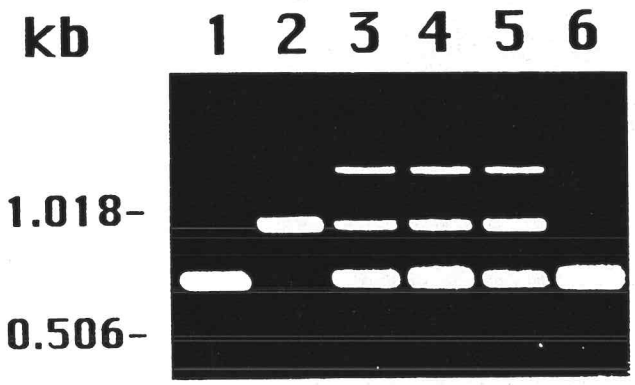


Figure 4. A marker linked to the rice wide compatibility locus. The lower band from this marker (arrow) cosegregates with the wide compatibility allele (WC+) derived from javanica rice (Jv). A different sized band is found in japonica rice (Jp) which lacks wide compatibility (WC-). The band found in indica rice (In) is indistinguishable from that of javanica. The WC+ marker is found in several California cultivars including A301, L202 and L203.

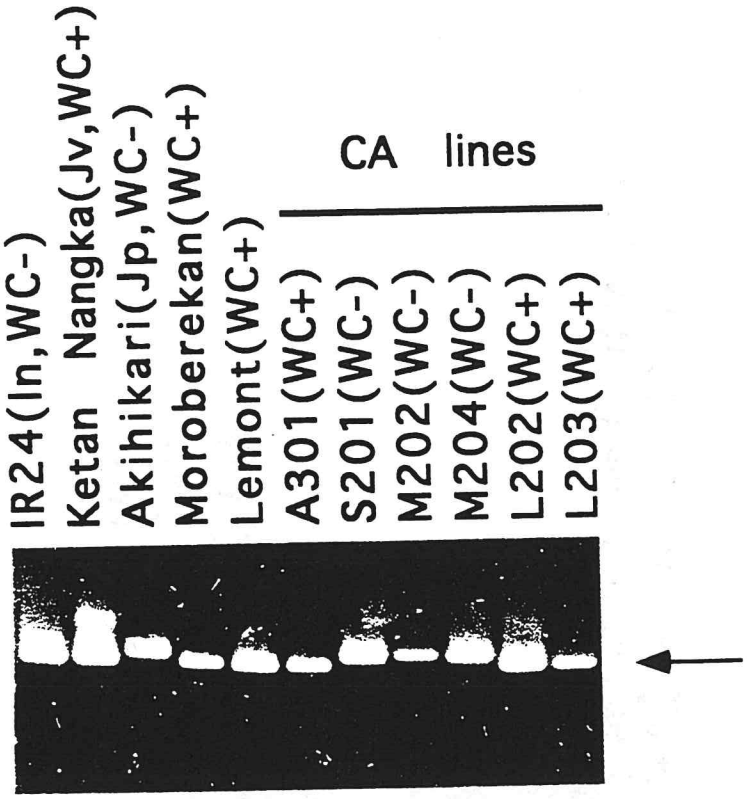
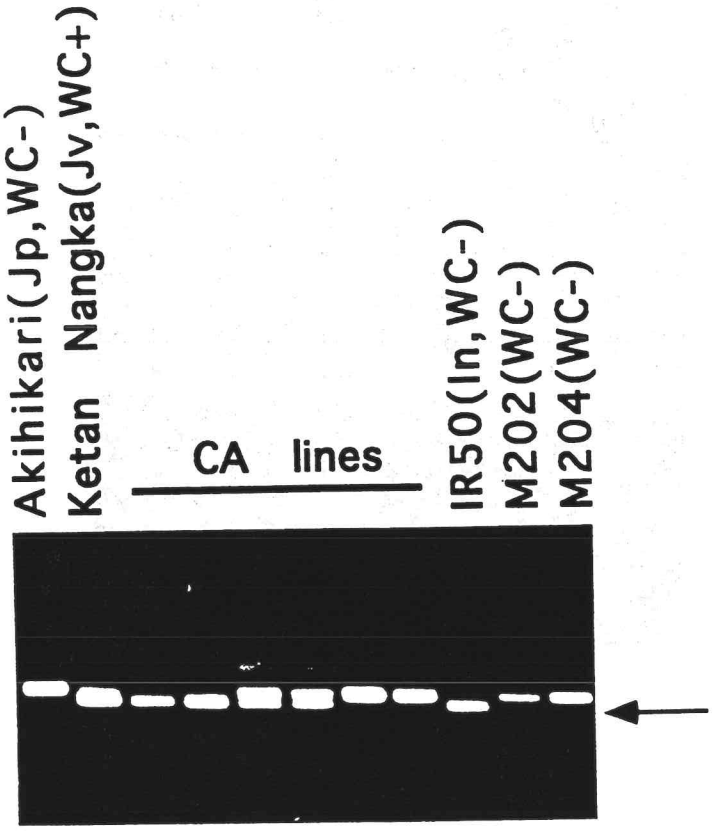


