GENETIC DIVERSITY AND SPECIES RELATIONSHIPS IN THE *ORYZA* COMPLEX AND GLUFOSINATE TOLERANCE IN RICE

A Dissertation

by

LAURA KELLY VAUGHAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Biochemistry

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May 2005

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ABSTRACT

Genetic Diversity and Species Relationships in the *Oryza* Complex

and Glufosinate Tolerance in Rice. (May 2005)

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The weed red rice is a major problem in rice producing areas world wide. All of the red rice in commercial rice fields in the United States has traditionally been considered to be the same species as commercial rice, *Oryza sativa*. However, using DNA markers it was found that most of the red rice with black hulls was sufficiently divergent to be considered a separate species. This includes TX4, a red rice ecotype that has been reported to have considerable natural tolerance to the herbicide glufosinate.

TX4 is closely related to samples that have been classified as *Oryza rufipogon*. However, it was shown that both the TX4-like red rice from commercial fields and most of the *Oryza rufipogon* accessions in the US National Small Grains Collection are more accurately classified as *Oryza nivara*. This is significant since *Oryza rufipogon* is regulated under the Federal Noxious Weed Act, while *Oryza nivara* is not.

Oryza nivara closely related to TX4 was found to be widely distributed across the rice production areas of Texas and was also found in Arkansas, Louisiana, and Mississippi. Of 240 samples from across Texas, 23 samples from six different counties were identical with TX4 with all 18 DNA markers tested.

The reported glufosinate tolerance of TX4 is a potential problem since this same herbicide would be used in conjunction with genetically modified (GM) that is being developed as a method of red rice control. Thus, field, greenhouse and tissue culture studies were conducted to evaluate the degree of glufosinate tolerance in TX4. TX4 typically was severely damaged by glufosinate, but not efficiently controlled. Even with the maximum number of herbicide applications at the proposed maximum label rate, TX4 often re-sprouted and produced viable seed. Herbicide tolerance was found to be variable, but appears to be sufficient to present a problem with the use of the GM glufosinate resistant varieties currently under development, particularly when combined with variation in the response of “sensitive” varieties.

DEDICATION

This dissertation is dedicated to my family and friends, both near and far. Without their love and support none of this would have been possible. In particular, my parents, who expected the best from their daughters and were supportive in our own personal quests. I would also like to dedicate this dissertation to the memory of my grandfather, Newton Kelly Vaughan, who gave me so much more than my name.

ACKNOWLEDGEMENTS

I would like to acknowledge all those who made this work possible. A special thanks to Dr. Park and Dr. Chandler for their support and guidance and my committee members for all their help along the way. Thanks to Grace Walker, who was instrumental in all the microsatellite analysis and keeping everything organized; Brian Ottis, Greg Steele, and all the members of Dr. Chandler's laboratory for all their assistance in the field and greenhouse work; Dr. Nicki Ayres for keeping the laboratory running in top form; and Dr. Connie Bormans for her friendship and assistance. I would also like to thank Dr. Peng Yu for introducing me to the world of protein purification, Mrs. Jenny Johnson for her help with the HPLC analysis, and Macair Dobo for his friendship and support.

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CHAPTER I

INTRODUCTION

Rice as a Model Crop

Rice is the world's most important food crop. It is particularly important for the tropical and subtropical regions, where it provides between 30 and 60% of calories for the majority of the population (Giri and Laxmi 2000). In the US, rice is mainly produced in Arkansas, California, Louisiana, Mississippi, Missouri and Texas. Over 3 million acres are in rice production in the US, most of which is exported, and has a value of over 1 billion dollars annually.

In addition to being an important food source, rice is also the model crop for cereal grain research. Rice has a relatively small genome size (430 Mb compared to 16,000 Mb for wheat), well developed genetics, and can be readily transformed. There is also a syntenous relationship between rice and other grass species, as first noted by Moore et al. (1995). Due to its model crop status and world wide importance, a consortium of researchers from across the globe set out to sequence the entire genome of the rice cultivar Nipponbarre (Bennetzen 2002). The goal of this project was to obtain the full sequence of all 12 chromosomes at a 99.99% level of accuracy and to anchor it to genetic and physical maps. This would allow for the identification of the actual genes that are linked to important traits, as has been done in humans and in plants such as *Arabidopsis*. The project was originally scheduled for completion in 2008 (Normile and Pennisi 2002). However, in 2002 Monsanto and Syngenta both published rough drafts of the rice

This dissertation is in the style and format of Weed Science.

genome. Neither sequence had complete genome coverage, but both companies made their sequences available to the consortium. In the same year, a draft sequence was also released of the *indica* subspecies of rice obtained using a rapid shotgun cloning method. (Yu et al. 2002) These events lead to a shift in strategy by the sequencing consortia to more rapid data release than was originally planned (http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/status.pl).

Seed Banks and Genetic Resources for Researchers

Wild species account for over 70% of the genetic variation in the *Oryza* genus (Tanksley and McCouch 1997). In the early 1900's an effort was made to begin the collection and preservation of these important wild resources. Worldwide there are over 215,000 entries in germplasm collections, with the largest rice collection maintained by the International Rice Research Institute (IRRI). However, wild species make up only 10-15% of the accessions in these collections (Tanksley and McCouch 1997).

Accessions of the wild species such as *Oryza glaberrima*, *Oryza nivara*, *Oryza rufipogon* and *Oryza spontanea* have already been utilized for rice crop improvement to increased yield, and for disease, stress and insect resistance (e.g. Jones et al. 1997; Hammer et al. 2003). The impact of this work on agriculture is illustrated by the fact that the 2004 World Food Prize was awarded to Dr. Monty Jones of the African Rice Center (WARDA) for his work using crosses between *Oryza sativa* and *Oryza glaberrima* to dramatically improve rice yields in Africa (www.warda.org).

Red Rice Is a Major Weed Problem

Red rice is a pernicious weed in rice production areas across the southern United States and in other major rice production areas of the world (Webster 2000). It is characterized by red pericarp, excessive vegetative growth, seed dormancy and shattering. Red rice reduces yield by competing with commercial rice varieties for light and nutrients and its seeds also contaminate the harvested grain (Kwon et al. 1991; Pantone and Baker 1991). Red rice has been estimated to be responsible for the loss of over 50 million dollars annually to US rice producers (Gealy et al. 2002).

Traditionally, all red rice in the United States has been classified as *Oryza sativa* – the same genus and species as commercial rice (Diarra et al. 1985; Langevin et al. 1990; Kwon et al. 1992). Because of this close genetic relationship, it is not surprising that herbicides that kill red rice also kill standard cultivated rice. Other reasons that red rice is difficult to control by traditional methods include the fact that red rice seed tend to shatter (seeds fall from the plant when mature) and that red rice seeds can remain dormant in the soil for many years (Gross and Brown 1939). Once red rice is introduced into a field and becomes established, the field is likely to be permanently contaminated. Red rice increases production costs and large areas have become so infested with red rice that land has been taken out of production (Huey and Baldwin 1978).

Relationship Between Red Rice and Other *Oryza* Species

Oryza sativa is a member of the '*Oryza* complex' section of the genus *Oryza* (Vaughan et al. 2003). The '*Oryza* complex' also includes a number of wild species. The most directly relevant of these for this study are *Oryza rufipogon, Oryza nivara* and weedy intermediate types which are similar in habitat, phenology and morphology.

Traditionally, taxonomic classification within the "*Oryza* complex" has been based on a detailed set of morphological characteristics (Hammer et al. 2003; McCouch 2004; Li et al. 2004). In addition to phenotypic characteristics, such as wild/weedy rice having red seed coats and high tiller production, one of the major means of distinction is the method of reproduction. Generally, the term *Oryza rufipogon* is used strictly *(sensu stricto)* to refer to the perennial rhizomatous form of wild rice and *Oryza nivara* is used to identify similar annual forms of wild rice (Oka 1991; Khush 1997; Martin *et a*l. 1997; Yamanaka et al. 2003). However, other workers use the term more broadly *Oryza rufipogon* (*sensu lato*) as a general term to describe both the annual and perennial forms (Morishima et al. 1992; Vaughan et al. 2003).

As cautioned by Vaughan and Morishima (2003), accurate nomenclature is an important issue since the understanding and correct interpretation of research depends on the germplasm being identified properly. For the purposes of this dissertation, wild perennial rice will be referred to as *Oryza rufipogon* (with the *sensu stricto* implied) and the wild annual form as *Oryza nivara*, unless noted to reflect classification by other sources.

The advent of molecular markers has both reinforced and challenged the traditional taxonomic classification of numerous species and has sparked a debate about the use of molecular markers for the study of the phylogenic relationship of seed plants (e.g. Kellogg 1998; Nickerson and Drouin 2003; Duvall and Ervin 2003). The *Oryza* genus is no exception. Several different types of markers have been used to validate the morphologically based taxonomic classification, with most of the classification agreeing with the traditional (Ge et al. 1999, Vaughan et al. 2003). However, genetic analysis does not always support traditional analysis. Analysis based on the transposable element *Tourist* provides a perfect example. The presence of a specific tourist element in both the AA (*Oryza sativa* complex) and the FF (*Oryza longistamminata*) genomes indicated that these two genomes were closely related; in direct contradiction to all other morphological and molecular based taxonomic research (Vaughan et al. 2003).

Of most direct relevance to this study, DNA markers have been used to examine genetic relationships within the *Oryza* complex (Motohashi 1997; Kanazawa 2000; Cheng et al. 2003; Yamanaka et al. 2003) and to distinguish between *Oryza rufipogon* and *Oryza nivara* (Cheng et al. 2003; Park et al. 2003: Yamanaka et al. 2003). While some markers such as the retrotransposon pSINE1-r2 have been shown to be useful taxonomically, they were only approximately 75% accurate (34/46) in distinguishing annual from perennial accessions (Yamanaka et al. 2003).

At least two different factors may be responsible for the lack of correspondence between genetic and taxonomic classification. First key phenotypic traits, such as formation of rhizomes, may actually be controlled by a small number of genes, which are most likely not linked to the markers utilized for genetic classification (Hu et al. 2003).. A second factor that has been

suggested to contribute to the confusion surrounding the proper classification of these species is genetic changes and outcrossing that occur in response to selective pressure. In the wild *Oryza rufipogon* largely reproduces vegetatively via rhizomes. While it does set some seed, since the perennial *Oryza rufipogon* has a higher degree of outcrossing than do the annual members of the *Oryza* complex, many of these may be the result of natural outcrossing (Jackson 1997; Vaughan et al. 2003; Yamanaka et al. 2003; Gao 2004). However, the plants are maintained as seed in typical germplasm collections. Therefore, there is very strong selection pressure for either outcrossing or other genetic changes that would favor increased seed production (Oka 1991; Morishima 2001).

Red rice, the American version of weedy rice, has traditionally been accepted to be the same species as cultivated rice, *Oryza sativa*. This classification is supported by a low fertility barrier, with red rice and cultivars easily producing hybrids in field and green house conditions. As detailed in Chapter II, recent work by our lab has challenged this belief and demonstrated that much of the black-hulled red rice in producer's fields is sufficiently divergent to be considered another species. The proper classification of this divergent group is the subject of Chapter III.

Interest in Herbicide Tolerant Rice

The 1990s brought about at new revolution in plant improvement, the so called "Gene Revolution" for which Dr. Norman Borlaug was awarded a Nobel Peace Prize. Just as the Green Revolution revolutionized world wide food production in the early 20th century, the "Gene Revolution" is expected to have a similar impact on food production in the 21st century (Huffman 2004). The process behind the "Gene Revolution" is the genetic modification of plants

to contain a foreign gene or segment of DNA. These crops have become known as genetically modified (GM) organisms and have been the subject of worldwide debate.

Since the weed red rice is such a severe problem in commercial fields and is difficult to control by traditional methods, there has been interest in using herbicide tolerant rice varieties as an alternate method of control. It has been predicted that implementation of GM herbicide resistant rice could cut general herbicide usage by 3.8 million pounds per year, with approximately 49 million dollars saved through cost reductions (Gianessi et al. 2002).

Currently, three different types of herbicide tolerant rice varieties are under commercial development. The most advanced of the herbicide tolerance systems is CLEARFIELD™. These are non-GM rice cultivars resistant to imazethapyr, a potent inhibitor of acetolactate synthetase (ALS). CLEARFIELD™ varieties were planted in commercial fields for the first time in the summer of 2001. A substantial amount of work has also been done on Roundup Ready™ rice, GM rice that contains a bacterial gene that confers resistance to glyphosate. Of most direct importance to this study, however are LibertyLink™ rice varieties. These varieties contain the bacterial BAR gene which provides tolerance to the herbicide Glufosinate (also known as phosphinothricin (PPT), Liberty or Basta) (Oard et al. 1996).

Glutamine Synthetase – The Target Enzyme of Glufosinate

Glutamine synthetase (GS, EC 6.3.1.2) is a primary enzyme in nitrogen metabolism and has been proposed to be the product of one of the oldest functioning genes. The physiologically relevant reaction catalyzed by GS is the ATP dependent formation of glutamine from ammonia and

glutamate in the presence of a divalent metal ion (either magnesium or manganese) (Purich 1998).

$$NH_4^+ + \text{Glutamate} + \text{ATP} \xrightarrow{Mg+2\ (Mn+2)} \text{Glutamine} + \text{ADP} + P_i$$

Most of our knowledge about GS is based on studies of the bacterial enzyme. The bacterial enzyme, referred to as GSI, is a 620-650 kDa dodecamer consisting of two donut shaped hexamers stacked on top of each other. X-ray crystallography revealed that the 12 active sites are at the interfaces between the monomers. The active site has been described as a bifunnel with ATP and glutamate binding on opposite sides and the divalent metal binding at the joint of the bifunnel (Figure 1-1). Ammonia binds after ATP binding near the glutamine site (Eisenberg et al. 2000).

Much of the work on the eukaryotic form of GS, referred to as GSII, has been on the enzyme from brain tissue (Eisenberg et al. 2000). This enzyme ranges from 350 to 550 kDa in size. In contrast to the dodecomeric structure of the bacterial enzyme, eukaryotic GS is believed to be octameric in structure, with two layers consisting of four sub-units each. There is currently no three dimensional structure of the eukaryotic enzyme and the number of active sites is a matter of debate. However, a number of sequence similarities, including residues in the active sites, allowed Eisenberg et al. (1987) to propose a model for the eukaryotic enzyme that is based on the structure of the bacterial enzyme (see also Forde and Cullimore 1989).

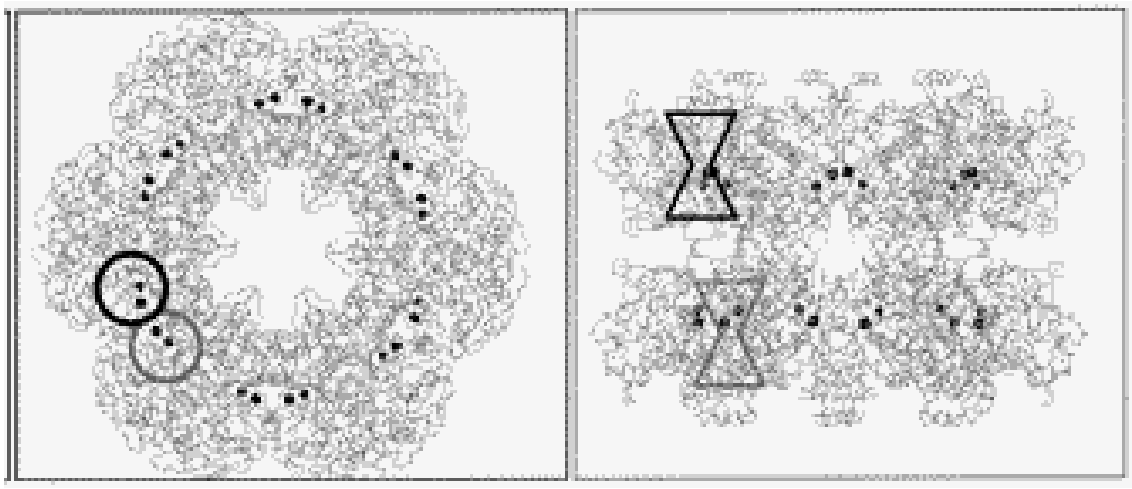

Figure 1-1. Dodecameric structure of prokaryotic glutamine synthetase. Overhead and side view of the active dodecameric glutamine synthetase of *Salmonella typhimurium*. The active site on the upper hexamer is indicated by the black hourglass, while an active site on the lower hexamer is indicated by the gray hourglass. The two ammonium binding sites in each active site are indicated by the black dots. Adapted from Gill and Eisenberg 2001.

In higher plants GS functions with glutamate synthase (GOGAT) in the GS/GOGAT cycle to assimilate NH_4^+ produced by nitrate reductase (NR) and recycle NH_4^+ resulting from photorespiration and de-amination reactions (Figure 1-2). In contrast to the single form of GSI, and the two forms of GSII in mammals, up to six different forms of GSII, coded by multiple genes, have been identified in higher plants. In general there are two major classes of GSII in plants, $GSII_a$ (or GS1), which is localized in the cytoplasm, and $GSII_b$ (or GS2), which is located in the chloroplast (Lea 1997).

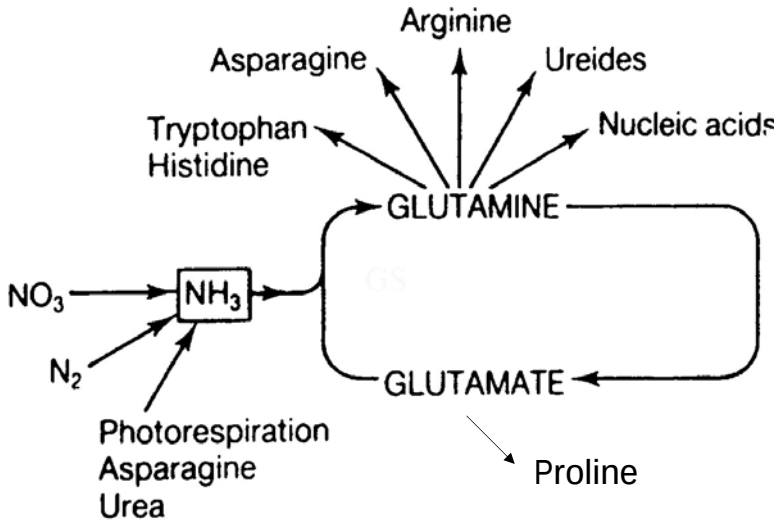

Figure 1-2. Flow of nitrogen through nitrogen assimilation and subsequent metabolism in the plant system. Adapted from Lea 1997.

The chloroplastic form, GS2, is coded by a single gene and is the major form of GS found in leaves. *Brassica napus* mutants lacking GS2 that can survive only in non-photorespiratory conditions (high CO_2, low O_2), demonstrate that GS2 is involved in the reassimilation of NH_4 produced by photorespiration (Wallsgrove et al. 1987).

Cytosolic GS (GS1) is the primary from of the enzyme found in the roots, etoliated leaves, and the only GS found in seeds thus far (Zhang et al. 2000). Different forms of GS1, coded by multiple genes have been identified on a tissue specific basis (Oliveira and Coruzzi 1999; Ishiyama et al. 2004a). It was recently shown that four of the five forms of GS1 in Arabidopsis

are differentially regulated by NH_4^+ availability. This helps to explain the complexity of the regulation of nitrogen metabolism (Ishiyama et al. 2004). Lack of mutants, probably due to the redundancy between the different forms, has complicated the elucidation of the exact function of the cytosolic enzyme. However, biochemical data, along with the localization of the enzyme have led researchers to postulate that it is involved in the production of glutamine for nitrogen transport (Cren and Hirel 1999).

In rice, three forms of glutamine synthetase have been relatively well characterized (Iyer et al. 1981; Sakamoto et al. 1989, Devine et al.. 1993). Two isoforms of GS1 are found in the cytoplasm, with one of those forms specific for the root, and GS2 is localized in chloroplasts. However, other less well-characterized isoforms also exist. For example, a second ammonia and nitrate inducible form of GS1 was recently detected in roots and has a different electrophoretic mobility than either the constitutive root form of GS1 or the cytosolic GS1 in leaves (Zhang et al. 1997). Whether this represents a different gene or a post-translational modification is not yet known.

In addition to GS1 and GS2, there was a recent report that plants also contain a form of glutamine synthetase that is more similar to bacterial glutamine synthetase than to the typical eukaryotic form of the enzyme (Mathis et al. 2000). This contradicts the previous belief that GSI was found solely in bacteria and GSII was found only in eukaryotes. The bacterial-like glutamine synthetase has thus far only been partially characterized in *Medicago* (accession CAB63844.1). However, it should be noted that the translated sequence of the rice cDNA clone RZ625 (AI978456) has very strong homology with the new form of glutamine synthetase from *Medicago* (E value = 2e –44).

Expression of GS2 is regulated by a phytochrome mediated pathway (Lea 1997) via phytochrome mediated interaction of cis-acting transcriptional regulatory elements in the promoter (Edwards and Coruzzi 1989; Oliveria and Coruzi 1999). GS2 is not directly regulated by nitrogen levels or by metabolite levels (Cren and Hirel 1999). In contrast, GS1 isoforms are regulated both by nitrate and NH_4^+ levels, as well as the levels of sugars and other metabolites (Oliveira and Coruzzi 1999; Ishiyama et al. 2004b).

In addition to direct effects, light also regulates the expression of both GS1 and GS2 through a type of carbon/nitrogen feed back regulation. Light activates photosynthesis which causes a build up of carbon skeletons. This in turn triggers nitrogen assimilation through the GS pathway. Addition of sugars in the absence of light can also cause the accumulation of both GS1 and GS2 transcripts (Oliveria and Coruzi 1999; Oliveria et al. 2001). In the presence of light, NH_4^+ is assimilated through GS into glutamine for further metabolism. In the dark, when carbon skeletons are limiting, glutamine is converted to asparagine for nitrogen storage. While we have a good understating of the basic points of regulation outlined above, the regulation of GS is obviously complex and relies on the interaction of many different genes and other factors, which are not fully understood.

Glutamine synthetase has also been implicated in a variety of plant stress responses. Lack of water and high levels of salt result in cessation of photosynthesis, and subsequent increase in photorespiration and production of reactive oxygen species (ROS) (Hasegawa et al. 2000; Zhu 2001). Production of proline, a downstream product of the GS cycle, is also triggered to adjust the osmotic imbalance in the cytosol. Rice plants over-expressing GS2 have increased levels of salt tolerance and an increase in photorespiration capacity (Kozaki and Takeba 1996; Hoshida et

al. 2000). Expression of antisense GS1 in tobacco has been shown to decrease proline production and significantly lower level of salt tolerance (Brugiere et al. 1999).

Effects of Inhibiting Glutamine Synthetase with Glufosinate

Glufosinate, or L-phosphinothricin (PPT), is a structural analog of glutamate and a potent inhibitor of GS. Recently, Gill and Eisenberg (2001) reported the crystal structure of *Salmonella typhimurium* GS bound to PPT (Figure 1-3). A model based on this structure predicts that when PPT is phosphorylated by ATP, it becomes an irreversible inhibitor by occupying the glutamate-binding site and disrupting the ammonia-binding site.

