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LIGNIFICATION AND STEM STRENGTH IN ORYZA SATIVA

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EXECUTIVE SUMMARY

Substantial progress was made during the tenure of this grant:

- Dr. Mae Mendoza (University of the Philippines, Los Baños, UPLB) and Mr. Joseph Bongon M.S. from her laboratory spent time at the University of Missouri observing molecular techniques, particularly the new technology of differential display. They also gained experience in the study of a model β-glucosidase, and became familiar with modern rapid techniques for protein purification (fast protein liquid chromatography, FPLC). Differential display will be applied in the future in Dr. Mendoza's laboratory to study rice lignification mutants.

- Substantial amounts of equipment and supplies for molecular studies were purchased and transferred to Dr. Mendoza's laboratory.

- The *A. tumefaciens cbg*-encoded β-glucosidase was partially characterized and kinetic parameters were determined.

- Several rice β-glucosidases were found. Two cytosolic β-glucosidases appear not to be involved in lignification or stem strength (on the basis of their low specificity for coniferin). On the other hand, two β-glucosidases from the wall-bound fraction displayed substrate preference for lignin precursors and were putative candidates for lignification control. Their purification is not yet complete because of stability problems. To bypass the stability problem, rice genomic libraries were prepared in a lambda vector for direct screening for the β-glucosidase gene(s). These libraries will shortly be transferred to Dr. Mendoza's laboratory for further screening.

- Glucosidase activity was measured in normal and brittle-culm (lignification-defective) rice lines.

- The differential display technique of Liang and Pardee was successfully applied to demonstrate the presence of leaf-specific and root-specific mRNAs in rice. We succeeded in isolating a root-specific gene (of unknown function) which has the potential to be very useful in the genetic engineering of that crop. Screening a rice genomic library allowed us to identify and map a clone which contains this gene. In addition, we isolated and characterized a leaf-specific partial cDNA which proved to be homologous to the glycine decarboxylase H subunit *(gdcH)* which had been reported previously in dicots but not in monocots. It too will prove invaluable for leaf-specific expression of desired genes in rice.

- Mr. Bongon observed this powerful technology in my laboratory and will be able to use it in Dr. Mendoza's laboratory to characterize single gene rice mutants which are defective in stem lignification.

- As a result of this research collaboration, the basis for molecular work been established in Dr. Mendoza's laboratory. We hope to continue the interaction in the future.

RESEARCH OBJECTIVES

The original long-term goals of this research were to isolate genes which control key enzymes of the rice lignin biosynthetic pathway, to identify factors which control their expression and, ultimately, to manipulate gene expression to enhance resistance to lodging. We chose to concentrate on a lignin-precursor specific β-glucosidase believed to be active only in lignifying tissues.

The research was proposed because it relates to lodging, one of the most significant problems facing rice breeders seeking to improve yield. The beneficial consequences of recent accomplishments in the rice breeding programs at the International Rice Research Institute (IRRI), in which genes controlling resistance to specific pests or diseases have been successfully introduced into high-yielding cultivars by wide crossing, have been partially offset by the concomitant introduction of weak culm structure. Since lignification contributes to culm strength, and because brittle-culm mutants known to be defective in lignification were available from IRRI, we felt that studies of the biochemistry and molecular biology underlying lignification would provide useful insights into culm strength.

The importance to development can be seen clearly. While plant physiology, rice genetics and plant breeding are the strengths of IRRI and while enzymology is a strength at the University of the Philippines, Los Baños (UPLB) there has been less emphasis on plant molecular biology. This research sought to address that need by providing molecular training and experience for a Philippine national. Originally, we had hoped to bring a postdoctoral fellow from Dr. Kush's laboratory to the USA for training. However, it proved more useful and productive to have Dr. Mae Mendoza, a faculty member at UPLB to spend time here, along with a M.S. student Mr. Joseph Bongon from her laboratory. During the course of the work, we made the exciting finding that differential display allowed the ready demonstration of tissue-specific gene expression in rice. Two genes were isolated. The first was shoot-specific (*gdcH*) and the second was root-specific (of unknown function). In light of the potential value of such genes in the future genetic engineering of rice for desirable traits, some effort was made to characterize these two genes and incorporate that study into the fabric of this project.