Figure 5. The marker was used to identify segregants that are likely to contain the wide compatibility locus. The six California lines are segregating for the marker that is linked to the wide compatibility locus. Lanes 1 and 2 indicate the band sizes for plants lacking and containing the linked marker, respectively. Lanes 3 and 4 contain the band size that is predictive of wide compatibility. Lanes 5 and 6 contain DNA from heterozygotes, and lanes 7 and 8 contain DNA from segregants lacking the predictive band. These California lines are from a japonica/javanica background and have been crossed to an indica (lane 9). Lanes 10 and 11 contain DNA amplified from the japonica-type parents of the segregants.



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PCR template-DNA isolated quickly from monocot and dicot leaves without tissue homogenization

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A protocol is presented that utilizes potassium ethyl xanthogenate to liberate DNA from leaves without the need for tissue homogenization. This quick, single-tube method requires only 1/3 cm^2 of tissue and yields enough DNA to serve as template for 20 polymerase chain reactions. The resulting amplification patterns are indistinguishable from those generated from rigorous large-scale DNA extractions. Because 100–200 samples can be processed per day by a single person, this method allows for the rapid screening of large mapping populations. This protocol was developed for use with rice, but also works well with other species including barley, oat, corn and bean.

Although many quick protocols have been published for small-scale isolation of DNA, certain characteristics of rice and other monocots result in inconsistent yields and poor amplification with the polymerase chain reaction (PCR). Because rice leaves are very fibrous, protocols specifying homogenization of leaf tissue (1) are laborious and undependable when scaled down. Quick protocols that amplify DNA directly from leaf tissue (2, 3, 4) have failed to rupture the tough cell wall of rice leaves sufficiently to liberate template DNA. Fast methods for the extraction of DNA from single seeds of cotton (5) rye and barley (6) and rice (7) have been published. In our hands, these procedures did yield sufficient quantities of rice DNA for PCR (about 100 ng per half-seed). However, PCR amplification was inconsistent, presumably due to the presence of inhibitors like starch. As a result, we designed a quick, small-scale prep that utilizes potassium ethyl xanthogenate (PEX; 8) to dissolve cell walls, degrade proteins and inhibit DNase activity (8). No tissue homogenization is required in order to yield enough DNA for 20 PCR amplifications from a small amount of leaf tissue.