As reviewed in Devine et al. (1993), inhibition of GS under conditions that support photorespiration (high light, low CO_2) leads to rapid accumulation of ammonia and cessation of photosynthesis. Plant death has traditionally been attributed to the high levels of ammonia that accumulate (Devine et al. 1993; Lea 1997). However, data from several laboratories indicate that the decrease of glutamine and subsequent accumulation of glyoxylate, an intermediate of photorespiration and a RUBISCO inhibitor, causes an interruption in photosynthesis that ultimately leads to plant death (Evstigneeva et al. 2003; Sauer et al. 1987; Wallsgrove et al. 1987).

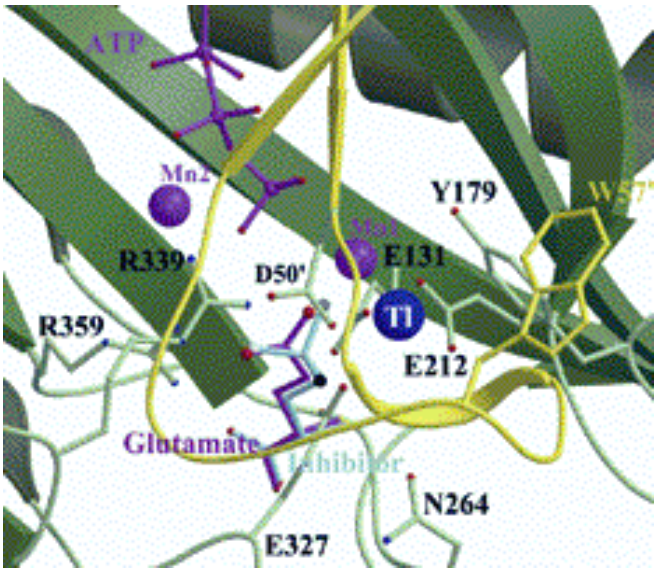

Figure 1-3. Active site of *Salmonella typhimurium* GS bound to PPT or glutamate, manganese and ATP. Adapted from Gill and Eisenberg 2001.

As would be expected, environmental conditions have a major effect on the toxicity of glutamine synthetase inhibitors. In general, intense light and high temperature would be expected to increase metabolic demand for glutamine synthetase (Lam et al. 1996). Thus, it is not surprising that higher temperature resulted in injury symptoms developing sooner after glufosinate treatment than lower temperatures (Petersen and Hurle 2001). As expected, light also has a significant effect on the efficacy of glufosinate (e.g. Sellers et al. 2004). Wild and Manderschied showed that NH_4^+ accumulation associated with PPT toxicity in plants was light dependent (Wild and Manderschied 1984). Ammonia accumulation only occurs with the presence of light, with plants treated after dark having a lower accumulation of NH_4^+ 72 hours after treatment than those treated in the afternoon (Sellers et al. 2004).

Time of day (TOD) has been shown to affect the toxicity of glufosinate. In velvet leaf, at least 4 hrs of light needed to achieve maximum toxicity (Martinson et al. 2002). The TOD effect has been shown to be due to the ammonium accumulation and the activity of the GS enzymes, not to adsorption or translocation (Sellers et al. 2004). Relative humidity also has an effect on the efficacy of glufosinate. Exposure to high humidity (95%) within 12 hours of either side of application increased efficacy when compared to applications under low humidity (40%). C-14 labeled glufosinate revealed that this difference was not due to altered adsorption or uptake at the different humidity levels. Instead, there was a difference in translocation of glufosinate at high and low relative humidity (Coetzer et al. 2001; Ramsey et al. 2002).

While most of the work on glufosinate in plants has focused on its role as a herbicide, low concentrations of the compound have also been shown to have growth hormone like activity. Toldi et al. (2000) were able to induce microshoots from rice seedlings grown on low concentrations of glufosinate. A growth regulator effect was also seen in snapdragon, where addition of 0.5mg/L glufosinate to the regeneration media increased shoot regeneration in agrobacterium transformed plants by 56% (Hoshino and Mii 1998). Other work has also shown that the growth of grape embryogenic calli was stimulated by the addition of 0.5mg/L glufosinate to the media (Herbert-Soule et al. 1995).

Glufosinate Resistance in Plants

The bacterial BAR gene provides herbicide tolerance in a variety of plant species by coding for the production of phosphinothricin N-acetyltransferase which enzymatically inactivates glufosinate and related compounds (Mazur and Falco 1989; Evstigneeva et al. 2003). However,

glutamine synthetase isoforms in plants are also known to vary substantially in their sensitivity to glufosinate (Ridley and McNally, 1985). In particular, it has been shown that a single amino acid substitution in pea glutamine synthetase can confer resistance to L-methionine sulfoximine (a structurally similar inhibitor of glutamine synthetase) yet the enzyme retains almost full enzymatic activity at high levels of ammonia in vitro (Clemente and Marquez 1999a). A naturally occurring mutation in maize GS1 has recently been shown to provide high levels of tolerance to glufosinate in field conditions. RT-PCR of the GS from the resistant cell line revealed a 12 nucleotide substitution which resulted in the alteration of 10 amino acids when compared to the native form of maize GS (unpublished observation, GenBank accession number AY339214).

Amplification of the GS1 gene can also provide glufosinate tolerance. In 1984, Donn et al. reported selection of alfalfa suspension cultures that were 20-100 fold more resistant to glufosinate than wild type cells. It was subsequently shown that glufosinate tolerance in these lines was due to an 4-11 fold amplification of the GS1 gene, resulting in a 3 to 7 fold increases in the amount of GS1 protein (Donn et al.. 1984). Similarly, tobacco cells selected in tissue culture for resistance to the herbicide Basta (active ingredient phosphinothricin) contained 20x more GS protein than did the wild type cells (Ishida et al. 1989).

Yamaya et al. (1990) utilized the phosphinothricin analog methionine sulfoximine (MSX) for the selection of resistant cells in tobacco suspension cultures. The resistant cell line obtained had an I50 of 4.65 μM, compared to the I50 of 0.18 μM for the wild type. Surprisingly, this line had only a 1.5x increase in GS activity. However, two-dimensional PAGE analysis revealed that the tolerant cell line had two major proteins at 40 kDa that were not present in the wild type cell line.

This is similar to other work in tomato and pine which has demonstrated that exposure to PPT induces novel forms of GS (Avilia et al. 1998; Perez-Garcia et al.. 1998).

Some Red Rice in Commercial Fields Is Naturally Glufosinate Tolerant

Herbicide resistant crops provide a powerful weed management tool particularly for weeds such a red rice that are difficult to control by traditional methods. However, there are significant reasons for concern about transfer of herbicide tolerance genes into weed species and about selection of herbicide tolerant weeds.

Gene transfer may be a particular problem for rice since hybridization levels of 50% or greater between cultivars and red rice has been documented in field trials with even greater levels shown in the greenhouse (Langevin et al. 1990). After hybridization, transgenes, such as the BAR gene, which confers glufonsinate tolerance, could become part of the red rice genetic base. Since red rice falls to the ground when mature and can remain dormant in the soil for many years, any field where red rice occurs could be contaminated with transgenes indefinitely (Ellstrand et al. 1999).

Concern about the use of herbicide resistant commercial rice varieties is further complicated due to the fact that some ecotypes of red rice already have substantial levels of tolerance to the same herbicides that will be used with GMO crops. The most notable example is TX4, which has substantial tolerance to glufosinate, the herbicide used with Liberty-Link^{TM} GMO rice (Noldin et al. 1999a, 1999b).

In the original study, 19 red rice ecotypes collected from Arkansas, Louisiana, Mississippi and Texas were screened under greenhouse conditions. All of the ecotypes, with the exception of TX4, were effectively controlled (>93% "control") with a single application of 0.56 kg ai ha^{-1} glufosinate. Only 46% control (54% of treated plants surviving) was obtained with TX4 under these conditions. Effective control of TX4 required at least 1.12 kg ai ha^{-1} of herbicide (Noldin et al. 1999b).

A recent poster reported the results from screening 160 red rice ecotypes. The red rice ecotype TX4 was again noted to be especially tolerant to 0.55 kg ai ha^{-1} of glufosinate (only 56% control). However, tolerance was also seen in the red rice accessions 17C, LA3, MS4, 2B, 4A, and 20E (Gealy et al. 2000).

TX4, and other types of red rice, have also been found to be tolerant to two applications of glufosinate under field conditions in experiments performed in Arkansas (Gealy and Black 1998). In these experiments TX4 produced an average of 40% as much dry matter after two applications of 0.42 kg ai ha^{-1} glufosinate as untreated plants. Interestingly, TX4 also produced approximately 40% as much dry matter after two applications of 0.83 kg ai ha^{-1} glufosinate. A second red rice ecotype, StS, produced an average of 20% of control levels of dry matter after two applications of 0.42 kg ai ha^{-1} glufosinate, but produced only very low levels with higher concentrations.

Although the level of glufosinate tolerance in TX4 has been investigated by several groups, it has been the subject of controversy (Gealy and Black 1998; Gealy et al. 2000; Wheeler et al. 2000; Wheeler and TeBest 2001). Most researchers have claimed that TX4 does not have enough

tolerance to be a significant issue for weed control despite finding that TX4 was not effectively killed by glufosinate. Conversely, others have reported that TX4 has significant tolerance to glufosinate. The controversy and actual levels of herbicide resistance of TX4 will be investigated further in Chapter V.

Herbicide "Sensitive" Accessions Sometimes Show Tolerance to Glufosinate

While substantial glufosinate tolerance has been noted in TX4, and lower levels have been observed in other red rice ecotypes, these are exceptions to the general sensitivity of red rice to glufosinate. Thus under field conditions, glufosinate normally gives very effective weed control. The left side of Figure 1-4, which was taken from Steele 2000, shows typical results from field studies designed to evaluate the use of glufosinate in conjunction with Liberty-Link^{TM} rice. Even though a type of straw-hulled red rice from Arkansas was planted in rows perpendicular to the GM rice to supplement the natural population of red rice in the experimental plot, excellent control of the red rice was seen after two applications of only 0.29 kg ai ha^{-1} glufosinate (Steele 2000). The Liberty-Link^{TM} rice was healthy and the space between the rows was clear.

Figure 1-4. Side by side comparison of glufosinate application to red rice and Liberty-Link™ rice. The left side is from 1998 and the right from 1999. The same protocol and source of red rice were used for both studies. Cultivars are planted in the vertical rows and red rice in the horizontal rows. (From Steele 2000, used by permission).

However, in other years much less effective control was obtained even using the same herbicide application protocol and the same source of red rice (Steele 2000). This is illustrated on the right side of the figure where the perpendicular rows of red rice were able to re-sprout after herbicide application and completely fill the spaces between the rows of Liberty-Link™ rice. As might be expected, poor control is correlated with cool cloudy conditions that would be expected to minimize the lethality of glufosinate by reducing photorespiration. However, other factors may also have been involved.

Herbicide Tolerance Can Be Induced in "Susceptible" Types in Tissue Culture

It has recently been shown that glufosinate tolerance can be efficiently induced by tissue culture of rice plantlets on sub-lethal doses of glufosinate (Toldi et al. 2000). In this study, rice plants with coleoptiles 5-8 mm in length were excised from the scutellum 3-7 days after germination and placed on hormone-free media containing glufosinate. In a narrow concentration range, glufosinate was found to induce the formation of multiple shoots on up to 40% of the plantlets. When several hundred of these shoots were grown and transferred to pots in the greenhouse, 78% survived two applications of a glufosinate (phosphinothricin) based herbicide that killed all of the control tissue cultured and greenhouse grown plants not previously exposed to the herbicide. The glufosinate tolerant plants were fertile, but passed only transient glufosinate tolerance on to their progeny. The transient protection of the progeny was proposed to be due to the accumulation of glutamine synthetase isoforms during seed filling analogous to the well-studied maternal effects on development seen in *Drosophila* embryos (Rivera-Pomar and Jackle, 1996). In agreement with this suggestion, plants derived from the selection of seeds on glufosinate were reported to have approximately 2x higher GS activity than did those not selected on PPT (Toldi et al. 2000).

CHAPTER II

IS ALL RED RICE FOUND IN COMMERCIAL RICE REALLY *Oryza sativa*?[*]

Overview

All red rice found in commercial rice in the United States has traditionally been classified as *Oryza sativa* ssp. *indica*. This assumption was tested by analyzing red rice samples collected from across the southern United States rice belt with 18 Simple Sequence Length Polymorphism (SSLP) markers distributed across all 12 chromosomes. The results clearly demonstrate that the traditional classification of red rice is inadequate. Some red rice is closely related to *Oryza sativa* ssp. *indica* cultivated rice. However, other red rice is more closely related to *Oryza sativa* ssp. *japonica*. Most importantly, some red rice samples collected from Arkansas, Louisiana, Mississippi, and Texas form a distinct group that includes a number of *Oryza nivara* and *Oryza rufipogon* accessions from the National Small Grains Center. In particular, red rice samples from three states were identified for all 18 markers as being identical to the *Oryza rufipogon* accession IRGC 105491.

These different classes of red rice are intermingled across the southern U.S. rice belt and within individual production fields. *Oryza sativa* ssp. *indica*-like red rice and *Oryza rufipogon*-like red rice have been found within a single 9 m^2 collection site. While the classification of red rice as *Oryza sativa* ssp. *indica*, *Oryza sativa* ssp. *japonica* or *Oryza rufipogon* using DNA markers is

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[*] This chapter has been previously published. Vaughan, L.K., B.V. Ottis, A.M. Prazak-Havey, C.A. Bormans, C. Sneller, J.M. Chandler, and W.D. Park. Is all red rice found in commercial fields really *Oryza sativa*? Weed Science. 49: 468-476. Used with permission.

generally in agreement with classification based on simple morphological traits, readily observed morphological traits alone are not sufficient to reliably classify red rice. Because red rice is much more diverse than previously assumed, its diversity must be considered when developing red rice management strategies.

Introduction

The weed red rice has traditionally been classified as *Oryza sativa* L. based on phenotypic characteristics (Diarra et al. 1985; Langevin et al. 1990; Kwon et al. 1992). In the United States, red rice is loosely grouped into two subclasses: straw-hulled and black-hulled, with straw-hulled being the most common. Within these subclasses, characteristics such as seed color and awning have been used to classify red rice into different ecotypes. There are no accurate estimates of how many different ecotypes exist. In 1850, the USDA identified four different ecotypes (Craigmiles 1978). Since then over 50 different types have been identified in the rice producing regions of the United States (Lago 1982; Noldin et al. 1999a).

Recently, genetic methods such as Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), and Randomly Amplified Polymorphic DNA (RAPD) have been used to differentiate between *Oryza sativa* subspecies *indica* and *japonica* and to look at the levels of diversity within rice subspecies (Mackill 1995; Olufowote et al. 1997; Parsons et al. 1997; Zhu et al.1998; Li et al. 2000; Virk et al. 2000). The phylogenetic relationship, or the genealogy, of the different *Oryza* species has also been examined using genetic methods (Aggarwal et al. 1999; Ge et al. 1999). The only molecular examination of cultivated rice and its weedy relatives used Korean weedy rice and a limited number of

cultivated accessions (Cho et al.1995; Shu et al. 1997). These studies used RFLP and RAPD technology to show that the weedy rice could be divided into *Oryza sativa* ssp. *japonica* and *Oryza sativa* ssp. *indica*-like groups, with some intermediate accessions having characteristics from both groups.

The high rate of fertility in crosses supports the traditional argument that red rice and cultivated rice are both *Oryza sativa*. However, not everyone agrees with this traditional classification. It has been argued that red rice is actually *Oryza barthii*, *Oryza longistaminata*, *Oryza rufipogon*, *Oryza perennis*, or *Oryza punctata* (Parker and Dean 1976; Craigmiles 1978; Kwon et al. 1991; Oka 1991). These arguments are based on morphological characteristics, but the high degree of variation and lack of a clear classification system makes it difficult to definitively categorize red rice.

Gene flow, or hybridization, is a major concern in rice because of the close relationship between the crop and its weedy relatives. Hybridization levels of 50% or more between cultivars and red rice have been documented in field trials with even greater levels shown in the greenhouse (Langevin et al. 1990). This phenomenon in itself creates a “red rice complex” which is dynamic and constantly evolving. Gene flow will become an important issue with the introduction of genetically modified crops into the natural population. Any modified genes placed in cultivated crops can be expected to flow into wild relatives in a short period of time. In the case of rice, these modified genes could then become part of the red rice genetic base. If this occurs, any field where red rice occurs could be contaminated with the foreign gene indefinitely (Ellstrand et al. 1999).

Field trials that evaluate herbicide efficacy and red rice competition typically use local populations of red rice, or a limited selection from different rice producing areas. Often, these experiments use only straw-hulled red rice (Khodayari et al. 1987, Kwon et al. 1991, Sankula et al. 1997a, Sankula et al. 1997b). Because of the diversity of the red rice complex, these samples may not be an adequate representation of the overall spectrum of red rice ecotypes. While accurate for the time and location of a specific study, these studies may be misleading if used to develop red rice control strategies for other areas.

Proper classification of red rice is important for the development of effective red rice control strategies. However, a complete understanding of the actual diversity of the red rice complex cannot readily be gained with traditional phenotypic characterization. Genetic analysis will give a clearer picture of the true diversity in natural red rice populations, and also provide tools that can be used to monitor gene flow from cultivated rice to red rice. The objective of this study was to genetically classify selected populations of red rice, and also to gain insight on the population structure and diversity of red rice in the southern United States rice belt.

Materials and Methods

Samples

A collection of mature red rice seed was obtained from rice fields in Brazoria County, Texas during the summer of 1998, just prior to the harvest of the predominant commercial cultivar, Cypress. Three fields were sampled, one in the northwestern section of the county and two in the southeastern. The upper southeast field is approximately 11 km from the northwest field. The lower southeast field is 3 km south of the upper southeast field. Within each field, panicles were collected from red rice plants in discrete 'patches' or small areas of infestation, approximately 9 m^2. Several patches in different parts of each field were sampled. Global positioning system (GPS) data were recorded for each sample taken. From each of the patches sampled, two to six plants were then selected for marker analysis based on differences in seed phenotype. Southern red rice collected and characterized in 1992 and 1993 from Arkansas, Louisiana, Mississippi, and Texas was also examined (Noldin et al. 1999a). These samples are identified by a two-letter code for the state and the number of the ecotype (e.g. TX2). Annual, black hulled *Oryza nivara* and *Oryza rufipogon* samples were obtained from the National Small Grains Collection (NSGC) in Aberdeen, Idaho and are identified by their NSGC accession number (http://www.ars-grin.gov/ars/PacWest/Aberdeen/nsgc.html). *Oryza rufipogon* International Rice Genomic Center (IRGC) accession 105491 (Xiao et al. 1998; http://singer2.cgiar.org), the *Oryza sativa* ssp. *indica* and *japonica* cultivars were supplied by Dr. Anna McClung from the USDA/ARS Station in Beaumont, TX.

Genetic Analysis

Twenty seeds from each sample were pooled. The DNA was extracted and analyzed using microsatellite, or Simple Sequence Length Polymorphism (SSLP), markers (Williams 1994). Eighteen different primer pairs[1] distributed across the 12 chromosomes were used for the analysis. Markers used include: RM102 (1), RM5 (1), RM166 (2), RM110 (2), RSus1 (3), RM143 (3), RM241 (4), RM153 (5), RM146 (5), WAXY (6), RSus2 (6), RM162 (6), RM118 (7), RM152 (8), RM242 (9), RM171 (10), RM20-L (11), OSM90 (12). Numbers in parentheses indicate the chromosome on which the marker is located. All of these markers are part of an integrated DNA map of rice that covers all 12 of the rice chromosomes (Temnykh et al. 2000). Primer pairs that have a low degree of polymorphism in the samples tested (two or three alleles) as well as those having a high degree of polymorphism (up to twelve alleles) were used to provide less biased results. PCR reactions for each microsatellite primer pair were carried out following guidelines listed on the RiceGenes web site (http://ars-genome.cornell.edu/rice) in a Stratagene Robocycler[2]. PCR products were separated by electrophoresis on 5% denaturing polyacrylamide gels. Gels were stained with SYBR® Green fluorescent dye[3] and the bands were visualized on a STORM® Imaging System[4] according to manufacturer instructions.

Data Analysis

The SSLP data are represented by two methods. The first is a simple color-coded tabular compilation of the allelic data for each red rice accession and the *Oryza sativa* cultivars. This has the advantage of allowing one to see the distribution of polymorphism across the 12 chromosomes and the degree of polymorphism at each marker. It is complementary to the

statistical analysis discussed below. In Figure 2-1 each row displays the data for an individual accession; i.e. the first row represents *Oryza sativa* ssp. *japonica* var. Nipponbare. Accessions that belong to the same type (e.g. red rice) are grouped together. The columns of the chart represent the data for each of the 18 microsatellite markers. For example, the first row represents the data for the microsatellite marker RM102, which is located on chromosome one. The letters in each column reflect the relative size of the amplified product for that particular marker, with (A) being the largest. For each marker the alleles were color-coded based on the standards for each type; Nipponbare for the *japonicas* (coded yellow), Taichung Native 1 (TN1) for the *indicas* (coded blue), and MS3 for red rice (coded red). Alleles that were common between the *Oryza sativa* ssp. *indica* and *japonica* cultivars were coded green. Alleles that were common between MS3 red rice and the *Oryza sativa* ssp. *indica* or *japonica* cultivars were coded purple and orange respectively. Other alleles that are common for each type are coded with a secondary color, such as pink for the second major allele for red rice in RM110.

The second method used to represent the data (Figure 2-2) is based on analysis of the SSLP data with genetic distance calculations (Gizlice et al. 1996). Genetic distances (GD) between all pairs of accessions were calculated as GD =1 –A/N where A is the total number of SSLP alleles shared by two accessions and N is the total number of SSLP loci scored for the two accessions. These values can range from zero (all alleles in common) to unity (no alleles in common). The lower the GD between two samples, the more related the accessions.