The objectives therefore crystallized into:

- Characterize the *A. tumefaciens* coniferin-specific β-glucosidase.
- Determine whether cognate β-glucosidases exist in rice.
- Measure β-glucosidase activities in wild-type and brittle-culm rice lines.
- Develop techniques (differential display) which would allow β-glucosidase genes to be demonstrated and isolated from lignifying tissues. We wished particularly to make this technology available for the later study of rice brittle-culm mutants defective in lignification.
- Construct rice genomic libraries.
- Characterize a shoot-specific gene expressed in rice leaves.
- Characterize a root-specific gene expressed in rice roots.

A particularly innovative aspect of this proposal was the application of differential display techniques to rice. This method which had been developed by Liang and Pardee (1992) for demonstrating differences in patterns of gene expression in animal tissues had not been applied successfully to plants. In our hands it provided a powerful and relatively inexpensive tool for demonstration of such differences and led to the isolation of two tissue-specific genes.

METHODS AND RESULTS

Objective 1. Characterization of the A. tumefaciens β-glucosidase.

The *E. coli* strain DH5α(pBR5.5-2) containing the cloned *A. tumefaciens cbg* gene, was grown at 37° C and harvested at mid-exponential phase. Cells were lysed in a buffer containing phenyl methyl sulfonyl fluoride (PMSF) and clarified by high speed centrifugation (100,000 x g / 30 min). Glucosidase activity was assayed with coniferin or 4-nitrophenyl-β-D-glucoside as substrate. Hydrolysis of coniferin was followed by measurement of the appearance of coniferyl alcohol (measured colorimetrically with Fast Blue RR). Hydrolysis of 4-nitrophenyl-β-D-glucoside was followed directly at A_{420}.

The pH optimum, temperature stability and K_m values were determined (Table 1). Surprisingly, the enzyme was very active at high pH.

Table 1: Coniferin-specific β-glucosidase activity from *A. tumefaciens*

pH	Activity (ΔA_{490} / 30 min)
5.0	0.002
6.8	0.071
8.0	0.238
9.5	0.387

Even though the enzyme activity was significant under these conditions, the levels of protein expressed from pBR5.5-2 were low. Attempts to over-express the gene in one of the PET vectors did not yield higher levels. However, enough enzyme was available to measure Michaelis constants. The K_m values were calculated for coniferin and for 4-nitrophenylglucoside. They were 190 μM and 8 μM respectively.

Objective 2: Demonstration of cognate β-glucosidases in rice.

The assay technologies were taken to UPLB and applied to rice stem samples. Extracts, after ammonium sulfate fractionation, yielded cytosolic and wall-bound enzyme fractions. The cytosolic enzymes were fractionated on Sephadex G-200 into two distinct peaks. These enzymes displayed good activity with 4-nitrophenyl-β-D-glucoside but poor activity with coniferin, suggesting that they were not involved with lignin biosynthesis. On the other hand, the wall-bound enzyme fraction was found to possess the following properties:

- There were two β-glucosidase isozymes.
- They were very strongly bound to ion exchange columns (> 0.6 M salt needed for elution)
- They possessed good activity with coniferin as substrate.

Unfortunately, these isozymes were particularly unstable and purification proved difficult.

Objective 3: Glucosidase activities in normal and brittle-culm mutants of rice

Studies were initiated by Dr. Mendoza on differences between β-glucosidase activities in wild-type and brittle-culm rice lines which are defective in lignification. Such studies formed the major portion of the theses of two graduate students at UPLB. Analyses are not yet complete, but in summary the relationship is complex and will require further study. The results are being presented by Dr. Mendoza at the Federation of Asian and Oceanian Biochemists Congress in Sydney, Australia this September. When abstracts for the presentations become available, they will be forwarded to USAID. Although the enzymes were not purified to homogeneity, substantial purification was achieved by conventional means, and a paper is in preparation.

Objective 4. Development of differential display techniques.