We tested our protocol on young leaves from bean (*Phaseolus vulgaris*), barley (*Hordeum vulgare*), corn (*Zea mays*), oat (*Avena sativa*), rice (*Oryza sativa*), triticale (a wheat/rye hybrid), wheat (*Triticum aestivum*) and durum (*T.durum*). Upon emergence from the soil, the first true leaf was collected on ice in the greenhouse and subsequently stored at −80°C. Older rice tissue, up to 8 weeks after emergence, also was collected. Frozen segments of leaf totalling 1/3 cm^2 were selected and placed in the bottom of a small microcentrifuge tube containing 100 μl of extraction buffer (8). This buffer consisted of 6.25 mM PEX (potassium ethyl xanthogenate or carbonodithioic acid, o-ethyl, potassium salt from Fluka), 100 mM Tris–HCl (pH 7.5), 700 mM NaCl, 10 mM EDTA (pH 8). Dipping the hydrophobic leaf tissue in 70% ethanol prior to placing it in buffer helped to keep it fully submerged. The samples were incubated in a water bath at 65°C for 5 min. Hot samples were transferred directly to a speed-vac and vacuum infiltrated for 2 min (rice, bean, corn and barley) or 6 min (oat, triticale, wheat and durum). After treatment, the leaf tissue was somewhat translucent and the buffer noticeably

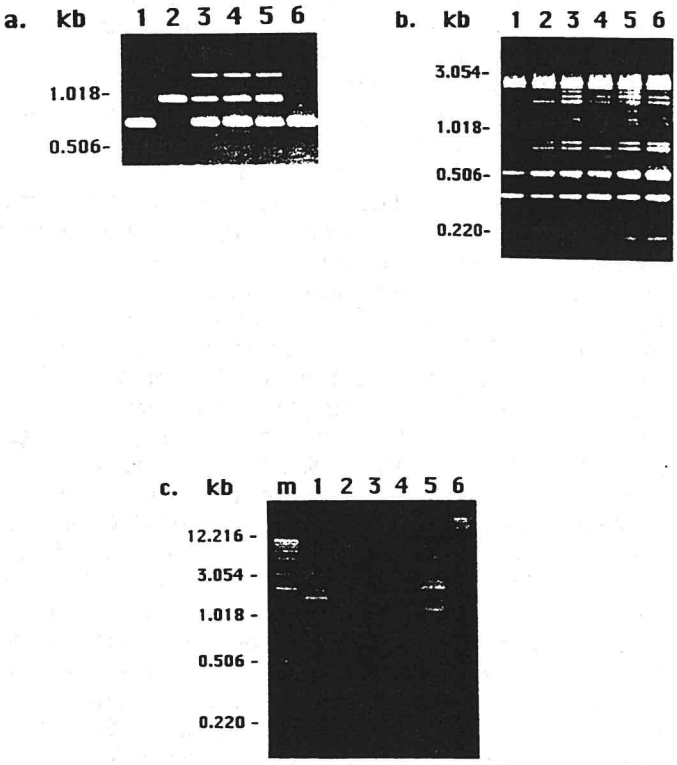

Figure 1. Separation of PCR products on gels containing 1% Synergel (Diversified Biotech) and 0.6% agarose (BRL) in 0.5× TBE buffer. **A)** Lane 1 and 2 contain DNA amplified from large-scale isolations of parental plant DNA. Subsequent lanes contain DNA amplified from PEX isolations of DNA from F_2 plants. Amplification with 24-base STS primers B7 and B8 (7) resulted in single PCR products from parental classes of homozygotes (lanes 1, 2 and 6) and the two parental plus one heteroduplex product from heterozygotes (lanes 3, 4 and 5). The heteroduplex is a PCR artifact that does not migrate in accordance with its actual molecular weight (12). **B)** Lanes are in the same order as those in A. Amplification with 10-base RAPD primer A02 (Operon, Inc.) produced no polymorphisms among the samples. **C)** RAPD primer AB09 generated discrete amplification products from bean (lane 1), barley (lane 2), oat (lane 3), corn (lane 4), rice (lane 5) and triticale (lane 6). Lane m contains 1 kb ladder.