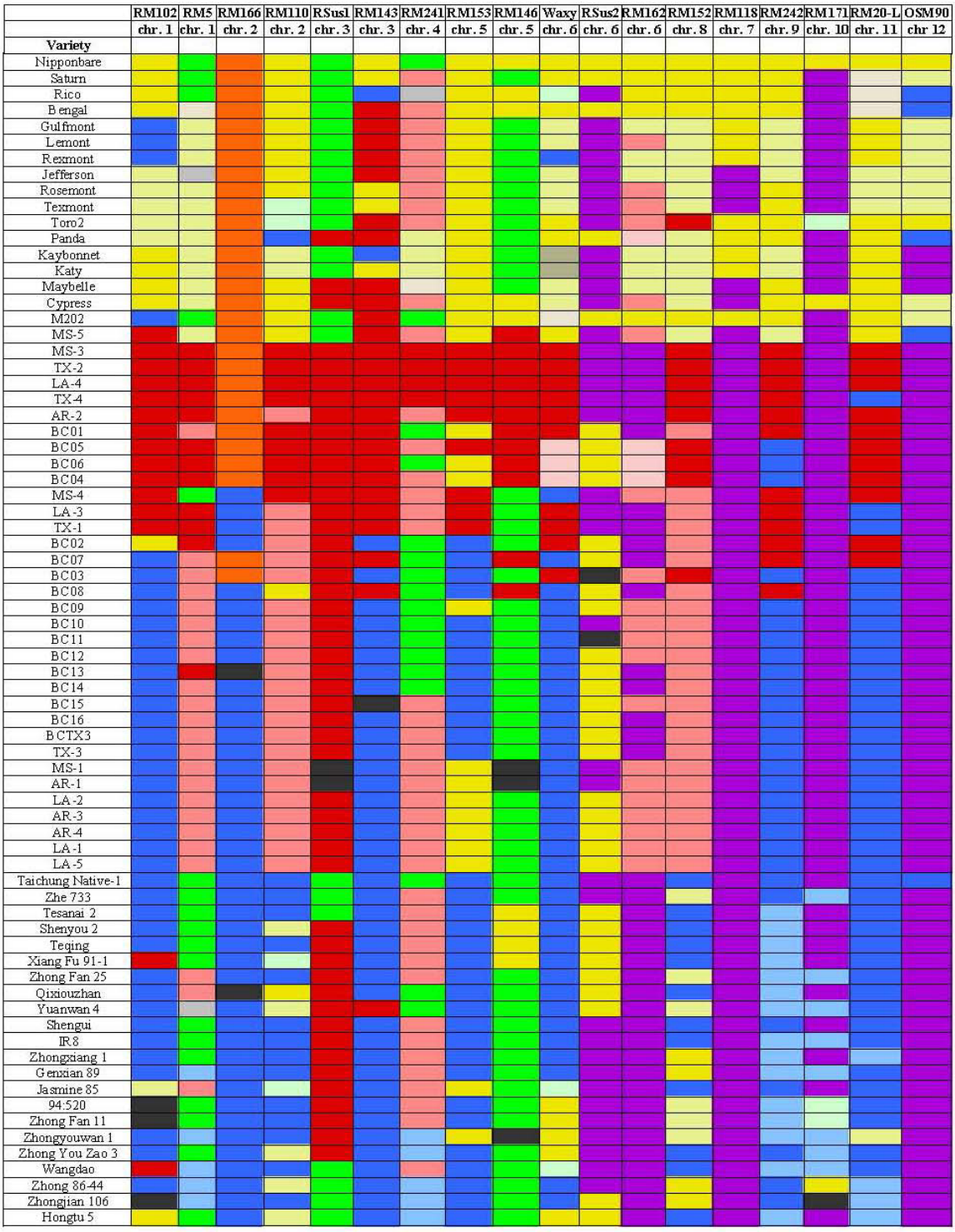


Figure 2-1. Color-coded simple sequence length polymorphism (SSLP) data for *Oryza sativa* cultivars and red rice. Columns represent data for each SSLP marker assayed and rows represent each accession. Each column is independently scored based on allele sizes. Color codes reflect the predominant allele for each of the three major types, red for red rice, yellow for Oryza sativa ssp. japonica cultivars and blue for *Oryza sativa* ssp. *indica* cultivars. See text for further explanation.

To group accessions, the matrix of GD values was subjected to hierarchical cluster analysis using the average linking method. A cluster was defined as having a within group average GD that was 85% or lower than the overall average GD. Clusters were considered to be independent if the average GD between a given cluster and the most related cluster was greater than the overall average GD. Genetic distances were calculated with a SAS IML program while clustering was performed with PROC CLUSTER of SAS (SAS Institute Inc. 1997).

The GD matrix was also subjected to a Multidimensional Scaling (MDS) analysis. MDS is similar to principle component analysis (PCA), which has been used to show the relationship between the different *Oryza* species (Xiao et al. 1998). Both start with a dissimilarity matrix and use Eigen analysis to summarize and condense the variance within the GD matrix to a few dimensions. The MDS has an advantage over PCA by providing dimension specific solutions, giving a better two-dimensional representation of the 18 dimensional data. MDS then uses an iterative process to find a set of coordinates in Euclidian space that best represents the original distances in the GD matrix (Gizlice et al. 1996). The axes of the graph are centered around the average of the dimensions, which is set at zero. Thus the linear distance between two points in Figure 2-2 estimates the actual GD between those two points. MDS analysis was performed using SAS software. MDS has been used in wheat (*Triticum vulgaris* L), soybean (*Glycine max* L.) and common bean (*Phaseolus vulgaris* L) to characterize genetic diversity within different sets of cultivars (Beebe et al. 1995; Gizlice et al. 1996; Johns et al. 1997; Skroch et al. 1998; Stachel 2000).

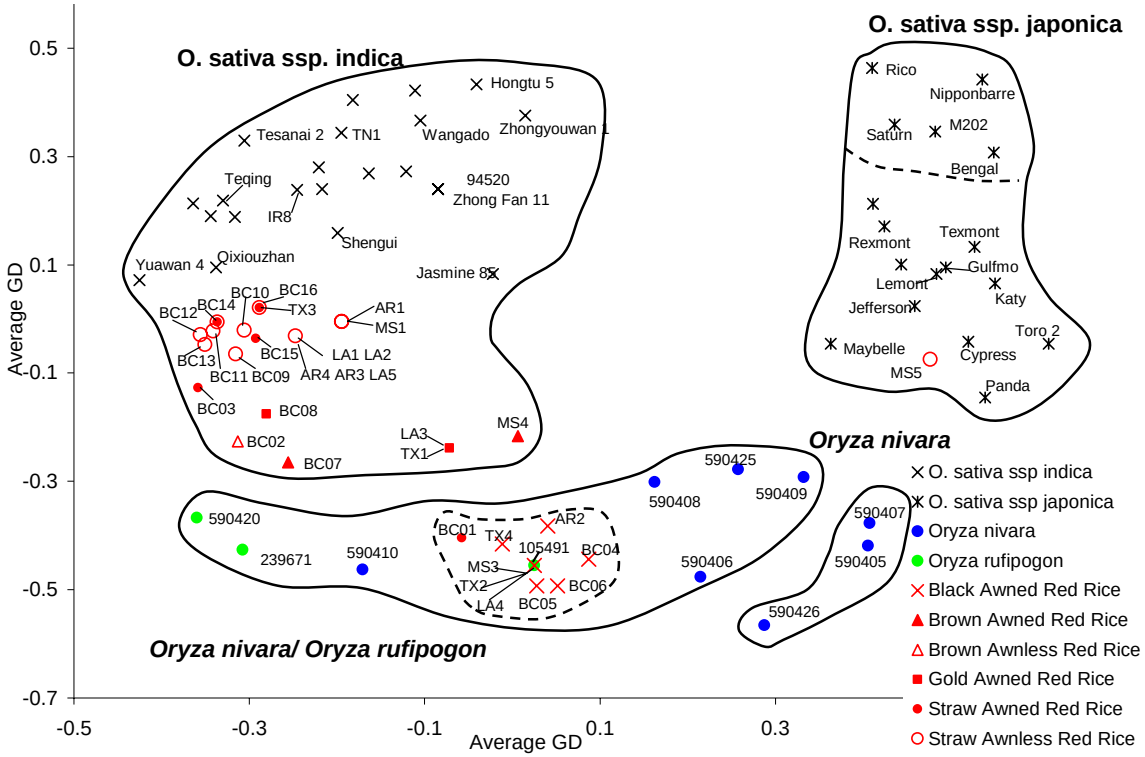


Figure 2-2. Multi-dimensional scaling (MDS) of SSLP data for *Oryza* species and red rice accessions. Plot of SSLP data using coordinates from the first two dimensions of multidimensional scaling analysis of the genetic distance matrix. The linear distance between two samples estimates the actual genetic distance between the samples using the X or Y-axis as the scale. Samples that were in the same cluster are circled with a solid line, while dashed lines indicate subgroups with a cluster. Symbols (see key) are used to differentiate samples based on type and morphology. See text for further explanation.

Results and Discussion

Genetic Identification of Red Rice

The 17 southern red rice ecotypes collected in 1992 and 1993 and the Brazoria County red rice ecotypes collected in 1998 were examined alongside a representative collection of *Oryza sativa* commercial cultivars from the United States and Asia and black hulled *Oryza nivara* and *Oryza rufipogon* accessions available from the NSGC. As can be seen in Figure 2, these samples fall into four main groups. The statistical analysis (Figure 2-2) clearly shows the distinction between the four major groups of red rice and the different *Oryza* species, while the color-coded data in Figure 2-1 demonstrate that the allelic differences that distinguish the red rice from the cultivar groups are widely distributed across the rice genome, rather than being restricted to particular chromosomes. The overall average GD for the entire data set is 0.627 (Figure 2-2, Table 2-1).

As expected, the commercial cultivars from the United States cluster with the *Oryza sativa* ssp. *japonica* cultivar, Nipponbare (Temnykh et al. 2000). The GD within this group is 0.472. This group is easily distinguished from the *Oryza sativa* ssp. *indica* and *Oryza nivara/ Oryza rufipogon* groups by a GD of 0.786 and 0.778, respectively. Consistent with previous DNA marker analysis, the *japonica* group can be further separated into two subgroups (Mackill 1995). These are illustrated by the heavy dashed line in Figure 2 and correspond to "temperate *japonica"* cultivars, such as the medium grain variety Nipponbare, and "*javanica*" (tropical *japonica*) cultivars such as the long grain variety Lemont. The GD between these two subgroups is 0.593, which is slightly below the standard cut-off of 0.627 (the distance between two groups must be

Table 2-1. Average genetic distance (GD) within and between *Oryza sativa* cultivars, *Oryza nivara*, *Oryza rufipogon* and red rice ecotypes in Figure 2-2. To be considered independent a group must have a within group GD that is less that 85% of the overall GD of 0.627 (0.533) and the GD between that group and the next most related group must be greater than the overall GD of 0.627.

	GD
Overall	**0.627**
Oryza sativa ssp. *japonica* cultivars	0.472
Temperate *japonica* cultivars	0.306
Tropical *japonica* (*javanica)* cultivars	0.393
Temperate *japonica* vs. tropical *japonica* (*javanica)*	0.593
Oryza sativa ssp. *indica* cultivars and *indica*-like red rice	0.402
Oryza sativa ssp. *indica* cultivars	0.359
Oryza sativa ssp. *indica*- like red rice	0.272
Oryza sativa ssp. *indica*- like straw-hull red	0.143
Oryza sativa ssp. *indica*- like gold/brown-hull	0.394
Oryza sativa ssp. *indica*- like straw-hull vs.	0.408
Oryza nivara	0.525
Oryza nivara/ Oryza rufipogon	0.500
Oryza rufipogon- like red rice	0.265
Oryza sativa ssp. *indica* cultivars vs.	
Oryza sativa ssp. *japonica*	0.786
Oryza sativa ssp. *indica*- like red rice	0.478
Oryza sativa ssp. *indica*- like straw-hull red	0.447
Oryza sativa ssp. *indica*- like gold/brown-hull	0.557
Oryza nivara/ Oryza rufipogon	0.702
Oryza rufipogon- like red rice	0.729
Oryza nivara	0.793
Oryza sativa ssp. *indica* cultivars and *indica*-like red rice vs.	
Oryza sativa ssp. *japonica*	0.786
Oryza nivara/ Oryza rufipogon	0.676
Oryza rufipogon- like red rice	0.669
Oryza nivara	0.775

Table 2-1 continued.

	GD
Oryza sativa ssp. *japonica* cultivars vs.	
Oryza sativa ssp. indica- like red rice	0.787
Oryza sativa ssp. *indica*- like straw-hull red	0.785
Oryza sativa ssp. *indica*- like gold/brown-hull	0.787
Oryza nivara/ Oryza rufipogon	0.778
Oryza rufipogon- like red rice	0.754
Oryza nivara	0.782
Oryza rufipogon/ Oryza nivara vs.	
Oryza sativa ssp. *indica*- like red rice	0.643
Oryza sativa ssp. *indica*- like straw-hull red	0.683
Oryza sativa ssp. *indica*- like gold/brown-hull	0.540
Oryza rufipogon- like red rice	0.441
Oryza nivara	0.736
Oryza rufipogon- like red rice vs.	
Oryza nivara/ Oryza rufipogon subgroup	0.602
Oryza nivara	0.791

greater than the overall GD for those groups to be considered independent). The similarity between these subgroups may be due to the fact that some of the cultivars tested have parents from both subgroups.

All of the red rice samples, with the exception of MS5, are easily distinguished from all of the *japonica* cultivars (Figure 2-2). Noldin et al. (1999a) reported that MS5 was similar to the cultivars Lemont and Maybelle in that it had long straw-colored glabrous seeds and resisted shattering, but that it still maintained the weedy characteristics of excessive plant height and dry matter production. The genetic data supports the observation of Noldin et al. (1999a) that MS5 was more similar to U.S. commercial cultivars than to other ecotypes in their collection and the suggestion that MS5 might be the result of a cross between red rice and a commercial cultivar.

As expected, other than Nipponbare, all of the Asian cultivars tested clustered around the *Oryza sativa* ssp. *indica* cultivars TN1 and IR8 with a mean GD of 0.359 (Figure 2-2, Table 2-1). Consistent with the traditional classification of red rice as *Oryza sativa* ssp. *indica*, the *indica* cluster also includes a number of the red rice samples. The *Oryza sativa* ssp. *indica*-like red rice subgroup has a GD of 0.272 and includes samples from both the Brazoria County collection and from the southern red rice collection. The *indica* cultivars and *indica*-like red rice form one large group at the standard statistical threshold (Figure 2-2). However, there are several markers (RM5, RM10 and RM152) that can distinguish the red rice samples from the *indica* cultivars (Figure 2-1).

The data discussed thus far are consistent with the traditional classification of red rice as *Oryza sativa*. However, surprisingly we found that a number of red rice samples collected from

Arkansas, Mississippi, Louisiana, and Texas formed a group distinct from either the *indica* or *japonica* subspecies of *Oryza sativa* (Figure 2-2). These red rice samples cluster with several accessions of *Oryza nivara* and *Oryza rufipogon* with a GD of 0.500 (Table 2-1). In particular, it should be noted that three of the red rice samples (LA4, MS3, and TX2) were identical (18 out of 18 loci) to *Oryza rufipogon* accession 105491. This is a greater degree of similarity than seen among any of the commercial cultivars tested with a marker set that can readily distinguish between the F3 sibling cultivars Lemont and Gulfmont. The GD of 0.643 between this *Oryza nivara*/*Oryza rufipogon* group and the *Oryza sativa* ssp. *indica*-like red rice strongly indicates that these are two distinctly different types of red rice (Table 2-1).

Within the *Oryza nivara/Oryza rufipogon* cluster, these red rice samples and *Oryza rufipogon* 105491, form a very closely related subgroup (GD 0.265). This subgroup is quite distinct within the *Oryza nivara/Oryza rufipogon* cluster as shown by the average GD of 0.602 between the red rice and *Oryza rufipogon* 105491 and the other accessions in the cluster. Both the tight relationship within this subgroup and the high GD between other accessions of *Oryza nivara* and *Oryza rufipogon* support the argument that the red rice and *Oryza rufipogon* 105491 share a recent common ancestor or represent a particular type of *Oryza rufipogon* that is well suited to conditions found in the rice fields in the southern United States.

The close relationship between the red rice and *Oryza rufipogon* 105491 is particularly interesting because of the amount of attention that has been devoted to this particular accession of *Oryza rufipogon*. Previous DNA analysis suggests that *Oryza rufipogon* IRGC 105491 may represent an ancestral type of rice (Tanksley and McCouch 1997). *Oryza rufipogon* 105491 has also been used as a source of genes to increase the yield of elite rice varieties (Xiao et al. 1998).

While some *Oryza nivara* accessions grouped with *Oryza rufipogon*, three of the *Oryza nivara* accessions formed a separate cluster (GD 0.525). The GD of 0.736 between this *Oryza nivara* cluster and the *Oryza nivara*/*Oryza rufipogon* group indicates that they are independent groups. The lack of a clear distinction between the two species is not surprising since previously published data indicate that the difference between *Oryza nivara* and *Oryza rufipogon* is complex (Oka 1991; Khush 1997; Martin et al. 1997; Xaio et al. 1998; Joshi et al. 2000). Proper classification of some accessions has also been a matter of debate. For example, NSGC accession 590425 was originally identified as *Oryza rufipogon* but was reclassified as *Oryza nivara* in 1999 (http://www.ars-grin.gov/ars/PacWest/Aberdeen/nsgc.html). A few published reports indicate that the annual type of *Oryza rufipogon*, the type used in this study, is actually *Oryza nivara*, while most others show no such differentiation (Oka 1991; Khush 1997; Martin et al. 1997).

The grouping of *Oryza nivara* and *Oryza rufipogon* accessions together and the presence of the independent *Oryza nivara* group both indicate that additional work is needed to re-evaluate the taxonomic classification. This is of particular importance because of the classification of *Oryza rufipogon*, but not *Oryza nivara*, as a noxious weed by the United States Department of Agriculture.

Regardless of whether the red rice in the United States that falls into the *Oryza nivara/ Oryza rufipogon* group should actually be considered *Oryza nivara* or the noxious weed *Oryza rufipogon*, the fact remains that these ecotypes are sufficiently distinct from both the *indica* and *japonica* subspecies of *Oryza sativa* to be considered a different species. These *Oryza nivara/*

Oryza rufipogon-like red rice ecotypes may be a useful source of genetic variation for rice improvement as has been shown for both *Oryza nivara* and *Oryza rufipogon* (Xiao et al. 1998).

Another important issue related to this new data involves herbicide tolerance. Noldin et al. (1999b) reported that the red rice ecotype Texas 4 (TX4) has significant levels of tolerance to the herbicide glufosinate [2-amino-4-(hydroxymethylphosphinyl)butanoic acid]. So far, TX4 is the only glufosinate tolerant red rice ecotype to be reported. However, TX4 differs from the several other *Oryza nivara/ Oryza rufipogon* red rice accessions and from the putative rice ancestor *Oryza rufipogon* 105491 by only three SSLP markers (Figure 2-1). Since the red rice in this cluster are so closely related, (GD 0.265), other accessions might also have a tolerance to glufosinate. More biochemical and physiological data are needed to address this question.

Genetic Diversity in Red Rice

Southern Rice Belt

The 17 red rice ecotypes originally identified by Noldin et al. (1999a) were classified based on collection site and a small set of morphological characteristics. When these same samples were classified based on the SSLP analysis, nine different “classes” or “DNA marker-types” were identified (Table 2-2). The ecotypes LA1, LA2, LA5, AR3, and AR4, which are straw-hulled awnless types, gave the same banding pattern with all 18 SSLP markers (Figure 2-1) and thus all belong to the same DNA marker-type. As noted above, *Oryza rufipogon* 105491, LA4, MS3, and TX2, which are awned black-hulled accessions, belong to the same DNA marker-type.

As shown in Table 2-2 and Figure 2-2, the phenotypic classification is largely in agreement with the DNA analysis. However, readily observed morphological traits such as hull color and the

presence or absence of awns would not allow one to determine, for example, that MS5 is distinctly different from other straw-hulled awnless types.

Brazoria County

Forty-three red rice samples from the Brazoria County collection were examined. DNA analysis of these samples revealed 17 different DNA marker-types, of which 16 differed from any of those identified in the southern red rice samples (Figure 2-2). Of the 17 different types, 13 are most closely related to the *Oryza sativa* ssp. *indica* group and four to the *Oryza nivara*/*Oryza rufipogon* group.

Of the 17 DNA marker-types identified, nine were found in the southern and eight were found in the northern areas of the county, with some found in all three fields (Figure 2-3). Both *indica*-like and *Oryza nivara/Oryza rufipogon*-like red rice were found in both areas of the county. For example, in the lower southeast field both *Oryza nivara/Oryza rufipogon*-like types BC04 and BC06 and straw-hulled *indica*-like types BC14 and BC10 were found (Figure 2-3c). BC14, a straw-hulled *indica*-like red rice, was the predominant type, and was found in multiple patches in both the southern and northern parts of the county (Figure 2-3 a, b, and c).

In some cases, all of the red rice plants at a single patch belong to the same DNA marker-type, as illustrated by the four patches in the upper southeast field (Figure 2-3b). In other cases, plants from a single collection site were from very different DNA marker-types. For example, patch 1 in the lower southeast field contains *indica*-like BC14 and BC13 types, as well as the *Oryza nivara/Oryza rufipogon*-like type BC06 (Figure 2-3c). Because only a few representative plants from each patch were chosen based on a cursory examination of morphological characteristics,

these results should be an underestimation of the number of DNA marker-types per field or patch. A more detailed analysis, with more samples and/or more SSLP markers would likely identify an even greater number of DNA marker-types.

Phenotypic vs. Genotypic Classification

For the most part, the Brazoria County samples follow the same trend as the southern red rice collection with the awned, black-hulled red rice closely related to *Oryza rufipogon* accession 105491 and awnless, straw-hulled red rice closely related to the *Oryza sativa* ssp. *indica* cultivars. However, there are a few exceptions. Marker-type BC01, which grouped with *Oryza nivara/ Oryza rufipogon*, has the straw-colored hulls typically found in commercial varieties. In general, the gold and brown-hulled types seem to be intermediate between the straw-hulled *indica*-like and *Oryza nivara*/*Oryza rufipogon*-like groups (Figure 2-2, Table 2-2). The within group GD for the intermediate gold/brown group is 0.394. The GD between the gold/brown-hulled group and the straw-hulled *indica*-like red rice is 0.408. However, it should be noted that this GD is substantially less than that which separates the four major groups in Figure 2 (Table 2-1).

Table 2-2. Southern and Brazoria County Texas red rice origin, phenotype and DNA marker types. [a] Accessions are grouped based on phenotype. Nomenclature for the Brazoria County (BC) individual samples follows the format BC-patch number-plant number (e.g. BC-20-11). [b] DNA marker-type indicates samples that have identical SSLP patterns. SR- Southern red rice collection. BC- Brazoria County, TX collection.