Liang and Pardee (1992) recently described a differential display method for the identification and characterization of genes which are expressed in one tissue but not in another. Such a technique would be invaluable for the demonstration of the presence of lignin-specific transcripts in lignifying tissues and for the identification of differences in lignin-defective mutants. We have acquired that technology, applying it to a model system of rice roots and leaves. The principle of the method is illustrated in Figure 1.

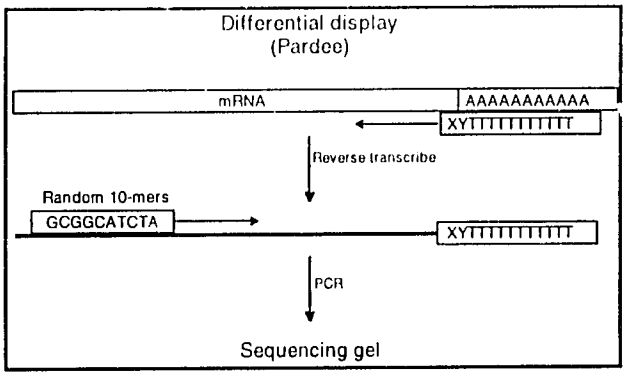

Figure 1: Differential Display Protocol

Total mRNA populations from the two tissue types were prepared by conventional means. They were then subjected to reverse transcriptase action, using as primers a $(T)_{12}XY$ oligomer and a selected random 10-mer. After 30 rounds of PCR, the products were analyzed on a sequencing gel. Tissue-specific gene expression is shown by the presence of bands in one PCR preparation but not in the other. Figure 2 illustrates the results obtained by the use of $(T)_{12}MC$ and CCGAATTCC on rice root and leaf RNA samples.

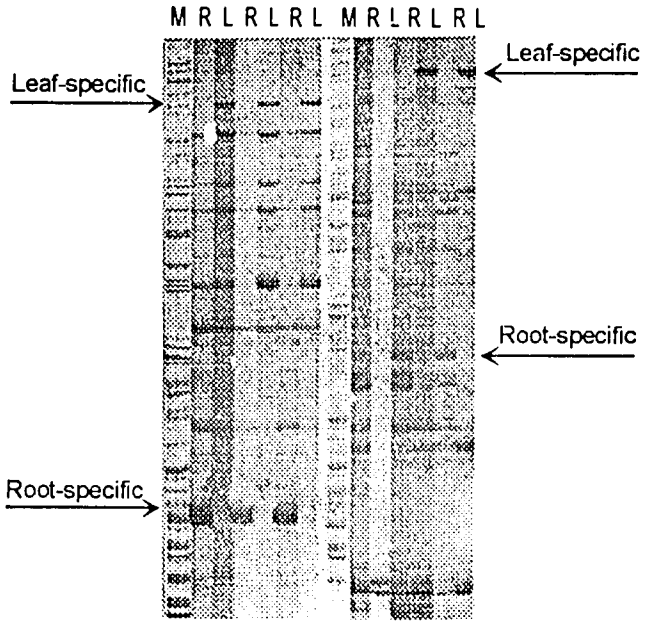

Figure 2: Display of mRNA differences between rice roots and rice leaves

Reproducible differences were noted for two bands. The first was a leaf-specific cDNA fragment of 386 base pairs. This was isolated, reamplified and cloned into the pCRII vector (InVitrogen) to give the recombinant plasmid pATF1. A second 222 base-pair cDNA was present in roots, but not in leaves. This band was isolated, reamplified and cloned into the AT vector to give pATF4.

Tissue specificity was confirmed by Northern blotting and by RNase protection assays. For both techniques, strong signals were obtained for pATF4 in root tissue, but not in shoot tissue. Conversely for pATF1, strong signals were seen from the leaf but none from the root. We conclude that expression of the genes is tightly regulated in a tissue-specific manner. Their properties are described below.

Objective 5. Construction of rice genomic libraries

Because of the instability of the wall-bound β-glucosidases, we decided to prepare rice genomic libraries which could be searched for β-glucosidase activity. Such libraries would also provide genomic clones for further characterization of the shoot-specific and root-specific cDNAs isolated by differential display.