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green, indicating that cell lysis had occurred. The samples were returned to the 65°C water bath for 15 min or longer, during which time a second set of samples was processed. After incubation, the samples were vortexed for 10 sec, the leaf segments were removed and 10 µl of 3 M sodium acetate (pH 5.2) plus 200 µl of cold ethanol were added for precipitation. Tubes were incubated until the alcohol became viscous, about 10 min, in a dry ice/ethanol bath before centrifugation for 20 min on the high setting of a microcentrifuge. All liquid was then removed from the samples and barely visible amounts of clear residue (containing DNA) remained scattered up the side of the tube. The precipitate was then resuspended by vortexing in 20 µl of modified TE buffer (10 mM Tris−HCl, pH 7.5 and 0.1 mM EDTA, pH 8). The samples were centrifuged (as before) for 5 min to pellet any debris before the liquid was transferred to a new tube for storage at −80°C. DNA was used directly in PCR without quantification. However, initial quantification of DNA yield may be useful if this protocol is to be applied to other plants. For the purpose of this report, rice DNA concentrations were determined for fifteen representative samples. Fluorometry detected 6 ng of DNA or less as the total yield for the 20 µl final volume. The DNA yield from 8-week-old rice tissue was slightly less than from younger plants.

PCR conditions for RAPD markers (random amplified polymorphic DNA) were similar to those reported by Williams *et al.* (9). One µl of DNA sample was added to 24 µl of PCR mix (10 mM Tris−HCl, pH 8.2; 50 mM KCl; 100 µM each of dATP, TTP, dCTP and dGTP; 2.0 mM $MgCl_2$; 400 nM ten-base primer; 40 u/ml Taq DNA polymerase). DNA was amplified under rapid cycling conditions (3 hr total) in a Perkin Elmer model 480 thermocycler. A preliminary denaturation step of 94°C for 1 min was followed by 3 cycles of 94°C for 1 min, 35°C for 1 min and 72°C for 2 min. This was followed by the rapid cycling phase consisting of 32 cycles of 94°C for 10 sec, 35°C for 30 sec, and 72°C for 1 min plus a final extension step at 72°C for 5 min (11). The PCR mix for STSs (sequence tagged sites; 10) was the same as that for RAPDs except that two 24-base primers were used at 500 nM concentration. The STS amplifications began with a denaturation step of 94°C for 1 min that was followed by 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, plus an extension step at 72°C for 5 min. Only 5% of all rice samples failed to amplify with either RAPD or STS primers. In general, amplification products from PEX DNA and large-scale rice DNA isolations were indistinguishable (Fig.1a and b), with the exception of an occasional primer showing decreased product with PEX DNA. However, equivalent amplification was achieved by lowering the annealing temperature by 5°C (STSs) or by increasing primer concentration to 500 nM (RAPDs). DNA isolated from young and old rice tissue worked equally well in PCR (data not shown). In determining the usefulness of this protocol with plants other than rice, 4 RAPD primers were tested. Discrete and reproducible amplification products were detected in reactions containing DNA from bean, barley, oat, corn and triticale (Fig. 1c). Although ample DNA to serve as PCR template was isolated from wheat and durum, only a high molecular weight smear was detected after amplification and electrophoresis. In our experience, amplification of PEX DNA can often be improved by altering DNA concentration and PCR conditions or by using primers known to permit good amplification in the target species (the 4 primers that we tried may be suboptimal for *Triticum*).

Map-based cloning efforts require high resolution genetic analysis of a few tightly linked DNA markers in large segregating populations. This work can be greatly facilitated by the PEX DNA protocol which yields small quantities of DNA which amplify well in PCR. The amplification patterns generated from the PEX DNA were both reproducible and comparable to patterns generated from DNA isolated in large preps that included tissue homogenization, phenol and chloroform extractions, and multiple precipitation steps. This method is applicable to other projects in which small quantities of DNA must be isolated from many individuals.

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