Sample[a]	**Hull color**	**Awning**	**Seed size**	**DNA marker-type[b]**
BC-19-P45	Straw	Yes	Medium	BC16
BC-16-P14	Straw	Yes	Medium	BC15
BC-7-P44	Straw	Yes	Medium	BC14
BC-18-P27	Straw	Yes	Medium	BC03
BC-3-P23	Straw	Yes	Medium	BC01
AR-1	Straw	None	Short	SR07
AR-3	Straw	None	Medium	SR08
AR-4	Straw	None	Medium	SR08
LA-1	Straw	None	Medium	SR08
LA-2	Straw	None	Medium	SR08
LA-5	Straw	None	Medium	SR08
MS-1	Straw	None	Medium	SR08
TX-3	Straw	None	Medium	SR09
BC-20-P43	Straw	None	Medium	BC16
BC-3-P45	Straw	None	Medium	BC14
BC-1-P1	Straw	None	Medium	BC13
BC-11-P28	Straw	None	Medium	BC12
BC-9-P25	Straw	None	Medium	BC11
BC-5-P14	Straw	None	Medium	BC10
BC-10-P19	Straw	None	Medium	BC09
MS-5	Straw	None	Long	SR06
LA-3	Gold	Yes	Medium	SR04
TX-1	Gold	Yes	Medium	SR04
BC-15-P27	Gold	Yes	Medium	BC08
MS-4	Brown	Yes	Medium	SR05
BC-3-P3	Brown	Yes	Medium	BC07
BC-15-P19	Brown	None	Medium	BC02
AR-2	Black	Yes	Medium	SR03
TX-4	Black	Yes	Medium	SR02
BC-1-P34	Black	Yes	Medium	BC06
BC-17-P46	Black	Yes	Medium	BC05
BC-5-P43	Black	Yes	Medium	BC04
Oryza rufipogon 105491	Black	Yes	Medium	SR01
LA-4	Black	Yes	Medium	SR01
TX-2	Black	Yes	Medium	SR01
MS-3	Black	Yes	Long	SR01

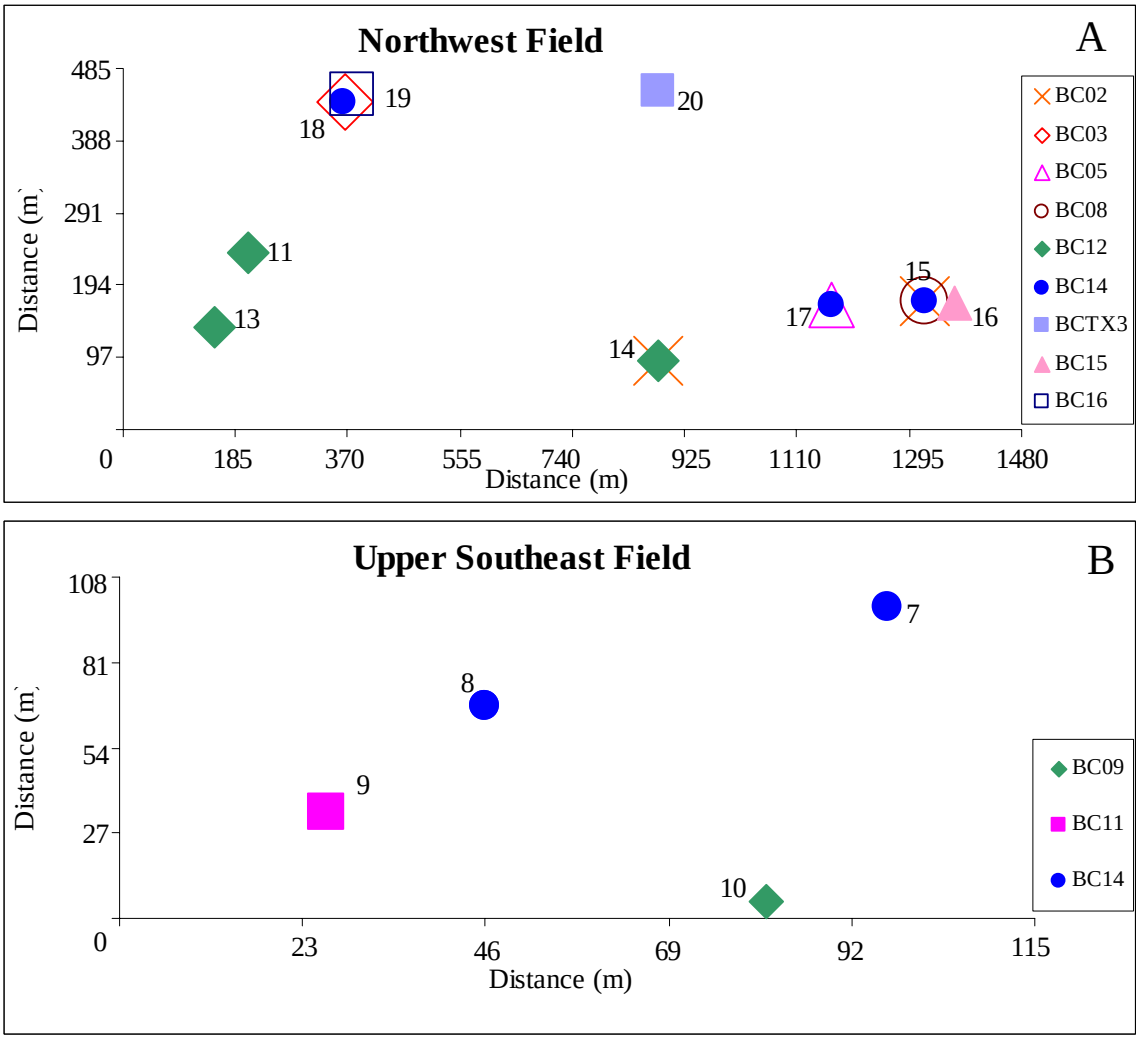


Figure 2-3. Distribution of DNA marker-types across the three fields in Brazoria County, Texas. Patch numbers are indicated on the graphs. Genetic marker-types identified in Brazoria County are designated as BC type number (e.g. BC2) and are identified in the figure key. A. Northwest field. B. Upper southeast field, C. Lower southeast field.

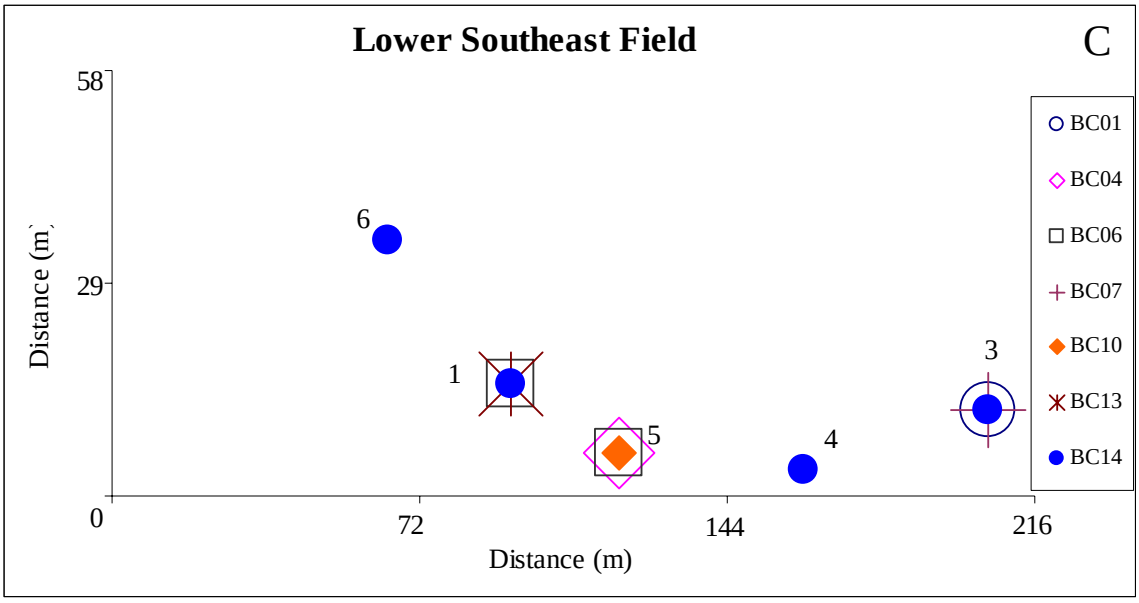


Figure 2-3 continued. C. Lower southeast field.

In some cases phenotypic data can be used to subdivide DNA-marker types. As mentioned above, three of the southern red rice samples were identical to *Oryza rufipogon* accession 105491 based on DNA marker data. Two of these, LA4 and TX2, have the same medium grain, black-hulled awned phenotype as *Oryza rufipogon* 105491. The third ecotype, MS3, has the black hull and awning, but has a long grain. A similar case can be seen in the Brazoria County DNA marker-type BC14. The majority of samples with this marker-type are straw-hulled and awnless. However, there are a few samples in BC14 marker-type that have awns. This additional splitting of DNA marker-types is not surprising since the SSLP markers used in the analysis are not known to be linked to awning, seed color or seed size. The use of DNA markers linked to these traits would be expected to reveal similar divisions.

Conclusion

Red rice has traditionally been classified as *Oryza sativa* ssp. *indica* and thought of as a single weed species. The results of this preliminary study show that this single-minded classification is inadequate, and that there are at least three genetically distinct types of red rice. Some red rice is appropriately classified as *Oryza sativa* ssp. *indica*. Other types of red rice, such as MS5, are more closely related to *Oryza sativa* ssp. *japonica* cultivars. Most importantly, some widely dispersed types of red rice are sufficiently distant from both *Oryza sativa* ssp. *japonica* and *Oryza sativa* ssp. *indica* to be considered a different species. These red rice accessions are very closely related to *Oryza nivara* and the noxious weed *Oryza rufipogon.* These different types of red rice can be found not only within all the rice producing states in the southern United States, but also within a single 9 m^2 collection site within a production field. Phenotypic classification can be used to provide an idea as to which red rice accessions belong to the *Oryza sativa* and

Oryza nivara/ Oryza rufipogon groups, but there are notable exceptions (BC01 and MS5) that illustrate the need for DNA markers to definitively classify red rice accessions.

Additional work is needed to more clearly determine the relationship of the weed red rice to *Oryza nivara*, *Oryza rufipogon* and other wild relatives of *Oryza sativa*. However, it is already clear that red rice is more diverse than previously assumed. This high level of divergence must be considered when developing and testing red rice management strategies. The *Oryza nivara/ Oryza rufipogon*-like red rice TX4, which has significant levels of natural tolerance to the herbicide glufosinate, illustrates the need to include a range of different types of red rice in herbicide studies, particularly those involving GMO rice (Noldin et al. 1999b). Considering the diversity of United States red rice, it is likely that there are also other tolerant types that will need to be managed thoughtfully in order to prevent the loss of agronomically important herbicides. However, United States red rice also represents a diverse genetic resource that likely contains a wide range of useful genes and which has the added advantage of already being adapted for growth in the southern United States.

CHAPTER III

IS THE BLACK-HULLED RED RICE IN COMMERCIAL FIELDS REALLY *Oryza rufipogon*?

Introduction

The red rice that is found as a weed in commercial fields has conventionally been loosely grouped into two subclasses: straw-hulled and black-hulled, with straw-hulled being the predominant form. Within these subclasses, characteristics such as seed color and awning or the location of the collection have been used to classify red rice into different ecotypes. While there are few accurate estimates of how many different ecotypes exist, over 50 different types of red rice have been identified thus far (Lago 1982; Noldin et al. 1999a).

As discussed in the previous chapter, all of the red rice in US commercial field has traditionally been classified as *Oryza sativa* L. based on phenotypic characteristics (Diarra et al. 1985; Langevin et al. 1990; Kwon et al. 1992). Work from our laboratory was the first research to challenge this classification (Vaughan et al. 2001). Our data suggested that not only is the red rice population in the US very diverse, but a portion of it may actually be sufficiently divergent to be considered a different species.

This discovery is important for two reasons. First, most of the research on herbicide resistance in red rice and on its competition with commercial rice has included only a limited number of ecotypes. In fact most researchers only use a few straw hulled ecotypes (for example see: Kwon, et al. 1992; Sankula et al. 1997a; Sankula et al. 1997b; Sagers and Naigemann 2002; Zhang et

al. 2003). Since red rice is quite diverse researchers should approach red rice research in a broader manner. In particular, experimental results obtained with limited number of red rice ecotypes may not apply to other areas of rice production that have significantly different red rice population.

The second important issue raised by our earlier work was that a portion of the red rice population was found to be very closely related to several wild rice samples that have been classified as *Oryza rufipogon*. We found that black-hulled red rice samples from Louisiana, Mississippi and Texas that were identical to the Asian accession *Oryza rufipogon* 105491 with all 18 DNA markers tested. These same markers can readily distinguish US commercial rice varieties, many of which are closely related. This is important since *Oryza rufipogon* is on the Federal Noxious Weed List (Vandiver et al. 1992, Plant Protection Act (PPA), www.aphis.usda.gov/ppq/weeds/weedhome.html). State, Federal permits and stringent isolation techniques are required to grow even a small number of *Oryza rufipogon* plants in strictly controlled laboratory settings. Regulation of this red rice under the Federal Noxious Weed Act could have serious economic impact. Farms and counties where *Oryza rufipogon* was found could be quarantined and APHIS could be blocked from issuing the phytosanitary certificates required for export.

Whether some of the red rice found in commercial rice fields is actually *Oryza rufipogon* is unclear. As discussed in the previous chapter, the same cluster of varieties that included *Oryza rufipogon* 105496 and some of the red rice samples from US commercial fields also contained samples that have been classified by the US National Small Grains Collection as *Oryza nivara*. *Oryza nivara* is not on the Federal Noxious Weed List and is completely unregulated.

This chapter will present the examination of a collection of *Oryza sativa* cultivars, *Oryza rufipogon* and *Oryza nivara* samples. It includes all of the samples of *Oryza rufipogon* and *Oryza nivara* that were available from the US National Germplasm Collection. Notably, it also includes a new group of perennial *Oryza rufipogon* that was recently collected from the wild in China and Vietnam and samples of *Oryza rufipogon* from the population in Florida described by Vandiver et al. (1992). Including perennial *Oryza rufipogon* samples is important because the classification "*Oryza rufipogon*" is used in two different ways. Some workers use it broadly to include all Asian wild red rice (Yamanaka et al. 2003; Vaughan et al. 2003).) Most authorities, however, use the term more strictly to include only perennial and rhizomatous red rice and classify annual red rice species as *Oryza nivara* (Oka 1991; Khush 1997; Martin et al. 1997; Yamanaka et al. 2003).

This chapter also includes an additional statistical analysis method. In the genetic distance method utilized in the previous chapter, marker data was used to calculate genetic distance matrix that was subsequently used for cluster analysis. In the new method, implemented by the program STRUCTURE (Pritchard et al. 2000), clusters are identified based on a model of the ideal population's allelic frequencies. Samples are then assigned to the proper population based on their allelic make up. Advantages of the model based method include the ability to assign samples to a particular population based on a limited number of markers, the ability to deal with link and unlinked markers, and the identification of individuals where the genotype is a mixture of two or more populations (Pritchard et al. 2000; Falush et al. 2003).

In addition to SSLP (microsatellite) markers short interspersed elements (SINEs) and miniature inverted-repeat transposable elements (MITEs) have also been utilized. These markers are

powerful tools for the examination of phylogenetic relationship on a species level since the probability of independent insertion into the same chromosome site in two different ecotypes is virtually zero (Nikaido et al. 1999; Tatout et al. 1999; Shedlock and Okada 2000; Hamdi et al. 1999). Additionally, the insertion is generally thought to be non-reversible. Recently both types of markers have been used in rice to examine the relationship of the members of the *Oryza* genus. Most notably, they have been used to differentiate between the annual and perennial members of the *Oryza* complex (Cheng et al. 2003; Kanazawa et al. 2000; Motohashi et al. 1997; Yamanaka et al. 2003).

The possibility of a large portion of red rice being closely related to the noxious weed *Oryza rufipogon* could have far reaching repercussions. The purpose of the research presented here is to clarify the classification of red rice from US producers' fields. In the process, the proper classification of *Oryza* species in the National Small Grains Collections will also be addressed.

Materials and Methods

Samples

In addition to the annual, black-hulled *Oryza nivara* and *Oryza rufipogon* samples analyzed previously (Vaughan et al. 2001), the straw-hulled, annual *Oryza rufipogon* and *Oryza nivara* samples from the National Small Grains Collection (NSGC) in Aberdeen, Idaho were also analyzed. The NSCG samples are identified by their accession number (http://www.ars-grin.gov/ars/PacWest/Aberdeen/nsgc.html). *Oryza rufipogon* international Rice Genomic Center (IRGC) accession 105491 (Xiao et al. 1998; http://singer2.cgiar.org) and the *Oryza sativa* ssp.

indica and *japonica* cultivars were supplied by Dr. Anna McClung from the USDA/ARS Station in Beaumont, TX. The collection of cultivars and red rice ecotypes originally analyzed in Vaughan et al. (2001) was also included in this analysis.

Two independent sources of *Oryza rufipogon* were obtained. Tissue from a new group of perennial *Oryza rufipogon* from China and Vietnam was collected by Asian colleagues of Dr. Allison Snow of The Ohio State University. DNA from these samples was graciously provided to our laboratory for analysis. Two samples of perennial rhizomatous *Oryza rufipogon* population in the Florida Everglades that was characterized by Dr. Vernon Vandiver of the University of Florida were also obtained. The *Oryza rufipogon* from the Everglades is the only verified source of the perennial, rhizomatous form in the United States (Vandiver et al. 1992).

Genetic Analysis

DNA was extracted as previously detailed (Vaughan et al. 2001). Two different sets of microsatellites were used for the analysis. The first set includes: RM102 (1), RM5 (1), RM166 (2), RM110 (2), RSus1 (3), RM143 (3), RM241 (4), RM153 (5), RM146 (5), WAXY (6), RSus2 (6), RM162 (6), RM118 (7), RM152 (8), RM242 (9), RM171 (10), RM20-L (11), OSM90 (12). The second set, which was used for the perennial *Oryza rufipogon* analysis includes: RM5 (1), RM166 (2), RSus1 (3), RM143 (3), RM153 (5), RM146 (5), WAXY (6), RSus2 (6), RM162 (6), RM152 (8), RM171 (10), and RM90 (12). Numbers in parentheses indicate the chromosome on which the marker is located. All of these markers are part of an integrated rice DNA map that covers all 12 chromosomes (Temnykh et al. 2000). Primer pairs that have a low degree of polymorphism in the samples tested (two or three alleles) as well as those having a high degree

of polymorphism (up to twelve alleles) were used to provide less biased results. PCR reactions for each microsatellite primer pair were carried out following guidelines listed on the RiceGenes web site (http://ars-genome.cornell.edu/rice) in a Stratagene Robocycler. PCR products were separated by electrophoresis on 5% denaturing polyacrylamide gels. Gels were stained with SYBR® Gold fluorescent dye and the bands were visualized on a Dark Reader®, according to manufacturer instructions.

The transposable element markers commonly referred to as short interspersed elements (SINEs) and miniature inverted repeat transposable elements (MITEs) were also used for genetic analysis. Both types have been used to distinguish between perennial and annual rice types (Cheng et al. 2003; Park et al. 2003) and are PCR based. The p-SINE1 family of transposable elements used included p-SINE1-r2, p-SINE-r30, and p-SINE-r34. The MITEs used belong to the *Stowaway* transposable element family and include: OSH45, F1-epsilon, and HSP82 (Kanazawa et al. 2000). PCR was preformed as previously described (Kanazawa et al. 2000; Cheng et al. 2003; Park et al. 2003) and results were visualized on a 1.5% agarose gel and visualized with SYBER® Gold on the Dark Reader®. Presence or absence of the SINEs and MITEs in the sequence amplified was established by comparing bands to molecular weight markers and the expected size of the sequence without the transposable element insert.

Data Analysis

The molecular marker data are represented by two methods. Both types of markers were independently subjected to each type of analysis. The first method is based on analysis of the

SSLP or SINE/MITE data with genetic distance calculations as described in the previous chapter. The dendograms are a visual representation of the SAS based clustering analysis.

The second method of analysis is a model based cluster analysis. This is in contrast to the above method where genetic distances are calculated based on the allelic identity of each sample. The program STRUCTURE (http://pritch.bsd.uchicago.edu; Pritchard and Wen 2003) was used for the model based analysis. The model is based on a Markov chain Monte Carlo (MCM) and Gibbs sampling techniques (see Pritchard et al. 2000). The algorithm attempts to identify distinct populations (K) and subgroups on the basis of allelic frequencies at each locus (or marker). The program also allows for admixed samples, where genotype may be a mixture of two or more populations. The analysis was based on the procedure described by Pritchard et al. (2000) and Pritchard and Wen (2003). Briefly, with a burn-in period of 20,000 and 100,000 iterations, the data was analyzed as an admixture population with independent allele frequencies for unlinked analysis. This was due to the fact that we have the prior knowledge that the markers we chose were not linked. The program was run for K = 1-6 (where K is the number of populations). The proper K was determined from the probability of that population number as described by Pritchard et al. 2000, Pritchard and Wen 2000 and Falush et al. 2003.

Results and Discussion

Clarification of Classification of Red Rice in U.S. Producers Fields

Verification of NSGC Oryza rufipogon *and* Oryza nivara *classification*

To further investigate the proper classification of the red rice samples from US commercial fields, the analysis was expanded to include the entire set of *Oryza rufipogon* and *Oryza nivara* samples available from the NSGC. The initial studies had only included *Oryza rufipogon* and *Oryza nivara* samples with red colored seeds and black hulls (Vaughan et al. 2001). All of the additional *Oryza rufipogon* and *Oryza nivara* samples from the NSGC introduced in this analysis are straw-hulled and some have white seed color.

Adding the straw-hulled samples of *Oryza rufipogon* and *Oryza nivara* from the NSGC did not change the number or basic structure of clusters obtained using genetic distance analysis from that shown in the previous chapter (Vaughan et al. 2001). However, some of the straw hulled "*Oryza rufipogon"* are clearly intermixed with the *Oryza sativa* ssp. *indica* cultivars (Figure 3-1). The GD of this group, 0.3952 is close to that of the original analysis of 0.402. The overall average GD for the entire data set is 0.5952 as compared to the GD of 0.627 from Vaughan et al. 2001.

Instead of clarifying the classification of the *Oryza rufipogon* 105491-like red rice from commercial fields, these results raise more questions. In particular, they raise questions about the proper classification of the purported *Oryza rufipogon* and *Oryza nivara* samples from the NSGC.