Rice DNA was prepared by conventional methods, partially digested with BamHI, sized by agarose gel electrophoresis and ligated to Lambda DASH II arms (Stratagene) using conditions recommended by the manufacturer. The ligation mixtures were transfected into XL1-Blue MRA (P2) *E. coli* for selection. Good transfection rates were observed (300,000 pfu/µl) and samples were screened as described below.

Objective 6. Characterization of the shoot-specific rice clone.

The shoot-specific cDNA insert in pATF1 was sequenced in both directions by the technique of Sanger *et al.* (1977). The sequence data are illustrated in Figure 3.

```
GGGAATTCGGCAACGTGGAGAGTGTGAA
GGCCACCAGCGACATCAACTCCCCCATC
TCCGGTGAGGTCGTCGAGGTTAACGACA
AGCTCTCTGAGACACCCGGCCTGATTAA
CTCAAGCCCGTACGAGGACGGGTGGAT
GATCAAGGTGAAGCCGAGCAGCCCGTC
GGAGCTGGACGCCCTGCTGGACCCGGC
CAAGTACACCAAGCACTGCGAGGAGGAA
GACGCTCACTAGGCT
```

Figure 3: Sequence of the shoot-specific cDNA from rice differential display

Comparison of the sequence with the GenBank database, using TFASTA gave strong homology at the protein and nucleotide level, with the known leaf-specific dicotyledonous gene GDCH. The homology at the nucleotide level is illustrated in Figure 4.

```
Glycine decarboxylase; H protein.
Flaveria pringlei . . .
73.8% identity in 233 bp overlap

                                                  10        20        30
leaf.s                                  GGGAATTCGGCAACGTGGAGAGTGTGAAGGCCACC
                                                || ||    || || ||||| || ||||||
z25856 GCCGGTGGCTCTGTGACCAAAGCCACCGGCTTTGGTGCAGTAGAAAGTGTCAAAGCCACC
       290       300       310       320       330       340

          40        50        60        70        80        90
leaf.s AGCGACATCAACTCCCCCATCTCCGGTGAGGTCGTCGAGGTTAACGACAAGCTCTCTGAG
       || ||| | |||||||| ||||| || ||| |||| ||||| |||   ||| | ||||||
z25856 AGTGACGTTAACTCCCCGATCTCTGGAGAGATCGTTGAGGTGAACTCGAAGTTGTCTGAG
       350       360       370       380       390       400

         100       110       120       130       140       150
leaf.s ACACCCGGCCTGATTAACTCAAGCCCGTACGAGGACGGGTGGATGATCAAGGTGAAGCCG
       || || ||  |||| || || |||||||| || || || |||||||| |||||||||||
z25856 ACTCCTGGTTTGATCAATTCGAGCCCGTATGAAGATGGATGGATGATTAAGGTGAAGCCA
       410       420       430       440       450       460

         160       170       180       190       200       210
leaf.s AGCAGCCCGTCGGAGCTGGACGCCCTGCTGGACCCGGCCAAGTACACCAAGCACTGCGAG
       |||| |||||||||||||||  |  |  |||   |     ||||||| |||  ||||||
z25856 AGCAACCCGTCGGAGCTGGATTCTTTAATGGGTGCAAAAGAGTACACAAAGTTCTGCGAA
       470       480       490       500       510       520

         220          230       240       250       260       270
leaf.s GAGGAAGA---CGCTCACTAGGCTCTTCCTCTCTGTTTTTTTTCTCTCCCATTTTGTCAC
       || |||||    ||||||||| |
z25856 GAAGAAGATTCTGCTCACTAGACAGACATGAACATAAATCATGTCTCATGATTTTTTTTT
       530       540       550       560       570       580
```

Figure 4: TFASTA comparison on the pAFT4 insert with the GenBank database

The homology is with the H-subunit of the glycine decarboxylase complex from the dicot *Flaveria pringlei*. This complex is of particular interest, in that it is encoded in the nucleus (Srinivasan and Oliver, 1992), but is expressed specifically in leaf mitochondria in a regulated fashion. The basis for this specificity has been shown to be the very tight regulation of the expression of the H-subunit *gdcH*. In the future we plan to isolate the *gdcH* promoter from rice. It should prove invaluable for the leaf-specific constitutive expression of desired transgenes.

Objective 6. *Characterization of the root-specific gene in rice*

The insert in pATF4 was sequenced in both directions, again by the technique of Sanger *et al.* (1977). The primary sequence of the insert is illustrated in Figure 5.

```
CAAGGGGCACGTATATCAATTAATTGTGT
GTCCCAATTAATTAGAGTCGATCCACTTG
ATATGTTCTTTTGTTAATTTGTATTATCAC
TCCATAGAGGAGTTGCTGTAGTAGTGCA
AAAGGTACATGCGGCCGCCGGCAGTATG
CATGTATTTCACTTCTGTTTCAGTATAATA
ATGCTATTCATATTTGTGGA
```

Figure 5: Sequence of insert from root-specific clone pATF4

Comparison of this sequence with the GenBank database using TFASTA, gave no homology to any known gene. In order, therefore, to further characterized this gene and begin its isolation, we hybridized the cDNA insert in pATF4 against the rice genomic libraries prepared as above. As Figure 6 illustrates, numbers of clones hybridized strongly to pATF4.

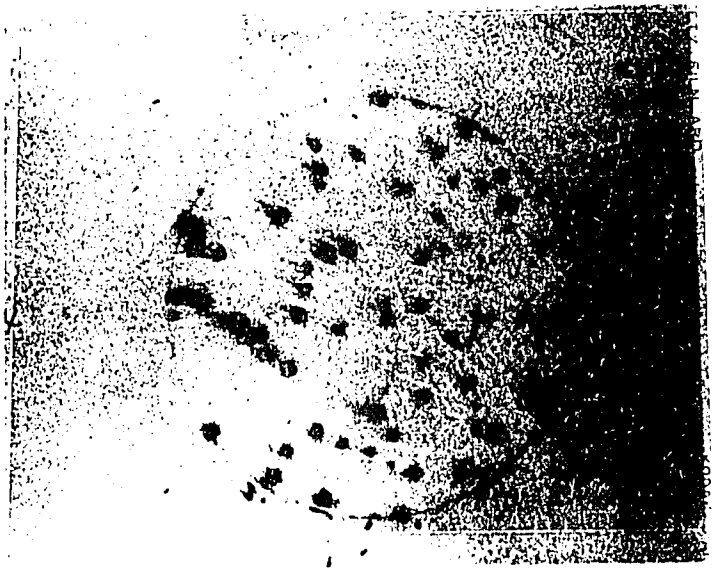

Figure 6: Hybridization of labelled root-specific clone pAFT4 against a rice genomic library

Two of these clones were isolated and characterized. A map of one of these clones (GR251) is illustrated in Figure 7.