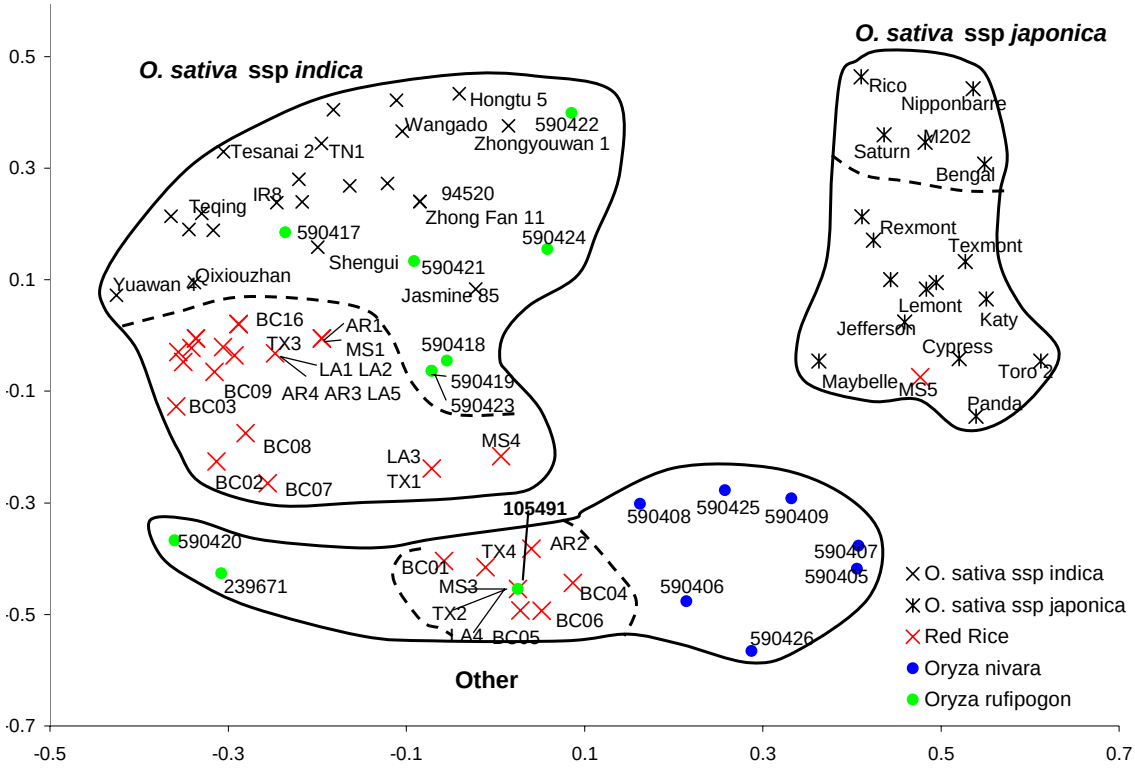


Figure 3-1. Multi-dimensional scaling (MDS) of SSLP data for *Oryza* species and red rice accessions. Plot of SSLP data using coordinates from the first two dimensions of multidimensional scaling analysis of the genetic distance matrix. The linear distance between two samples estimates the actual genetic distance between the samples using the X or Y-axis as the scale. Samples that were in the same cluster are circled with a solid line, while dashed lines indicate subgroups with a cluster. Symbols (see key) are used to differentiate samples based on type and morphology. Note the green "*Oryza rufipogon*" samples included with the *Oryza sativa* ssp. *indica* cultivars (black x's).

A certain amount of confusion in the NSGC collection would not be surprising since the classification of the *Oryza* species is difficult, particularly the closely related members of the "*Oryza sativa* complex" (Oka 1991; Khush 1997; Martin et al. 1997; Xaio et al. 1998; Joshi et al. 2000). It should be noted that some of the NSGC accessions have been reclassified. For example, NSGC accession 590425 was originally identified as *Oryza rufipogon*, but was reclassified as *Oryza nivara* in 1999 (http://www.ars-grin.gov/ars/PacWest/Aberdeen/nsgc.html).

Perennial Oryza rufipogon

One reason for confusion about the classification of red rice is that some workers use the term *Oryza rufipogon* in a general sense to refer to all wild Asian red rice (Yamanaka et al. 2003; Vaughan et al. 2003). Other workers use the term more strictly to refer only to the perennial rhizomatous form of red rice (Oka 1991; Khush 1997; Martin et al. 1997; Yamanaka et al. 2003). This is the definition used by the International Rice Research Institute (http://www.knowledgebank.irri.org/wildRiceTaxonomy/default.htm#rufipogon/Oryza_rufipogon.htm). Workers using *Oryza rufipogon sensu stricto* would classify the annual type of *Oryza rufipogon* used in this study as *Oryza nivara.*

To establish the proper classification of the red rice samples from US commercial fields, we obtained 28 perennial *Oryza rufipogon* samples that had been freshly collected from China and Vietnam (A. Snow personal communication). We also obtained two samples of the perennial rhizomatous *Oryza rufipogon* from the Florida Everglades that had previously been characterized morphologically (Vandiver et al. 1992).

As predicted by the strict definition of *Oryza rufipogon*, the perennial samples from Florida and China are in an independent group that is distinctly different from all but two of the annual samples in the analysis (Figure 3-2, Table 3-1). The two annual samples in this group, *Oryza rufipogon* 590422 and *Oryza nivara* 590405, are on the edge of the cluster. This indicates that they differ from the China and Florida samples by several alleles. These two samples may represent natural mixtures between annual and perennial types that have been forced into one group or another due to the MDS analysis inability to deal with admixed samples. This topic will be discussed further in subsequent sections of the text. Distance based statistical analysis of the SSLP data resulted in overall GD of 0.6427 and a within group GD of 0.3425 for the perennial *Oryza rufipogon*.

As in Vaughan et al. (2001) *Oryza sativa* ssp. *japonica* samples formed an independent group, with a within group GD of 0.5097. However, *Oryza sativa* ssp. *indica*, red rice similar to "*Oryza rufipogon"* 105491, and most of the annual NSGC samples, grouped together with a GD of 0.5192. This group is labeled as *Oryza sativa* ssp. *indica* / *Oryza nivara* in Figure 3-2.

That most of annual species classified as *Oryza rufipogon* by the NSGC grouped with *Oryza nivara* rather than with the Chinese and Florida accessions of perennial *Oryza rufipogon* is not surprising. This supports the view of many taxonomist that all of these annual wild rice accessions are more properly classified as *Oryza nivara* (Oka 1991; Khush 1997; Martin et al. 1997; Yamanaka et al. 2003). The NSGC web site lists all the *Oryza rufipogon* and *Oryza nivara* samples used in this study as being annual in their life form. Our experience with growing these samples in the greenhouse supports this classification and none of the samples formed rhizomes.

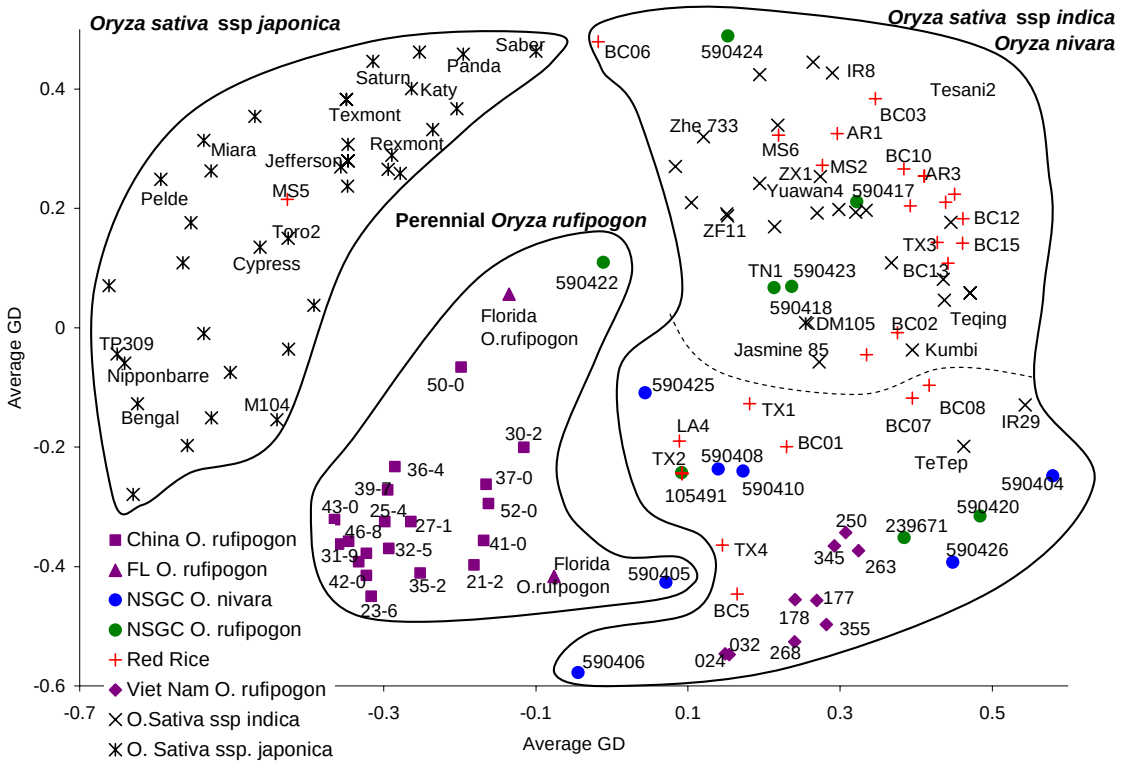


Figure 3-2. Multi-dimensional scaling (MDS) of SSLP data for *Oryza* species, including verified perennial *Oryza rufipogon*, and red rice accessions. Plot of SSLP data using coordinates from the first two dimensions of multidimensional scaling analysis of the genetic distance matrix. The linear distance between two samples estimates the actual genetic distance between the samples using the X or Y-axis as the scale. Samples that were in the same cluster are circled with a solid line.

Table 3-1. Average genetic distance (GD) within and between *Oryza sativa* cultivars, annual *Oryza nivara*, *Oryza rufipogon*, perennial *Oryza rufipogon* and red rice ecotypes in Figure 3-2. To be considered independent a group must have a within group GD that is less that 85% of the overall GD of 0.6465 (0.5165) and the GD between that group and the next most related group must be greater than the overall GD of 0.6465.

Overall GD	**0.6465**
Oryza sativa ssp. *indica*/ *Oryza nivara*	0.5165
Oryza sativa ssp. *japonica* & Perennial *Oryza rufipogon*	0.5563
Oryza sativa ssp. *japonica*	0.4804
Perennial *Oryza rufipogon*	0.3425
Oryza sativa ssp. *indica* vs. everything else	0.7626
Oryza sativa ssp. *japonica* vs. Perennial *Oryza rufipogon*	0.6866

It was surprising, however, that the perennial samples collected from Vietnam grouped with the annual *indica* and *Oryza nivara* samples rather than with the other perennial samples. Similar exceptions to the correspondence between DNA marker type and annual vs. perennial growth have been seen previously (Kanazawa et al. 2000; Yamanaka et al. 2003; Cheng et al. 2003). This could be due to the occurrence of intermediate types that are the result of natural hybridization in the wild populations (Oka 1991; Morishima 2001). These plants are reported to display the rhizomatous phenotype, but rhizomes appear to be controlled by a small number of genes (Hu et al. 2003). As will be discussed later, this grouping could also be due to a basic indica/ japonica differentiation that is present in wild species of rice.

The Vietnam samples are included in a subgroup within the larger *Oryza sativa* ssp. *indica*/ *Oryza nivara* group. This group contains black-hulled annual *Oryza nivara* and *Oryza rufipogon* samples, as well as the black, brown and gold-hulled red rice from Vaughan et al. (2001). The samples within this subgroup are substantially different that the neighboring *Oryza sativa ssp. indica* cultivars and straw-hulled red rice samples. However the GD of 0.5360 within this subgroup and the GD of 0.6346 between these samples and the *indica* cultivars and straw hulled red rice do meet the criteria for this subgroup to form an independent group.

In our previous work, annual *Oryza rufipogon* and *Oryza nivara* formed a group that was distinct from *Oryza sativa* (Vaughan et al. 2001). However, in the current study this group overlaps and joins with the *Oryza sativa* ssp. *indica* group. Other workers have also encountered difficulty in cleanly separating the annual *Oryza nivara* from the *indica* sub-species of *Oryza sativa* and from perennial *Oryza rufipogon* (Parsons et al. 1997; Sun et al. 2001; Ni et al. 2002; Cheng et al. 2003; Park et al. 2003; Ren et al. 2003; Vaughan et al. 2003; Yamanaka et al. 2003).

To be considered independent, a group must have a within group GD that is less that 85% of the overall GD and the GD between that group and the next most related group must be greater than the overall GD. Thus, the resolution of groups can be influenced both by the selection of markers, as well as the samples are included in the analysis. In particular, the presence of intermediate types can result in the fusion of groups. This will be discussed further below.

Model Based Cluster Analysis

Distance based methods, such as the GD and MDS method utilized above, are easy to apply and are relatively easy to understand. They are the most popular method of statistical analysis currently being utilized in the literature. Unfortunately, they have several disadvantages.

These methods are heavily dependent on the graphical method of visualization. Since all samples must be assigned to individual groups, these methods have difficulty dealing with samples that represent crosses between groups. There is also difficulty in the compression of 18 dimensions of relationships to 2 dimensions for graphical representation. Despite the MDS being the best fit of the data, complex relationships may not be visible in the compressed graph. It can also be difficult to assess the confidence in the clustering and also difficult to incorporate additional information into the analysis, such as hull color, awning, or GPS data (Pritchard 2000).

The GD/MDS analysis also does not have the capability to deal appropriately with missing data. Missing data for alleles are treated as identical scores. To circumvent this, as many missing data points as possible were eliminated, both with removal of samples, and the use of markers that amplified for all the samples included in the analysis. This however, can bias the analysis away

from null alleles, or microsatellites that are not present in a certain germplasm. Perennial *Oryza rufipogon* in particular has a high frequency of null alleles for an assortment of molecular markers (Vaughan et al. unpublished observations).

The Bayesian statistics based clustering program STRUCTURE has become a popular alternative for the analysis of complex relationships, as exemplified by the recent article in Science where STRUCTURE was used to analyze microsatellite data to determine the genetic structure of the purebred dog (Parker et al. 2004). This program allows for the analysis of both independent and admixed populations, linked and un-linked markers, as well as providing information on population make up, admixture values, and allelic frequencies, all with a relatively low number of markers (Falush et al. 2003).

STRUCTURE was first applied to investigate the population structure of the original data set from Vaughan et al. 2001. Model-based analysis revealed similar clusters to those obtained with genetic distance analysis (Figure 2-2). The order of the samples in the STRUCTURE graph reflects the groups found in the haplotype chart (Figure 2-1) from Vaughan et al. (2001) with the *Oryza sativa* spp. *japonica* samples first, followed by the 105941-like red rice, the *Oryza sativa* ssp. *indica*-like red rice and *Oryza sativa* ssp. *indica* cultivars. *Oryza rufipogon* and *Oryza nivara* samples from the NSGC are shown on the right at the end (Figure 3-3).

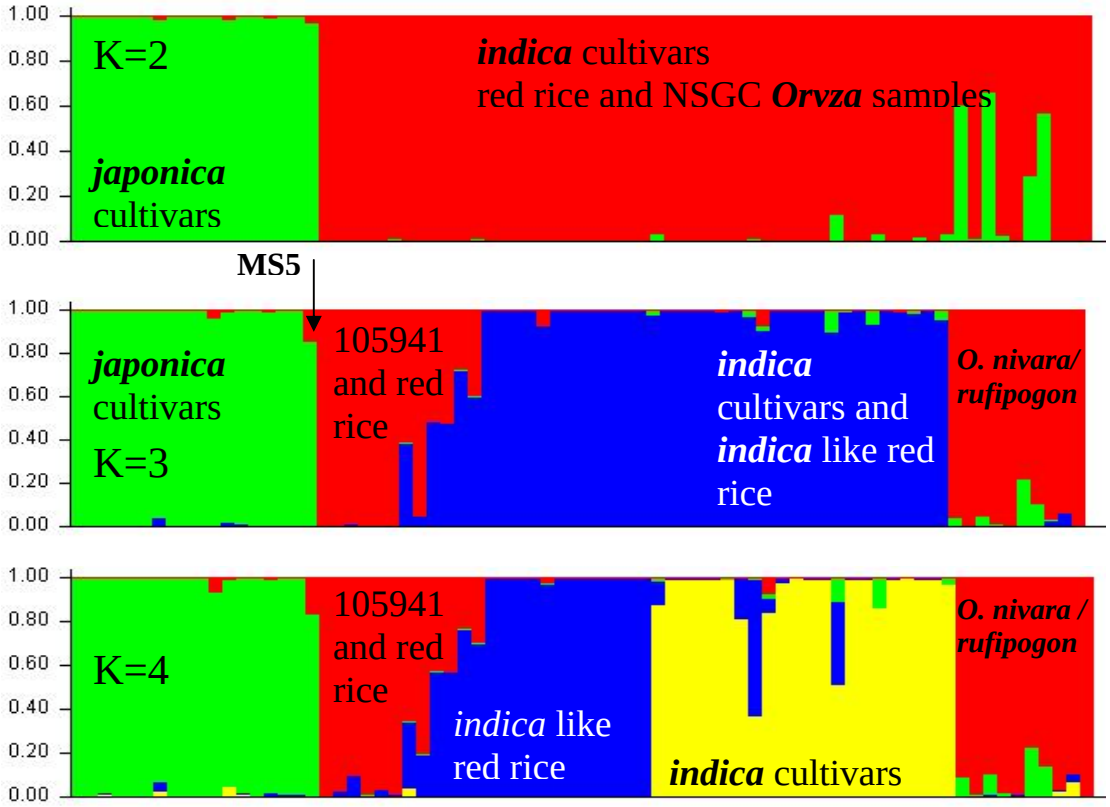


Figure 3-3. STRUCTURE analysis of the data from Vaughan et al. 2001. Each sample is represented by a single line that is divided into K colors. For each assigned K, a color represents a different population from the corresponding model. The length of the color represents the proportion of the genome in a sample that belongs to each population in the model. Samples are in the order given in Table 3-2. The K=3 graph has the *Oryza sativa* ssp. *japonica* samples indicated in green, the *Oryza rufipogon* 105491-like red rice in red, *Oryza sativa* ssp. *indica* and *indica* like red rice in blue and the NSGC *Oryza nivara*, *Oryza rufipogon* samples at the end of the analysis. MS5, the red rice that groups with the *Oryza sativa* ssp. *japonica* cultivars is indicated by the arrow on the K=3 graph. Graphs shown are a representation of at least 5 replications of each K value.

Table 3-2. Estimated probabilities for STRUCTURE analysis of the SSLP data for Figure 3-3. Three representative runs for each K value are shown. The LnP(D), Variation of [LnP(D)] and $\alpha 1$ values are used to estimate the proper K value for the data set. For this data set K=3 is chosen because the difference between the average for K=2 and K=3 is significantly less than the difference between K=3 and K=4. Also, the variation of [LnP(D)] is relatively low, and the $\alpha 1$ is relatively stable (Pritchard et al. 2000, Pritchard and Wen 2003 and Falush et al. 2003).

K	LnP(D)	Var [LnP(D)	$\alpha 1$
1	-3248.0	39.4	
1	-3248.0	39.5	
1	-3248.2	39.8	
2	-2595.5	87.4	0.0419
2	-2595.8	87.8	0.0416
2	-2595.4	87.3	0.0418
3	-2111.3	108.2	0.0392
3	-2112.9	111.0	0.0387
3	-2112.2	109.8	0.0386
4	-1879.4	125.8	0.0385
4	-1934.0	121.6	0.0293
4	-1880.2	127.8	0.0388
5	-1697.9	142.7	0.0356
5	-1695.2	136.7	0.0356
5	-1732.9	146.0	0.0361

In agreement with the GD and MDS analysis, STRUCTURE suggests that the population presented in Chapter II can validly be split into three groups. These groups largely correspond to *Oryza sativa* ssp. *japonica* cultivars (in green), *Oryza sativa* ssp. *indica* cultivars and the *indica*-like red rice (blue) and a mixed group of red rices (red) that includes *Oryza nivara*, the annual *Oryza rufipogon* accessions from NRGC, as well as *Oryza rufipogon* 105491 and the closely related red rice ecotypes from commercial fields.

If the analysis is pushed one step beyond the proper K value, to K=4, the *Oryza sativa* ssp. *indica*-like red rice group breaks out from the *Oryza sativa* ssp. *indica* cultivars to form an independent group (for K=4 *Oryza sativa* ssp. *indica*-like red rice remains blue and *Oryza sativa* ssp. *indica* cultivars are yellow). This supports the assignment of the proper K=3 since the *indica*-like red rice group was not an independent group in Vaughan et al. 2001. Similarly, if the K value is dropped below the proper K=3 to K=2, the majority of the red rice accessions group with the *Oryza sativa* ssp. *indica* samples, but the *Oryza sativa* ssp. *japonica* samples remain independent. This correlates with the hierarchical analysis discussed later.

Of particular interest is the red rice sample MS5, the only red rice to group with the *Oryza sativa* ssp. *japonica* cultivars, indicated by the arrow in Figure 3-3. In the previous GD and MDS analysis, this red rice ecotype grouped with *japonica* rice cultivars rather than with other red rice. STRUCTURE analysis confirms this general conclusion, but also reveals the amount of admixture, or chromosomal regions that can be attributed to different ancestors. MS5 is clearly mostly of *Oryza sativa* ssp. *japonica* heritage, but also has the characteristics of red rice; in agreement with the original description of this ecotype by Noldin (1999a). MS5 provides an

excellent illustration that red rice and cultivated rice do hybridize and that genes from cultivars can move in the red rice gene pool.

To further examine the relationship between different types of red rice STRUCTURE analysis was next performed with all the NSGC samples and with the perennial *Oryza rufipogon* samples from Florida, China and Vietnam included (Figure 3-4, Table 3-3, Table 3-4). Samples are ordered based on the MDS designation of clusters. The data indicate that these samples can validly be divided into four groups (K=4).

At K=4, *Oryza sativa* ssp. *japonica* samples form an independent group (blue) as do the perennial *Oryza rufipogon* samples from China and Florida (green). This was expected since these samples also formed independent groups in the GD/MDS analysis. However, in contrast to the GD/MDS analysis, the two subgroups within the large *Oryza sativa* ssp. *indica / Oryza nivara* group were resolved in the STRUCTURE analysis. As discussed below, this was not surprising since STRUCTURE is better able to deal with samples with mixed ancestry.

The group shown in yellow contains *Oryza sativa* ssp. *indica* cultivars and closely related red rice. Most of the red rice in this group has straw colored hulls as do all of the "*Oryza rufipogon*" samples from NSGC. As noted above, these samples also grouped with *indica* cultivars in the GD/MDS analysis. The group shown in red contains all of the *Oryza nivara* and *Oryza rufipogon* from the NSGC that had black hulls as well as the "*Oryza rufipogon*"-like red rice collected from commercial fields.

Of particular interest are NSGC samples 590422 and 590405, numbers 116 and 117 respectively. These the two annual samples grouped with perennial *Oryza rufipogon* from Florida and China in the GD and MDS analysis. The advantage of STRUCTURE analysis in dealing with admixed samples is clearly demonstrated here. These samples both had high levels of admixture, with approximately half their markers belonging to the *Oryza sativa* ssp. *indica* group (590422) or the NSGC/black-hulled red rice group (590405).