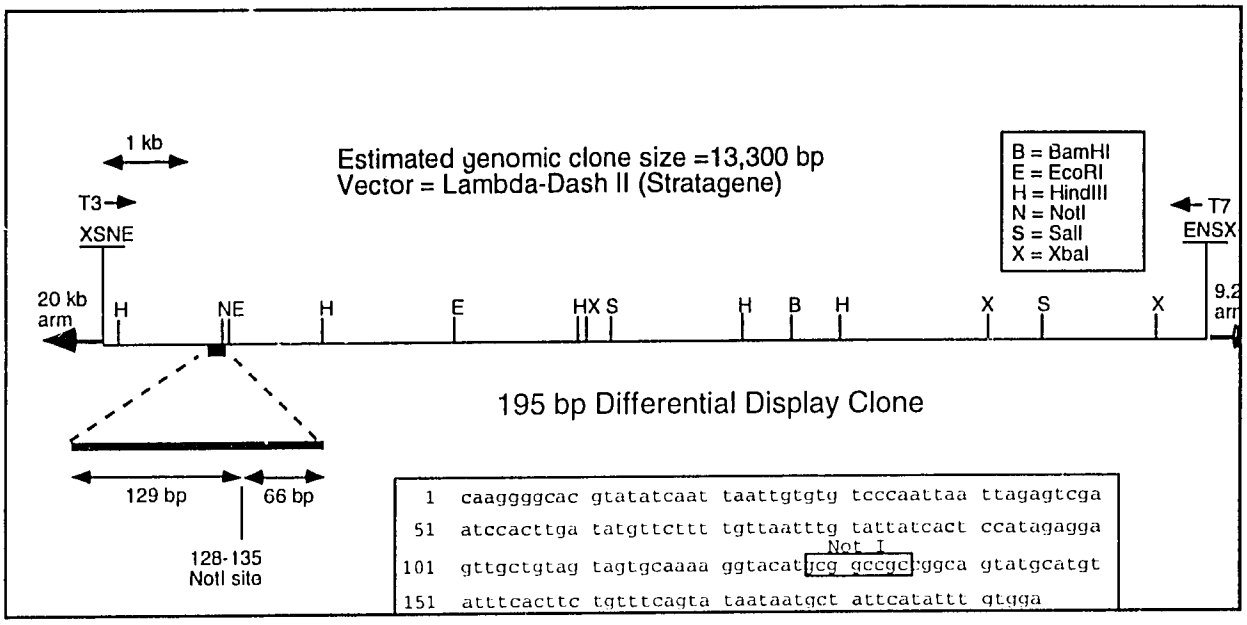

Figure 7: Restriction map of clone GR251 showing the location hybridizing to pATF4

Approximately 1.5 kb of the 3' end of the gene is present at the left-hand end of the insert, as indicated by the bar. Future studies will involve the complete sequencing of this region, the rescue of the 5' end of the gene, and the isolation of the promoter.

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

The project has been useful in that it initiated molecular approaches in Dr. Mendoza's laboratory at UPLB which will be useful in her future research. Dr. Mendoza spent a month in my laboratory in 1992 learning growth of recombinant *E. coli*, plasmid isolation, restriction enzyme digestion, lasmid mapping, and expression of the *cbg* gene in *E. coli*. Joseph Bongon from her laboratory subsequently spent 3 months in the laboratory, carrrying out FPLC experiments and observing the techniques used in differential display. As a result, Dr. Mendoza's labortatory is now well placed to employ molecular techniques in any project she chooses. On Dr. Mendoza's and Mr. Bongon's return to UPLB, supplies and basic equipment to continue the project were transferred. Among major purchases were micropipettors and molecular biological manuals with continuing updates to provide an ongoing technical resource

PROJECT ACTIVITIES / OUTPUTS

Some of this work was presented as a poster entitled "Cytokinin Changes in Developing Rice Grains and Development of Techniques for their Manipulation" Roy O. Morris, Michael Curtin, Joseph P. Dietrich, Richard Meilan and Hong Yang at the Rockefeller Annual Meeting, Bali, Indonesia, May 1994. The results of the β-glucosidase studies in rice mutants are being presented by Dr. Mendoza at the Federation of Asian and Oceanian Biochemists Congress in Sydney, Australia this September. When abstracts for these presentations become available, they will be forwarded. The work on the characterization of the root-specific genomic clone is being completed and will be published shortly. In regard to training aspects, Dr. Mendoza (UPLB) and Mr. Joseph Bongon from her laboratory participated actively in the project as described above.

PROJECT PRODUCTIVITY

The original goal of the project was to isolate the genes encoding β-glucosidase enzymes related to lignification in rice. This goal was not met. However, substantial progress was made in characterizing the rice β-glucosidases. Further, and most significant, two tissue-specific rice genes were isolated and partially characterized. These are the leaf-specific *gdcH* subunit gene and the root-specific gene of unknown function (pATF4). These two alone will provide valuable tools for researchers in this country and at IRRI and elsewhere to control the expression of desired transgenes in rice. We are currently communicating with Dr. Gurdev Khush at IRRI to determine his interest in their use.

FUTURE WORK

As indicated earlier, the most exciting aspect of this research was the unexpected finding of root-specific and shoot-specific gene expression in rice. This is the first such report of such expression in rice and should prove useful as transgenic plants are produced with traits which will improve resistance to pests, etc. for use in developing countries. We are actively exploring the preparation of root-specific constructs with the cytokinin biosynthetic gene *ipt* designed to enhance rice yield.

LITERATURE CITED

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