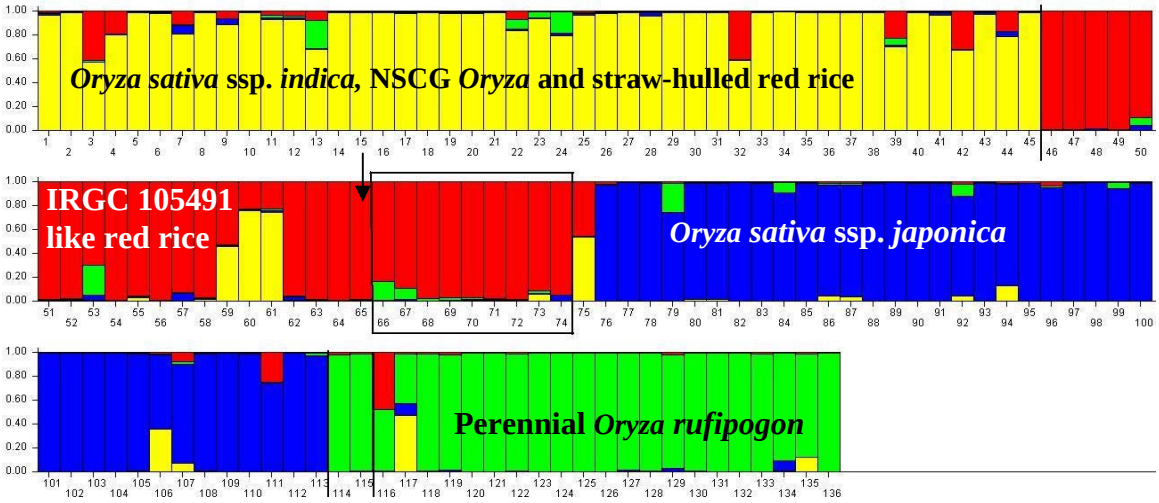


Figure 3-4. STRUCTURE analysis of 136 members of the *Oryza* species, including the verified perennial *Oryza rufipogon*. K=4 of annual *Oryza sativa* ssp. *japonica* (yellow) *Oryza sativa* ssp.. *indica* (blue), red rice (red), Vietnam perennial *Oryza rufipogon* samples (red in box) and the Chinese and Florida perennial *Oryza rufipogon* samples (green). Individual sample labels at bottom of each column correspond to the list in table 3-3. Graphs shown are a representation of at least 5 replications of each K value. Of particular note are samples 590420 #65 (indicated by the arrow), TX4 #57, 590405 #116, 590422 #117 and the box indicates the *Oryza rufipogon* samples from Vietnam.

Table 3-3. Sample list for Figure 3-4.

Variety ID	Structure ID	Variety ID	Structure ID	Variety ID	Structure ID	Variety ID	Structure ID
94:520	1	BC11	35	OR-VN- 178	69	RCO	103
AR1	2	BC12	36	OR-VN- 250	70	RSMT	104
BC02	3	BC15	37	OR-VN- 263	71	RXMT	105
BC13	4	J85	38	OR-VN- 268	72	SBR	106
GX89	5	KMBI	39	OR-VN- 345	73	SNTO	107
HT5	6	LA2	40	OR-VN- 355	74	SRRA	108
IAC102	7	MS2	41	TTP	75	STRN	109
IR8	8	MS4	42	BNGL	76	TP308	110
KDM105	9	MS6	43	CCDR	77	TR2	111
OR-590417	10	OR-590424	44	CDT	78	TXMT	112
OR-590418	11	TX3	45	CM101	79	YCB	113
OR-590423	12	AR2	46	CPRS	80	FLOR2	114
PI-408449	13	BC05	47	DXBL	81	FLOR3	115
QXN	14	BC06	48	GFMT	82	ON-590405	116
SHG	15	LA4	49	HMNI	83	OR-590422	117
SY2	16	ON-590406	50	JCNT	84	OR-C- 21-2	118
TN1	17	ON-590408	51	JEFF	85	OR-C- 23-6	119
TQNG	18	ON-590410	52	KBNT	86	OR-C- 25-4	120
TS2	19	ON-590425	53	KTY	87	OR-C- 27-1	121
WNGD	20	OR-105491	54	L202	88	OR-C- 30-2	122
XF-91-1	21	TX1	55	LMNT	89	OR-C- 31-9	123
Y4	22	TX2	56	LVCA	90	OR-C- 32-5	124
Z733	23	TX4	57	M103-1	91	OR-C- 33-4	125
Z-86-44	24	BC01	58	M104-1	92	OR-C- 35-2	126
ZF11	25	BC07	59	M201	93	OR-C- 36-4	127
ZF25	26	BC08	60	MDSN	94	OR-C- 37-0	128
ZX1	27	IR29	61	MRA	95	OR-C- 39-7	129
ZYW1	28	ON-590404	62	MS5	96	OR-C- 41-0	130
ZYZ3	29	ON-590426	63	NIPP	97	OR-C- 42-0	131
AR3	30	OR-239671	64	PCS	98	OR-C- 43-0	132
AR4	31	OR-590420	65	PLDE	99	OR-C- 46-8	133
BC03	32	OR-VN- 24	66	PNDA	100	OR-C- 50-0	134
BC09	33	OR-VN- 32	67	PNTL	101	OR-C- 52-0	135
BC10	34	OR-VN- 77	68	PRSL	102	OR-C- 56-3	136

Table 3-4. Estimated probabilities for STRUCTURE analysis of the SSLP data for Figure 3-4. Representative runs for each K value are shown.

K	Ln P(D)	Var Ln P(D)	α1
1	-3431.5	38.2	
1	-3431.4	38.0	
1	-3431.5	38.3	
1	-3431.5	38.2	
2	-2733.0	79.2	0.0357
2	-2759.1	117.4	0.0715
2	-2741.8	88.4	0.0504
2	-2733.2	79.3	0.0350
3	-2292.1	104.0	0.0393
3	-2291.3	102.8	0.0388
3	-2292.9	105.4	0.0389
3	-2261.4	115.1	0.0411
4	-1958.6	124.9	0.0351
4	-1958.0	123.6	0.0345
4	-1958.3	124.4	0.0340
4	-1958.5	124.1	0.0337
5	-1741.0	144.7	0.0349
5	-1734.6	135.6	0.0335
5	-1740.7	146.4	0.0338
5	-1736.8	138.9	0.0340
6	-1612.4	150.6	0.0320
6	-1609.0	144.5	0.0315
6	-1609.1	145.0	0.0320
6	-1611.4	148.8	0.0321
7	-1527.9	153.4	0.0298
7	-1544.7	185.1	0.0298
7	-1528.3	154.7	0.0299
8	-1476.1	173.6	0.0302
8	-1473.2	166.4	0.0291
8	-1475.3	166.1	0.0296

As in the MDS analysis the perennial *Oryza rufipogon* samples from Vietnam group with the black-hulled red rice and annual NSGC samples, including IRGC 105491. This group corresponds to subgroup below the dashed line in Figure 3-3. There is very little admixture in the Vietnam *Oryza rufipogon* compared to the other samples in the red population. This was unexpected since one of the possible explanations for the inclusion of the Vietnam samples in this group was that they represent natural mixtures between perennial and annual types. Apparently this is not the case. Unlike NSGC samples 590422 and 590405 (numbers 116 and 117), there is no indication of significant admixture in the *Oryza rufipogon* samples from Vietnam.

Even though the perennial *Oryza rufipogon* samples from Vietnam were not completely resolved from the annual samples in the analysis discussed thus far, they do have different alleles for some of the markers used and can be resolved with other analysis. As will be discussed further below, the deepest division in the data was not between *Oryza sativa* and the other *Oryza* species. Instead, in agreement with other recent work, we found that one of the most basic distinctions was between *Oryza sativa* ssp. *indica* vs. *Oryza sativa* ssp. *japonica*. Thus markers with only two alleles tend to reflect this difference rather than providing resolution between species.

One way to circumvent this problem is to remove the *Oryza sativa* ssp. *japonica* samples from the analysis and focus on resolving red rice from *Oryza sativa* ssp. *indica*. Under these conditions, STRUCTURE is able to cleanly resolve the perennial *Oryza rufipogon* from Vietnam (red) from both the annual species (blue, yellow and pink) and from the perennial *Oryza rufipogon* from China and Florida (green) (Figure 3-5, Table 3-5). These results, including the

resolution of perennial *Oryza rufipogon* into distinct groups, is in agreement with recent results from Cheng et al. (2003). These investigators were able to resolve four different groups of *Oryza rufipogon* using SINE markers. One of the three perennial groups identified by Cheng et al. 2003 was related to *Oryza sativa* ssp. *japonica* cultivars. All of the annual *Oryza rufipogon*, however, (a term stated by Cheng et al. 2003 to be synonymous with *Oryza nivara*) were closely related to *Oryza sativa* ssp. *indica.* It is interesting to note that the Vietnam samples only separate from the 105491-like red rice group at the proper population number of K=5. In contrast the *Oryza sativa* ssp. indica-like red rice separate out from the cultivars at a population of K=4.

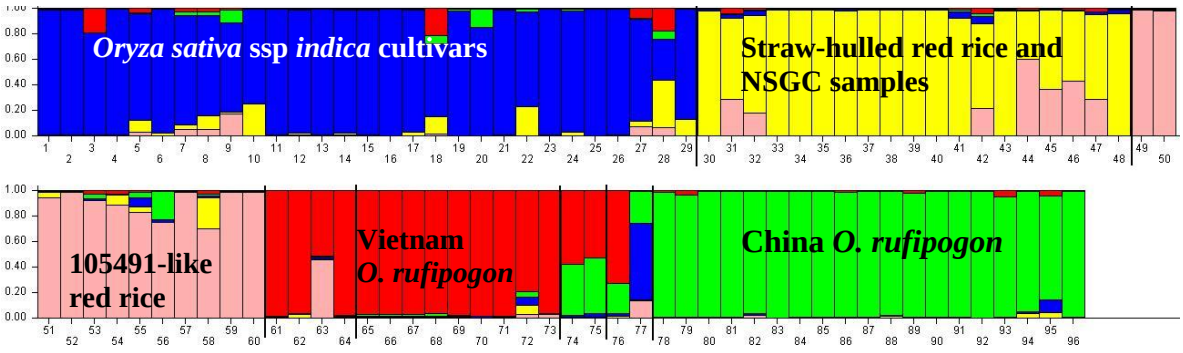


Figure 3-5. STRUCTURE analysis of red rice, *Oryza rufipogon*, *Oryza nivara* and *Oryza sativa* ssp. *indica* samples. This analysis differs from that in Figure 3-4 in that the *Oryza sativa* ssp. *japonica* cultivars have been removed. The proper population level in this analysis is K=5. In this analysis, samples 1-29 (predominantly blue) are *Oryza sativa* ssp. *indica* cultivars samples, samples 30-48 (predominantly yellow) are *Oryza sativa* ssp. *indica*- like straw-hulled red rice and straw-hulled NSGC samples, a samples 49-60 (pink) are the black-hulled *Oryza rufipogon* 105491-like red rice, samples 61-64 (red) are the NSGC samples 590404, 590426, 239671 and 590420, samples 65-73 (red) perennial *Oryza rufipogon* from Vietnam, samples 74 and 75 Florida *Oryza rufipogon*, samples 76 and 77 NSGC samples 590405 and 590422, and samples 78-96 (green) perennial *Oryza rufipogon* samples from China.

Table 3-5. Sample list for Figure 3-5. Samples are ordered based on MDS clusters.

Variety ID	Structure ID	Variety ID	Structure ID	Variety ID	Structure ID
94:520	1	AR4	34	OR-VN- 024	65
HT5	2	BC09	35	OR-VN- 032	66
IAC102	3	BC10	36	OR-VN- 177	67
IR8	4	BC11	37	OR-VN- 178	68
KDM105	5	BC12	38	OR-VN- 250	69
OR-590417	6	BC15	39	OR-VN- 263	70
OR-590418	7	LA2	40	OR-VN- 268	71
OR-590423	8	MS2	41	OR-VN- 345	72
PI-408449	9	MS4	42	OR-VN- 355	73
QXN	10	MS6	43	FLOR2	74
SHG	11	BC01	44	FLOR3	75
SY2	12	BC03	45	ON-590405	76
TN1	13	BC07	46	OR-590422	77
TQNG	14	BC08	47	OR-C- 21-2	78
TS2	15	TX3	48	OR-C- 23-6	79
WNGD	16	AR2	49	OR-C- 25-4	80
XF-91-1	17	BC05	50	OR-C- 27-1	81
Y4	18	BC06	51	OR-C- 30-2	82
Z733	19	LA4	52	OR-C- 31-9	83
Z-86-44	20	ON-590406	53	OR-C- 32-5	84
ZF11	21	ON-590408	54	OR-C- 33-4	85
ZF25	22	ON-590410	55	OR-C- 35-2	86
ZX1	23	ON-590425	56	OR-C- 36-4	87
ZYW1	24	OR-105491	57	OR-C- 37-0	88
ZYZ3	25	TX1	58	OR-C- 39-7	89
GX89	26	TX2	59	OR-C- 41-0	90
OR-590424	27	TX4	60	OR-C- 42-0	91
KMBI	28	ON-590404	61	OR-C- 43-0	92
J85	29	ON-590426	62	OR-C- 46-8	93
AR1	30	OR-239671	63	OR-C- 50-0	94
BC02	31	OR-590420	64	OR-C- 52-0	95
BC13	32			OR-C- 56-3	96
AR3	33				

Structure ID	Sample ID	F1 epsilon	F1 epsilon	HSP 82	HSP 82	OSH 45	OSH 45	r2	r2	r30	r30	r34	r34
1	O.R China 31-P	-	-	+	+	+	+	ND	ND	+	+	-	-
2	O.R Vietnam 177-P	-	-	+	+	+	+	-	-	+	-	-	-
3	O.R Vietnam 268-P-	+	-	+	+	+	+	-	-	+	-	-	-
4	O.R Vietnam 330-P?	+	-	+	+	+	+	-	-	+	+	-	-
5	FL OR-2-P	+	-	ND	ND	+	+	-	-	-	-	-	-
6	FL OR-3-P	+	-	+	+	+	+	-	-	-	-	-	-
7	W108-P	-	-	+	+	+	+	-	-	+	-	+	+
8	W1943-P	-	-	+	+	+	+	-	-	+	+	-	-
9	W1945-P	-	-	ND	ND	+	+	-	-	ND	ND	-	-
10	W1981-P	-	-	+	+	+	+	-	-	-	-	-	-
11	OR 590420-A	+	-	+	+	+	+	+	+	-	-	-	-
12	OR 590424-A	+	+	+	+	+	+	+	+	+	+	-	-
13	*O. sativa ssp.. indica* IR8-A	-	-	+	+	+	+	+	+	+	+	-	-
14	OR 105491-A	+	+	+	+	+	+	+	+	+	+	+	+
	O. sativa ssp. japonica Cypress	+	+	+	+	+	+	+	+	+	+	+	+
15	ON 590425-A	+	+	ND	ND	+	+	+	+	+	+	+	+
16	ON 590426-A	+	-	+	+	+	+	+	+	+	+	+	+
17	AR2-A	+	+	+	+	ND	ND	+	+	+	+	+	+
18	W0630-A	-	-	+	+	+	+	+	+	+	+	+	+
19	W106-A	+	+	+	+	+	+	-	-	+	+	+	+
20	W1681-A	+	+	+	+	+	+	+	+	ND	ND	+	+
21	W1238-P	+	+	+	+	+	+	+	+	-	-	+	+
22	W2005-P	+	+	ND	ND	+	+	+	-	-	-	+	+
23	W2007-I	+	+	ND	ND	+	+	+	+	-	-	+	+

Figure 3-6. SINE and MITE data for annual and perennial *Oryza* species. Haplotype chart for representative SINE/MITE patterns. Each marker is represented by two columns to illustrate both alleles. F1 epsilon, HSP82 and OSH45 are the MITE markers and r2, r30 and r34 are different pSINE markers. ND indicates no amplification product, + (blue) insertion present, - (red) insertion absent. Samples from Cheng et al (2003) and Kanazawa et al (2000) are indicated by the "W" in front of the number, Reproductive type designated by these authors, by Vandiver et al. for the Florida samples, by Alison Snow for the Vietnam and China *Oryza rufipogon*, or by NSGC are indicated by A (annual), P (perennial) or I (intermediate) following the name. Note that *Oryza sativa* ssp. *japonica* cultivars have the same amplification as IRGC 105491.

Resolving Annual and Perennial Types Using Transposable Element Markers

The type of molecular marker used can influence the resolution in genetic analysis (Parsons et al. 1997; Ravi et al. 2003; Ren et al. 2003). Thus, as a third method of examining the genetic relationships between these samples, the transposable element markers SINEs and MITEs were also utilized.

The SINE and MITE markers used for this analysis are among the few markers that have been reported to reproducibly distinguish the annual and perennial types of *Oryza rufipogon* (Kanazawa et al. 2000; Yamanaka et al. 2003). Data from samples examined with the MITE and SINE markers in Cheng et al. (2003) and Kanazawa et al. (2000) are also included in the analysis to provide a link between our research and published work from these groups. These samples are from the Japanese National Institute of Genetics and have been verified by the authors to be perennial, annual or intermediate. Following the nomenclature of Kanazawa et al. (2000) these are indicated by a "W" in their name. The other pSINE markers used by Cheng et al. (2003) would clearly have been useful, but the corresponding primer sequences were not publicly available at the time this work was performed.

In agreement with Kanazawa et al. 2000 and Cheng *et a*l. 2003, *Oryza sativa ssp. japonica* cultivars such as Cypress contain the full complement of retroelements used in this analysis (Figure 3-6) Since the acquisition of these elements is thought to be essentially irreversible, this is taken as an *indica*tion that *Oryza sativa* ssp. *japonica* is of relatively recent origin. This same pattern was also seen in *Oryza rufipogon* 105491 and closely related black-hulled red rice and in some of the red rice that groups very closely to *Oryza sativa* ssp. *indica* in GD/MDS analysis

(e.g. accession 590424). The presence of these elements in the annual species is consistent with data from Kanazawa et al. (2000) and Cheng et al. (2003).

The marker pPINE-r34 is generally present in annual species and absent in perennials. However, there are exceptions. Cheng et al. (2003) noted that many *Oryza sativa* ssp. *indica* accessions lack pSINE-34. Thus, it was not surprising that this element was also missing in IR8 and other *Oryza sativa* ssp. *indica* tested in the current study (Figure 3-6). pSINE-r34 was also present in two of the *Oryza rufipogon* samples from NSGC that are listed as annuals (accessions 590420 and 590424) and was missing in two of the perennial samples examined by Cheng et al. (2003), W1238 and W2005.

In previous work the MITE marker F1epsilon was able to correctly identify perennial or annual for 47/52 samples (Kanazawa et al. 2000). In the current study the presence of F1 epsilon also correlated with annual growth, but again there were exceptions (Figure 3-6). The annual *Oryza sativa* ssp. *indica* cultivar IR8 lacks F1 epsilon and the accession W0630 described by Cheng et al. (2003) as an annual type lacks this element. Interestingly, several samples were heterozygous for this marker including the perennial rhizomatous samples from Florida.

A pSINE marker in exon 10 of the gene for granule bound starch synthase, pSINE1-r2, has also been shown to be very useful in distinguishing annual from perennial accessions (Yamanaka et al. 2003, Cheng et al. 2003). Among the samples in the current study this marker failed to correctly predict only two samples (Figure 3-6). It should be noted that one of these (W0630) was also exceptional with F1 epsilon. The only other discrepancy was that this marker was heterozygous in W2005. Interestingly, W2005 was also was also exception with pSINE-r34.

No single marker completely distinguished the annual and perennial types. However two groups that largely correspond to annual and perennial types of wild rice could be easily distinguished by a combination of markers using either GD/MDS or STRUCTURE (Figures 3-7 & 3-8, Tables 3-6 & 3-7). As expected from their description as annuals in the NSGC database, most of the NSGC samples grouped with the annual types. Notably, this included both samples listed as *Oryza nivara* as well as annuals listed as *Oryza rufipogon* by the NSGC. In agreement with the descriptions provided by Dr. Snow, all of the perennial *Oryza rufipogon* from China and Vietnam grouped with the perennial rhizomatous rufipogon from Florida and with most of the samples listed as perennials by Cheng et al. 2003.

While the SINE/MITE analysis generally agrees with phenotypic description of annual vs. perennial growth pattern, there are a few exceptions. It seems surprising that *Oryza sativa* ssp. *indica* cultivars, such as IR8, grouped with perennial rather than annual types. However, as noted above, it is well established that some *indica* cultivars lack some of the DNA elements such as pSINE r-34 that typically distinguish annual types (Cheng *et al.* 2003). *Oryza rufipogon* 504220 is listed by as an annual by the NSGC, but grouped with the perennials. This accession had some insertions characteristic of annuals, such as pSINE-r2, but it lacked the diagnostic marker pSINE-r32 and was heterozygous for F1 epsilon.

The fact that *Oryza rufipogon* 590420 and IR8 contained only a subset of the markers characteristic of annual species is indicated by the mixed parentage, illustrated in the STRUCTURE analysis of these samples (Figure 3-7, lanes 11 and 13, respectively). Possible mixed ancestry is also indicated for W108 (lane 7) and *Oryza rufipogon* 590424 (lane 12) although in these cases the phenotypic description matches the grouping with DNA markers.

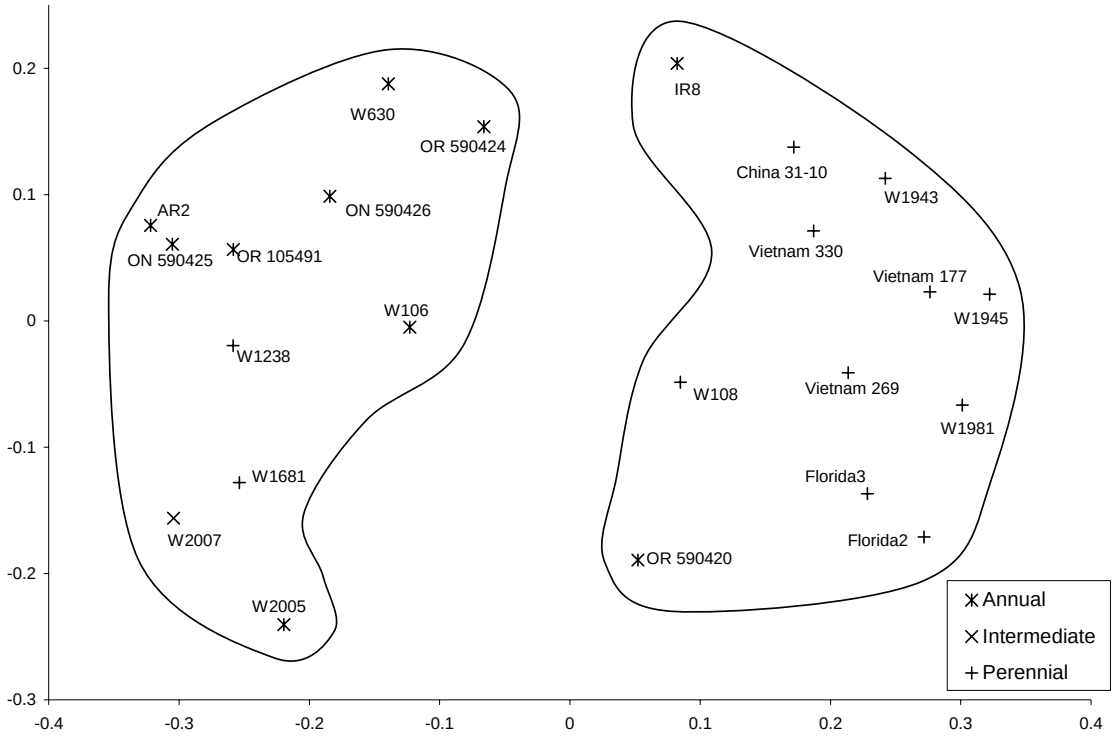


Figure 3-7. Multi-dimensional scaling (MDS) of SINE and MITE data for *Oryza* species and red rice accessions. Samples in the same cluster are circled with a solid line. Reproductive characteristics of the samples are indicated by the markers- see key.

Table 3-6. Average genetic distance (GD) within and between *Oryza sativa* cultivars, *Oryza nivara*, *Oryza rufipogon*, perennial *Oryza rufipogon* and red rice ecotypes in Figure 3-7. To be considered independent, a group must have a within group GD that is less that 85% of the overall GD and the GD between that group and the next most related group must be greater than the overall GD. Thus these two groups are independent.

	GD
Overall GD	**0.3219**
Perennial	0.1728
Annual	0.1695
Perennial vs. Annual	0.4654

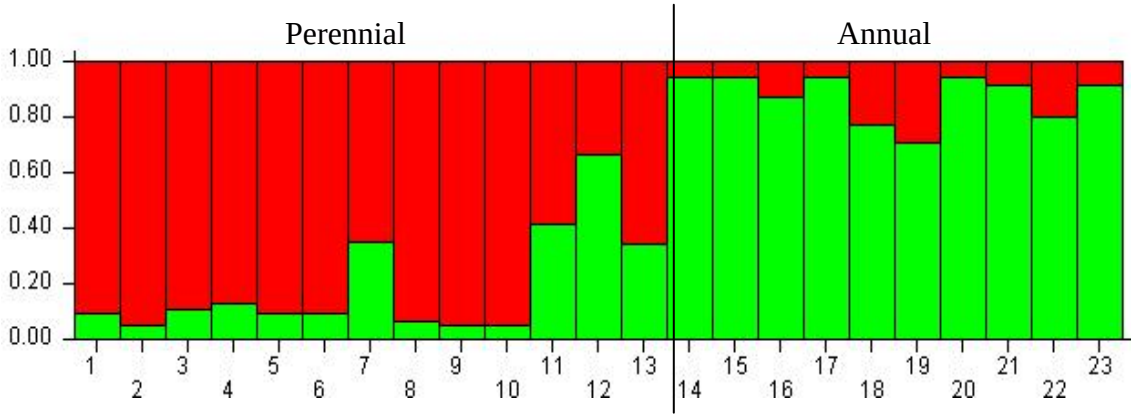


Figure 3-8. STRUCTURE analysis of SINE and MITE data for annual and perennial *Oryza* species. Perennial samples are indicted in red, with the annuals in green. Samples are in the order indicated by the haplotype chart Figure 3-6.

Table 3-7. Estimated probabilities for STRUCTURE analysis of the SSLP data for the data presented in Figure 3-8. Representative runs for each K value are shown.

K	Ln P(D)	Var Ln P(D)	α1
1	-124.7	1.3	
1	-124.7	1.3	
2	-90.9	18.7	0.3305
2	-90.8	18.7	0.3201
2	-91.2	19.3	0.3110
2	-90.7	18.6	0.3158
2	-91.7	20.4	0.3618
3	-88.5	32.8	0.1433
3	-89.3	33.9	0.1485
3	-89.5	34.0	0.1572
3	-89.2	34.3	0.1495
3	-89.0	33.1	0.1606

There are other samples from Cheng et al. (2003) and Kanazawa et al. (2003) in which the SINE and MITE data presented here do not coincide with phenotypic classification. W1238 and W2005 are classified as perennial by both Cheng and Kanazawa, but grouped with the annuals in this analysis. These samples both contain F1 epsilon, pSINE-r2 and pSINE-r34; the three makers most diagnostic of annual types in the current study. Cheng et al. (2003) were able to group these accessions with other perennial accessions using additional pSINE markers. However, the corresponding primer sequences for these markers were not publicly available when this work was done.

In general the results from the SINE/MITE analysis agree with those from the SSLP (microsatellite) analysis presented above. The most notable difference is that the perennial *Oryza rufipogon* samples from Vietnam group with the perennial samples from China and Florida in the SINE/MITE analysis, rather than with the other annual samples from the NSGC and with black hulled red rice from commercial fields as seen in most of the SSLP analysis. However, the results with SINE and MITE markers are in agreement with the SSLP analysis shown in Figure 3-5 in which confounding effects of *Oryza sativa* ssp. *japonica* cultivars were removed.

It is important to realize that the SINE and MITE markers used by Cheng *et a*l. (2003) and Kanazawa et al. (2000) were those that gave the greatest resolution between *Oryza* species. In contrast, essentially random markers were used for the SSLP analysis. Therefore, it is not surprising that the classification obtained would depend upon the marker set used. Similar results have been seen by others (Parsons et al. 1997; Ravi et al. 2003; Ren et al. 2003). Which result is most valid could be debated.

Dual Origin of Cultivated Rice

Cultivated *Oryza sativa* has traditionally been considered to originate from a single species. However, several groups have suggested that the two sub-species of *Oryza sativa*, *japonica* and *indica*, may have originated from the perennial and annual forms of wild rice respectively (e.g. Yamanaka et al. 2003). The idea that the cultivated *indica* and *japonica* species may have different origins is also supported by the occurrence of corresponding types in wild species and the infertility barrier often encountered in *indica*/ *japonica* crosses. This infertility barrier is less than is seen between *Oryza sativa* cultivars and some wild species, including *Oryza rufipogon* (in particular OR 105491) (Xaio et al. 1996; Vaughan et al. 2003). The dual origin hypothesis has also been supported by research utilizing the pSINE1 family of transposable element marker (Cheng et al. 2003; Yamanaka et al. 2003). These researchers suggested that *indica* varieties derived form the annual wild species while the *japonica* cultivars originated from the perennial species.

The microsatellite data from the present study also lends credence to the dual origin hypothesis. A simple alternative to the clustering methods discussed above is to look at the hierarchal nature of the clustering, illustrated in a phylogenetic tree (Figure 3-8). The deepest branches of the tree largely correspond to an *indica/japonica* split. The perennial samples from China and Florida *Oryza rufipogon* belong to the same branch of the tree as the *Oryza sativa* ssp. *japonica* cultivars while the annual *Oryza nivara* and *Oryza rufipogon* and Vietnam samples grouped with the *Oryza sativa* ssp. *indica* samples.

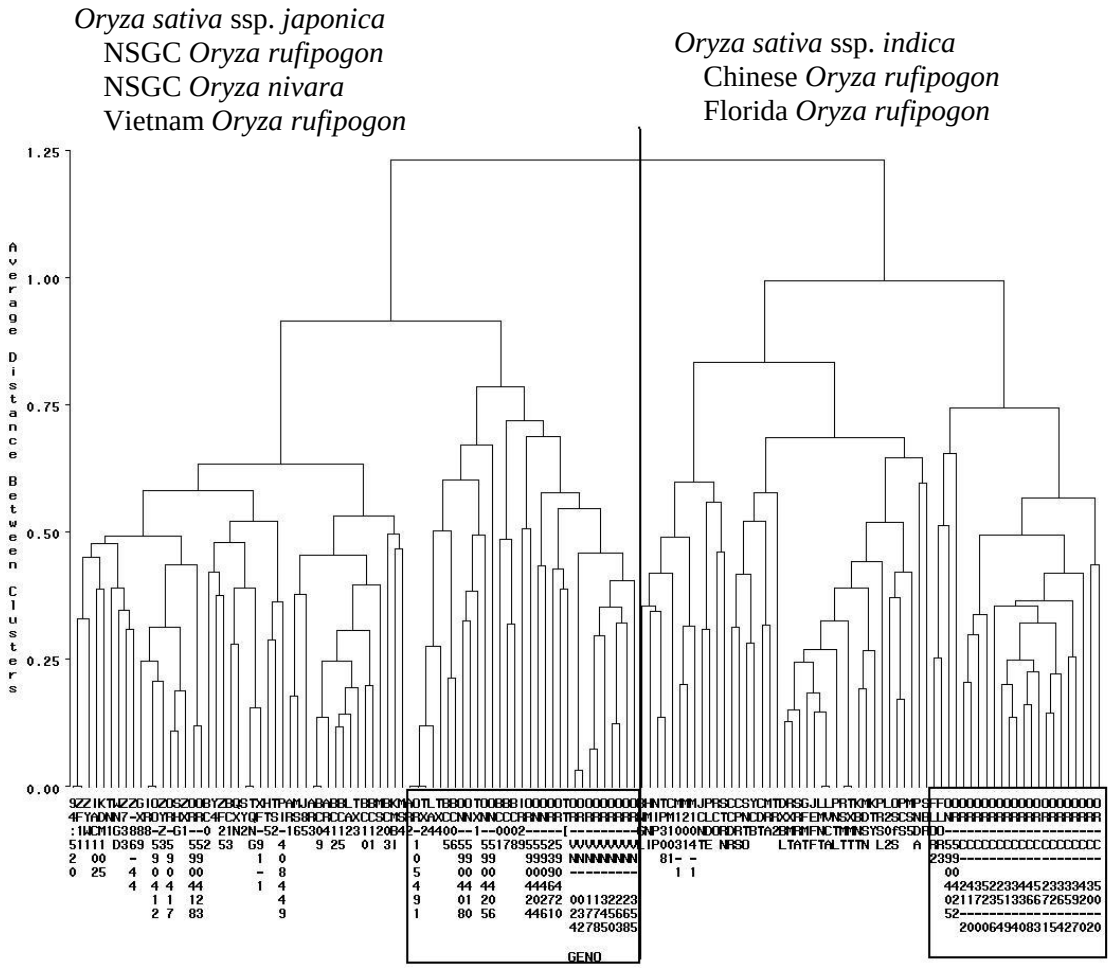


Figure 3-9. Phylogenetic tree of microsatellite data for *Oryza sativa* ssp. *japonica*, *Oryza sativa* ssp. *indica*, NSGC *Oryza rufipogon* and *Oryza nivara*, red rice and perennial *Oryza rufipogon* samples from the GS/MDS (Figure 3-4) and STRUCTURE (Figure 3-5) analysis. The heavy line indicates the division between the two main groups, *Oryza sativa* ssp. *indica* on the left and *Oryza sativa* ssp. *japonica* on the right. The box on the left includes the NSGC samples, red rice, and Vietnam *Oryza rufipogon* samples (those in the dashed line subgroup in the MDS analysis and the red group in the STRUCTURE analysis). The box on the right contains the perennial Florida *Oryza rufipogon* and China *Oryza rufipogon*.

STRUCTURE analysis also reveals a similar hierarchal structure of the *Oryza sativa* complex, with the annual *Oryza rufipogon* and *Oryza nivara* samples included in the same group as the annual *Oryza sativa* cultivars at the lower population number (Figure 3-5 above).

MDS and GD analysis further supports a high degree of separation between the two sub-species. In fact, the genetic distance between the two sub-species of *Oryza sativa* is equal to or greater than the distance between either of those groups and the perennial *Oryza rufipogon*. This trend is also seen in the GD analysis in Vaughan et al. (2001), where the difference between the two sub-species is also greater than any of the other between group distances.

The dual origin hypothesis has been a subject of much debate. However, our results are in agreement with an increasing amount of biochemical and molecular data that suggests that the origin of cultivated rice is polyphyletic (Cheng et al. 2003). The divergent origin of the *Oryza sativa* sub-species may also explain why the *Oryza rufipogon* samples from Vietnam are associated with the NSGC samples and TX4 type red rice. The phylogenic tree structure clearly illustrates that the Vietnam samples are rooted on the *Oryza sativa* ssp. *indica* side of the tree while the other perennial samples are on the *Oryza sativa* ssp. *japonica* side (Figure 3-9). This *indica/ japonica* differentiation in wild species of rice, as well as the high degree of diversity between geographically distinct populations has been reported by other investigators (Vaughan et al. 2003).

Conclusion

Despite the differences between the results with SSLP and SINE/MITE markers, certain conclusions can be drawn. In agreement with our previous results, the red rice from commercial fields can be split into three distinct groups. Much of the straw-hulled red rice is closely related to *Oryza sativa* ssp. *indica*. Rare ecotypes, such as MS5, are closely related to *Oryza sativa* ssp. *japonica* and appear to be the result of crossing with US rice cultivars. Some of the black-hulled red rice in commercial fields forms a third group.

Whether or not this group can be resolved from *Oryza sativa* ssp. *indica* and straw hulled red rice using GD/MDS analysis depends on the markers used and the samples are included in the analysis. However, it could consistently be resolved from *Oryza sativa* ssp. *indica* and straw-hulled red rice using STRUCTURE. That STRUCTURE allowed these two groups to be resolved was not surprising since some of the samples represent natural mixtures. STRUCTURE is a model based approach and can explicitly deal with samples with mixed ancestry. In MS/MDS analysis, however, samples are forced to belong to one group or another. Thus, mixtures are either forced into the most closely related group or, if present in sufficient numbers, cause groups to merge.

Some of the samples that are closely related to the black-hulled red rice in producer's fields have been classified as *Oryza rufipogon*. However, several pieces of data suggest that all of these accessions should be more accurately classified as *Oryza nivara*. First, the strict definition of *Oryza rufipogon* that is used by many workers is limited to perennial and rhizomatous plants (Oka 1991; Hush 1997; Martin et al. 1997; Yamanaka et al. 2003). All of the red rice in US

commercial fields are annuals plants and none appear to have rhizomes. Second, all of the red rice in commercial fields could be easily distinguished from the perennial rhizomatous red rice from Florida described by Vandiver et al. (1992) and from all of the samples from China provided by Dr. Snow. The perennial *Oryza rufipogon* samples from Vietnam were more closely related to some of the black-hulled red rice in commercial fields, but could still be distinguished; most notably by using SINE and MITE markers that have been previously established to resolve the annual species *Oryza nivara* from the perennial species *Oryza rufipogon*.

It should also be noted that substantial intermixing has already occurred between what we would classify as *Oryza nivara* and other types of rice. As noted above MS5 appears to be a natural hybrid between *Oryza nivara* and *Oryza sativa* ssp. *japonica* cultivars. STRUCTURE analysis also shows substantial levels of intermixing between *Oryza sativa* ssp. *indica*-like red rice and *Oryza nivara* (see Figure 3-4).

As will be discussed in Chapter IV, red rice samples that appear to represent natural mixtures of *Oryza nivara* and *Oryza sativa* ssp. *indica*-like red rice were also abundant in a new collection of red rice samples representative of fields across the rice production areas of Texas. Many of these samples have black hulls. Thus, even if one uses the broad definition of *Oryza rufipogon* that includes both annual and perennial species, it becomes very difficult to unambiguously resolve "annual *Oryza rufipogon*" from *Oryza sativa* ssp. *indica*. This difficulty in resolving *Oryza nivara* (annual *Oryza rufipogon*) from *Oryza sativa* ssp. *indica* has been encountered by several other groups (Parsons et al. 1997; Sun et al. 2001; Ni et al. 2002; Cheng et al. 2003; Park et al. 2003; Ren et al. 2003; Vaughan et al. 2003; Yamanaka et al. 2003).

Under the Federal Noxious Weed Act *Oryza rufipogon* is defined as an annual species (Plant Protection Act (PPA), www.aphis.usda.gov/ppq/weeds/weedhome.html). However, the annual species *Oryza nivara* is completely unregulated. Supporting this differentiation the US National Germplasm Collection (NSGC) contains samples of annual species that are labeled as *Oryza rufipogon* and other annual species that are labeled as *Oryza nivara*. However, this distinction does not appear to be scientifically valid. In all of the analysis presented above, samples from the NSGC labeled as *Oryza rufipogon* have always grouped with *Oryza nivara*. There are only two exceptions. First, some of the "*Oryza rufipogon*" samples from the NSGC clearly group with *indica* cultivars (see Figure 3-4). Second, only two samples from the NSGC grouped with the perennial *Oryza rufipogon* from Florida and China in the GD/MDS analysis. One of these was labeled as *Oryza rufipogon*, while the other was labeled as *Oryza nivara*. However, both showed substantial admixture upon STRUCTURE analysis (Figure 3-5).

There are several reasons why this apparent mixing may have occurred. The most obvious is the built in bias toward seed producing samples in the NSGC collection. True perennial wild rice reproduces mainly through the formation of rhizomes with very low and infrequent seed set (Yamanaka et al. 2003). However, in a germplasm bank based on seed, accessions must produce seed to propagate. There is also a tendency towards collecting samples with high seed set when the samples are periodically grown out to increase the number of seed due to depletion or age of the collection. Other authors have discussed examples of both instances (Jackson 1997; Vaughan, et al. 2003; Gao 2004). Other points of possible error are also introduced during the process of seed increase. These include the possibility of outcrossing and contamination. In fact, several of the different *Oryza* species samples from the NSGC were labeled with the warning "May contain red rice". Other factors include the dynamic nature of wild-weedy rice complex

present on the native habitat, mislabeling during storage or seed increase, and the possibility that the person making the original collection was mistaken in their classification.

Regardless of the source of the error, it is imperative that the samples in the NSGC be accurately classified since they might be used as standards for classification of unknowns. This is particularly important in the case of *Oryza rufipogon* and *Oryza nivara*. Since *Oryza rufipogon* is on the Federal Noxious Weed List and *Oryza nivara* is not, misclassification of red rice from producers fields based on faulty “standards” could have far reaching effects.

CHAPTER IV

TEXAS RED RICE GENETIC STRUCTURE AND IDENTITY

Introduction

As discussed in Chapters I and II, the weed red rice is generally divided into two broad groups based on hull color. Within these the straw and black-hulled types, other phenotypic characteristics such as the presence or absence of awns, as well as the location of original collection have been used to categorize red rice into different ecotypes (Diarra et al. 1985; Langevin et al. 1990; Kwon et al. 1992; Noldin 1999a). Thus far only two studies have utilized DNA markers to analyze the true genetic identity and diversity of red rice in U.S. producers' fields. These are the studies outlined in Chapter II (Vaughan et al. 2001) and a subsequent study by Gealy et al. (2002).

The work with molecular markers has demonstrated that the red rice population in the US is genetically very diverse. In the 60 different red rice samples that Vaughan et al. (2001) analyzed with 18 microsatellite markers, 25 distinct marker types were identified. Some of these were found in multiple locations in several states, while others were found only in a single location. In a similar screen of 89 red rice samples mainly from Arkansas with 18 microsatellite markers, Gealy et al. (2002) found 48 distinct marker types.

As discussed in Chapter II, most of the straw-hulled red rice from production fields is closely related to *Oryza sativa* ssp. *indica*, but some of the black-hulled red rice is sufficiently divergent

to be considered a different species. These black-hulled samples are closely related to some annual accessions that have been classified as *Oryza rufipogon.* However, as discussed in Chapter III, they do not meet the criteria of perennial and rhizomatous growth to be classified as *Oryza rufipogon sensu strictu* and are therefore more properly classified as *Oryza nivara*.

The diversity in red rice discovered in these studies directly contradicts conventional thought that there is a single generic type of red rice in US production fields. This convention has been reflected in the usage of only a narrow range of red rice for agronomic studies and weed control studies. Such studies typically utilize local populations of red rice or a very limited selection of straw hulled red rice (Khodayari et al. 1987; Kwon et al.. 1991; Kwon et al. 1992; Sankula et al. 1997a; Sankula et al. 1997b; Sagers and Naigemann 2002; Zhang et al. 2003). However, because of the diversity of the red rice complex, the use of such limited samples many not provide a realistic representation of red rice across the broader geographical areas.

All of the molecular studies on red rice in the US thus far have only included a small number of samples collected across a large geographical area. The only exception was a more focused collection from Brazoria county Texas (Vaughan et al. 2001). Even though rice cultivation in this part of Texas is relatively recent, starting only after World War II (J. Stansel, personal communication), several distinct types of red rice were found within individual fields (Vaughan et al. 2001).

In the current study the genetic profile of a large number of samples from a second county in Texas (Liberty County) was examined. Liberty County is on the other side of the coastal rice production area of Texas and has a longer history of rice production. A representative collection

of red rice from rice producing counties across the Gulf Coast of Texas and from an isolated production area on the Texas/Arkansas border was also examined.

The purpose of the work reported in this chapter was the investigation of the actual degree of genetic diversity of the red rice in Texas in a single field, a county, and the entire region. This will help identify a core collection of red rice accessions which would be particularly useful in herbicide research, since it has been shown that different red rice ecotypes have a varying response to several herbicides (Noldin et al. 1999b; Gealy et al. 2000).

Materials and Methods

A collection of approximately 700 red rice samples were obtained from rice fields in Liberty County, Texas during the summer of 1998, just prior to the harvest of the predominant commercial cultivar, Gulfmont. Panicles were collected from red rice plants in discrete 'patches' or small areas of infestation, approximately 9 m^2. Several patches in different parts of each field were sampled. Approximately 450 representative red rice samples along the Texas Gulf Coast were also collected between August 12 and August 20, 2003. Samples from Bowie County in Northeast Texas were collected on September 18, 2003. Seed from single plants were collected separately with a total number of 50 plants sampled from each of the following counties: Bowie, Chambers, Colorado, Fort Bend, Jackson, Jefferson, Matagorda, Waller, and Wharton. Global positioning system (GPS) data were recorded for each sample taken. MARPLOT^R was used to map the GPS data (http://www.epa.gov/ceppo/cameo/marplot.htm). All samples were cataloged and scored based on grain color, grain length and presence or absence of awns. The Texas State and Brazoria County samples from Vaughan et al. 2001 were

also included in the analysis. Genetic and data analysis were conducted as previously described (Vaughan et al 2001; Chapters II & III). GD and cluster analysis were used to identify samples in the same marker type, i.e. those undistinguishable based on the microsatellite markers utilized. Only one representative from each marker type was included in subsequent analysis. This was to prevent distortion of the cluster due to a single point having more weight than the other samples (Nylander 2001).

Results and Discussion

Hull color

As discussed in Chapter II, hull color was often an indicator of the genetic group to which a particular accession belonged. In general, straw-hulled red rice grouped with *Oryza sativa* ssp *indica* groups, while black-hulled red rice has been found to group with NSGC *Oryza nivara* accessions. The brown or gold-hulled samples have previously been considered intermediates between the straw and black-hulled types (Vaughan et al. 2001; Gealy et al. 2002).

Black-hulled red rice was found in every county examined, except Jackson (Figure 4-1, Table 4-1). The highest level of black-hulled red rice was found in Bowie County in northeast Texas, with 63% of the red rice samples having black hulls. Interestingly, in some counties only straw or black-hulled types were found. In other counties a range of colors was found including brown and gold-hulled accessions (Table 4-1). In most counties, brown and gold-hulled red rice was present at a relatively low level. However, brown-hulled samples were the predominant type found in Matagorda County.

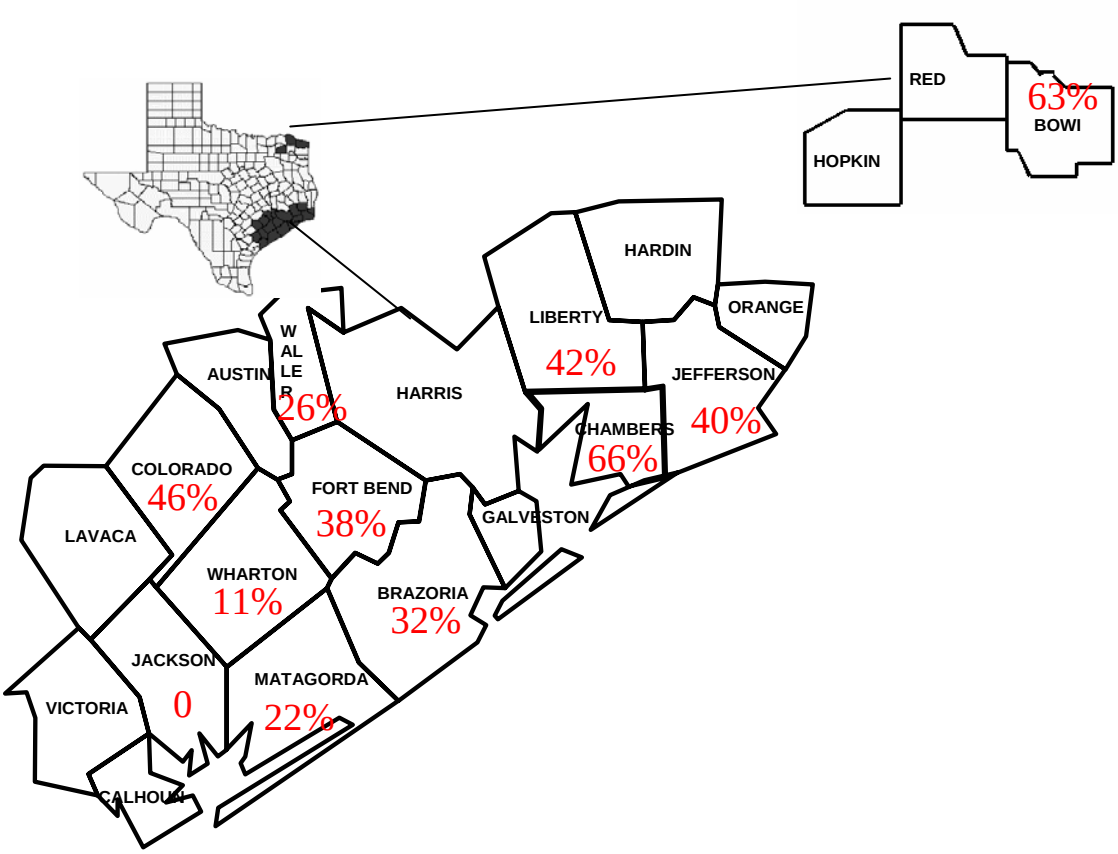


Figure 4-1. Percent of red rice collected from the various Texas counties that have black hulls.

Table 4-1. Hull color of red rice samples from Texas rice producers' fields by county. Percentage of Black, Gold, Brown and Straw colored samples collected from various production areas in Texas. [a.] Brown and gold-hulls were included with the black and straw-hulled samples respectively.

County	% Black	% Gold	% Brown	% Straw
Jefferson	40	4	9	47
Chambers	66	32	2	0
Fort Bend	38	0	0	62
Waller	26	18	24	32
Matagorda	22	21	55	4
Wharton	11	0	6	83
Colorado	46	2	6	46
Jackson	0	0	0	100
Bowie	63	0	8	29
Liberty[a]	26	-	-	74
Brazoria[a]	20	-	-	80

Table 4-2. Red rice samples from Liberty County and Texas red rice that were indistinguishable with the DNA marker set used in this study. Bold font indicates samples that were identified as indistinguishable from each other in the Liberty County analysis. Italicized font indicates samples that are used in the analysis to represent that particular group in subsequent analysis. Double lines are used to separate the different groups of samples. Groups with samples from Liberty County are referred to as Liberty County type 1- 19. Groups with members from various counties are referred to as Texas County (TXC) types 1- 18. Groups identical to TX4 and IRGC 105491 are identified as TX04 and 105491 respectively. County abbreviations are as follows: BC- Bowie County, CC- Chambers County, COC- Colorado County, FBC- Fort Bend County, JFC- Jefferson County, JKC- Jackson County, LBC- Liberty County, MDC- Matagorda County, WRC- Waller County, WTC- Wharton County.

LBC-01-15	***LC01***
LBC-01-26	**LC01**
LBC-01-24	***LC02***
LBC-01-29	**LC02**
LBC-01-12	***LC03***
LBC-01-17	**LC03**
LBC-01-18	**LC03**
LBC-01-19	**LC03**
LBC-01-21	**LC03**
LBC-01-22	LC03
LBC-01-25	**LC03**
LBC-01-27	**LC03**
LBC-04-34	LC03
LBC-01-14	***LC04***
LBC-01-23	**LC04**
LBC-01-28	**LC04**
LBC-07-24	***LC05***
LBC-07-43	**LC05**
LBC-01-31	***LC06***
LBC-03-08	**LC06**
LBC-01-45	***LC07***
LBC-03-05	**LC07**
LBC-03-06	**LC07**
LBC-01-36	**LC08**
LBC-06-1	***LC08***
LBC-06-43	**LC08**
LBC-01-48	**LC09**
LBC-02-25	**LC09**
LBC-03-47	**LC09**
LBC-07-05	**LC09**
LBC-08-01	**LC09**
LBC-08-36	**LC09**

LBC-10-16	**LC09**
LBC-10-48	**LC09**
LBC-12-37	**LC09**
LBC-13-28	**LC09**
LBC-18-44	***LC09***
LBC-13-12	LC09
LBC-14-01	LC09
LBC-07-22	***LC10***
LBC-11-15	**LC10**
LBC15-25A	***LC11***
LBC-17-06	**LC11**
LBC-01-02	LC12
LBC-01-09	*LC12*
LBC-15-25	*LC13*
LBC-16-07	LC13
LBC-08-37	***LC14***
LBC-08-42	**LC14**
LBC-08-43	**LC14**
LBC-01-20	**LC15**
LBC-01-34	**LC15**
LBC-03-25	*LC15*
LBC-03-48	**LC15**
LBC-04-09	**LC15**
LBC-05-25	**LC15**
LBC-07-48	**LC15**
LBC-08-14A	**LC15**
LBC-03-13	LC16
LBC-03-16	*LC16*
LBC-01-43	LC17
LBC-02-21	*LC17*
LBC-03-30	*LC18*
LBC-17-17	LC18
LBC-06-19	*LC19*
LBC-06-42	LC19

LBC-03-21	TX01
TX1	*TX01*
BC-01-39	TXC02
JFC-01-07	*TXC02*
JFC-01-43	TXC02
MDC-01-18	TXC02
WTC-01-38	*TXC03*
WtC-01-39	TXC03
WtC-01-40	TXC03
CC-01-06	TX04
CC-01-12	TX04
CC-01-15	TX04
CC-01-31	TX04
CC-01-36	TX04
CC-01-40	TX04
CC-01-47	TX04
CC-01-48	TX04
CC-01-50	TX04
COC-01-01	TX04
COC-01-05	TX04
FBC-01-20	TX04
FBC-01-28	TX04
FBC-01-33	TX04
FBC-10	TX04
JFC-01-08	TX04
JFC-01-12	TX04
JFC-01-14	TX04
JFC-01-33	TX04
JFC-01-38	TX04
LBC-12-17	TX04
MDC-01-02	TX04
TX4	*TX04*
FBC-27-2	TXC05
FBC-27-3	*TXC05*
JFC-01-42	TXC06
JFC-01-44	*TXC06*

FBC-01-02	*TXC07*
MDC-01-10	TXC07
WTC-01-02	*TXC08*
WtC-01-16	TXC08
WTC-01-43	*TXC09*
WtC-01-49	TXC09
BC-01-18	*TXC10*
BC-01-33	TXC10
COC-01-45	*TXC11*
COC-01-48	TXC11
WTC-17	*TXC12*
WtC-19	TXC12
WTC-14	*TXC13*
WtC-16	TXC13
MDC-01-28	*TXC14*
MDC-01-45	TXC14
WRC-4	*TXC15*
WRC-6	TXC15
LBC-03-28	TXC16
WRC-10	*TXC16*
WRC-7	TXC16
WRC-8	TXC16
BC-01-06	*TXC17*
LBC-11-13	TXC17
LBC-11-31	TXC17
LBC-13-02	TXC17
LBC-13-23	TXC17
LBC-16-35	TXC17
COC-01-14	*TXC18*
COC-01-02	TXC18
JKC-01-04	TXC18
JKC-01-15	TXC18
JKC-01-18	TXC18
JKC-01-27	TXC18
JKC-01-33	TXC18
JKC-01-44	TXC18
IRGC 105491	*105491*
BC-01-22	105491
BC-01-40	105491

Molecular Marker Analysis

Red Rice Diversity in Liberty County Texas

Analysis of the Liberty County samples offers several opportunities for the investigation of red rice diversity and genetic structure. In particular, the Liberty County collection allows for the investigation of red rice on the smallest and most basic unit of analysis, within a single field. The intense collection effort also allows for the investigation of red rice characteristics on the level of a large county that has been in rice production for over a century.

In Liberty County there were two main areas of collection approximately 27km apart, the southern field and the northern field. Patches, or small areas of red rice infestation, are identified by number (1-17) and GPS coordinates were taken to determine the distance between the patches. Within each patch, panicles from all red rice plants were collected and individually numbered to reflect patch and plant number (e.g. LC-01-01= Liberty County - patch one- plant one). In the northern collection location, 10 patches were collected. The closest patches (2 and 3) were 16m apart and the furthest (patches 8 and 17) were 415m apart. In the southern area, 7 patches were collected. Patches 15 and 16 were the closest (52m) and patches 10 and 15 were the most distant (379m) in this field. A third single small collection area (patch18) was located 21km and 34km respectively from the other two locations.

Molecular marker analysis revealed that most of the samples were unique, but some had the same marker patterns as other samples in the analysis. These samples with identical marker types are referred to as Liberty County ecotypes and are listed in Table 4-2. The samples listed in italics are those that are included in the analysis as representatives of their group. Some of these

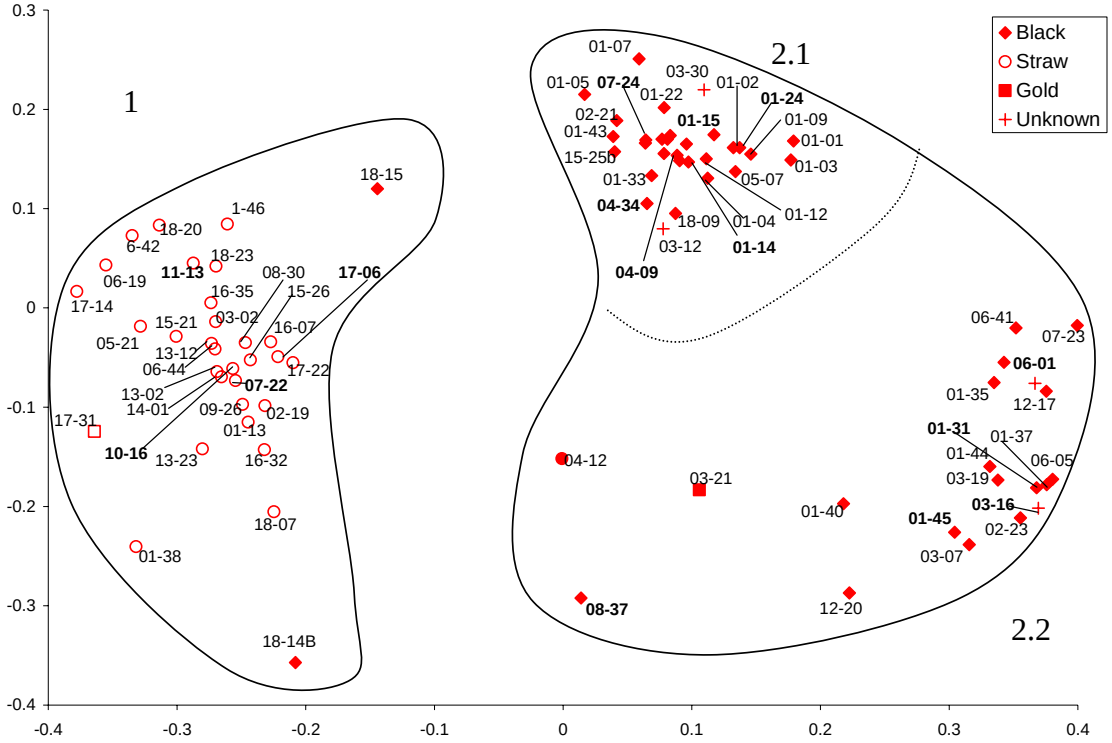


Figure 4-2. MDS of Liberty County red rice samples. Solid lines indicate clusters, with the dashed line separating sub-group 2.1 from the remainder of group 2. Symbol shape indicates hull color. Solid markers indicated awned samples, while empty markers indicate awnless samples. The patch and plant number for the samples are indicated. Sample IDs in bold indicate samples that are undistinguishable from other samples based on cluster analysis.

Table 4-3. Genetic distance data for Figure 4-2.

Overall GD	**0.3559**
85%	0.3025
Group 1- Straw-hulled samples	0.1599
Group 2- Mainly Black-hulled samples	0.2609
Sub group 2.1- Small group of Black-hulled, mainly LC patch 1	0.0818
Remainder of the Black-hulled samples	0.2302
Black-hulled (2) vs. Straw-hulled (1)	0.4928
LC patch one sub group (2.1) vs. remainder of Black-hulled group	0.4055

Liberty County ecotypes consisted of two samples from a singe patch; while others consisted of samples from up to nine different patches distributed across all three collection sites (LC09-patches 1, 2, 3, 7, 8, 10, 12, 13, and 18). The distribution of samples in Liberty County supports the findings from Vaughan et al. (2001) that some red rice ecotypes are restricted to a small area, while others can be found at multiple locations across a large area.

Genetic distance and MDS analysis of the molecular marker data revealed two distinct groups of red rice (Figure 4-2, Table 4-3) in Liberty County. One group consisted of a diverse collection of black, gold and brown-hulled samples. The other group consisted mainly of straw-hulled samples. Interestingly, although the majority of the samples from Liberty County are straw-hulled (75%, Table 4-1), the straw-hulled group is less genetically diverse than the black-hulled group (within group GD of 0.1600 compared to 0.2609).

Within the black-hulled group there was a tight cluster of closely related samples (subgroup 2.1, GD of 0.0818). Over half of the samples in this small cluster originated from patch one, indicating that this particular area of red rice infestation had a large number of closely related black hulled samples. However, this same small region (approximately 9 m^2) also contained other types of black-hulled red rice as well as three straw-hulled samples that map to different regions on the other side of Figure 4-2.

The northern field (patches 1-10) and southern field (11-17) both had samples in each of the major groups. However, the diversity of red rice within a field varied considerably. Patches 1-8 from the northern field, had samples that are located in both groups. In fact, of the 38 samples used for microsatellite analysis from patch one, 28 had unique marker types. In contrast, patches

9 and 10 only have samples from the black-hulled group. Red rice in the southern field was generally less diverse. Fewer patches that had samples in both groups (patches 12, 15 and 17) and more that are only found in the black hulled group (patches 11, 13, 14 and 16). Note that since many samples have identical marker patterns, not all of them are labeled in Figure 4-2.

A potential artifact in GD/MDS analysis is that a tight cluster of samples can distort the over-all analysis (Nylander 2001). To obtain a more "balanced" set of samples in the analysis, most of the closely related black-hulled samples from patch one were removed and the analysis was repeated. As before, strong evidence was again obtained for two distinct groups and none of the remaining samples moved between groups (data not shown).

STRUCTURE analysis of the Liberty County samples was generally consistent with GD/MDS. As expected, the proper population level was found to be K=2 (Figure 4-3, Table 4-4, Table 4-5). These two populations largely corresponded to the two groups identified in the GD/MDS analysis. However, many of the samples in the tight cluster on the right side of Figure 4-2 (subgroup 2.1) were found to be mixtures (Figure 4-3, samples 33-45). Notably, this includes many of the black-hulled samples from patch one.

Genetic analysis of the red rice samples from Liberty County support the findings from Brazoria County (Vaughan et al. 2001). In both instances, some ecotypes were only found in a single location, while other ecotypes were found in multiple locations. Analysis of the Liberty County samples also supported the existence of two main groups of red rice, which mainly correspond to straw and black-hulled phenotypes.

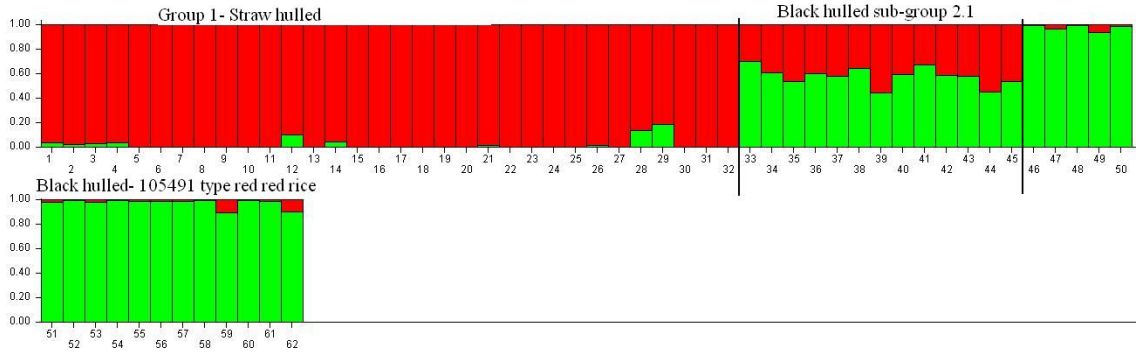


Figure 4-3. STRUCTURE analysis for Liberty County samples with black-hulled group thinned. Remaining samples from patch one are indicated in **BOLD** in Table 4-3.

Table 4-4. STRUCTURE info for above run.

K	Ln P(D)	Var Ln P(D)	α1
1	-1477.1	15.3	
1	-1477.2	15.4	
1	-1477.1	15.4	
1	-1477.1	15.3	
1	-1477.1	15.4	
2	-863.0	42.0	0.1405
2	-862.3	40.7	0.1389
2	-863.4	43.2	0.1387
2	-863.1	42.4	0.1383
2	-863.0	42.0	0.1380
3	-786.3	57.8	0.0913
3	-670.6	48.3	0.0423
3	-669.7	46.4	0.0430
3	-786.0	57.1	0.0910
3	-669.3	45.5	0.0423
4	-596.2	54.7	0.0384
4	-599.0	52.5	0.0393
4	-596.7	56.2	0.0389
4	-597.3	57.6	0.0389
4	-596.9	56.6	0.0386

Table 4-5. Sample list for Figure 4-3. STRUCTURE ID and MDS cluster are listed. ID indicates the county of collection (Liberty County- LBC), patch number and plant number, eg. LBC-01-01 is patch one plant one.

Structure ID	MDS Cluster	Variety ID	Structure ID	MDS Cluster	Variety ID
1	1	LBC-01-13	33	2.1	**LBC-01-03**
2	1	LBC-01-38	34	2.1	**LBC-01-04**
3	1	LBC-01-46	35	2.1	**LBC-01-05**
4	1	LBC-02-19	36	2.1	**LBC-01-15**
5	1	LBC-03-02	37	2.1	**LBC-01-16**
6	1	LBC-05-21-1	38	2.1	LBC-03-21
7	1	LBC-05-21-2	39	2.1	LBC-04-12
8	1	LBC-06-19	40	2.1	LBC-04-34
9	1	LBC-06-42	41	2.1	LBC-05-07
10	1	LBC-06-44	42	2.1	LBC-08-37
11	1	LBC-07-22	43	2.1	LBC-08-47
12	1	LBC-08-14B	44	2.1	LBC-15-25B
13	1	LBC-08-30	45	2.1	LBC-17-17
14	1	LBC-09-26	46	2	LBC-01-31
15	1	LBC-11-13	47	2	LBC-01-35
16	1	LBC-13-02	48	2	LBC-01-37
17	1	LBC-13-12	49	2	LBC-01-40
18	1	LBC-13-23	50	2	LBC-01-44
19	1	LBC-14-01	51	2	LBC-01-45
20	1	LBC-15-26	52	2	LBC-02-23
21	1	LBC-15-25A	53	2	LBC-03-07
22	1	LBC-16-07	54	2	LBC-03-16
23	1	LBC-16-32	55	2	LBC-03-19
24	1	LBC-16-35	56	2	LBC-03-28
25	1	LBC-17-14	57	2	LBC-06-01
26	1	LBC-17-22	58	2	LBC-06-05
27	1	LBC-17-31	59	2	LBC-06-41
28	1	LBC-18-07	60	2	LBC-07-23
29	1	LBC-18-15	61	2	LBC-12-17
30	1	LBC-18-20	62	2	LBC-12-20
31	1	LBC-18-23			
32	1	LBC-18-44			

Statewide Diversity of Texas Red Rice

Investigation of the diversity of red rice within an entire state is important for understanding the dynamics of the red rice complex on a larger geographical basis. For the investigation of the red rice complex across Texas, red rice samples were collected from the major rice producing counties (Bowie, Colorado, Chambers, Fort Bend, Jackson, Jefferson, Matagorda, Waller, and Wharton). To ensure that the study focused on the red rice most likely to contaminate commercial rice, red rice seed samples were collected just before the crop was harvested. Both black and straw-hulled red rice were collected from every county except Jackson (no black) and Chambers (no straw) Counties (Table 4-1).

The samples from these additional counties were combined with the Liberty County samples, as well as the Brazoria County and Texas State samples from Vaughan et al. (2001) for microsatellite analysis. From these samples, 139 different DNA marker patterns were identified. Many of these were represented by only one sample. However, some markers patterns were found in multiple locations (Table 4-2). Interestingly, 23 samples from across the rice production area of Texas were found that were indistinguishable from the red rice ecotype TX4 that was originally isolated near Katy, Texas (Noldin et al. 1999a, 1999b). The abundance of this particular ecotype may be significant because the founding member of this group, TX4, was has considerable resistance to the glutamine synthetase inhibitor glufosinate. This is discussed further in Chapter V.

The addition of the Brazoria County samples from Vaughan et al. 2001 into the analysis caused the inclusion of some Liberty County samples into previously identified groups. Additionally, half of the LC01 samples from the closely related group of black-hulled red rice in (Figure 4-2,

subgroup 2.1) were removed to prevent the over-representation of this small group from distorting the overall analysis (Nylander 2001).

GD/MDS statistical analysis of the state-wide samples revealed three groups of red rice. One group consists of largely straw-hulled type (group 1), while the other two contained predominantly black and brown-hulled samples (Figure 4-4). The within group GD of the straw-hulled group was 0.3766, the black-hulled group on the left side of the figure (group 3) was 0.1911 and the intermediate group (group 2) was 0.3648. The GD for the entire data set was 0.4811 (Table 4-6).

Five of counties (Brazoria, Bowie, Colorado Liberty and Matagorda) had representatives in each of the three groups (Figure 4-4B). Chambers County only had samples in the two predominantly black-hulled groups, while Ford Bend, Jefferson and Wharton Counties lacked samples in group 2. Jackson County was the only county with samples in only a single group. This was is not surprising since the Jackson County collection contained only straw-hulled samples (Table 4-1). Interestingly, even though Bowie County in northeastern Texas is geographically isolated from the other rice producing counties (Figure 4-1), it contains samples in all three groups.

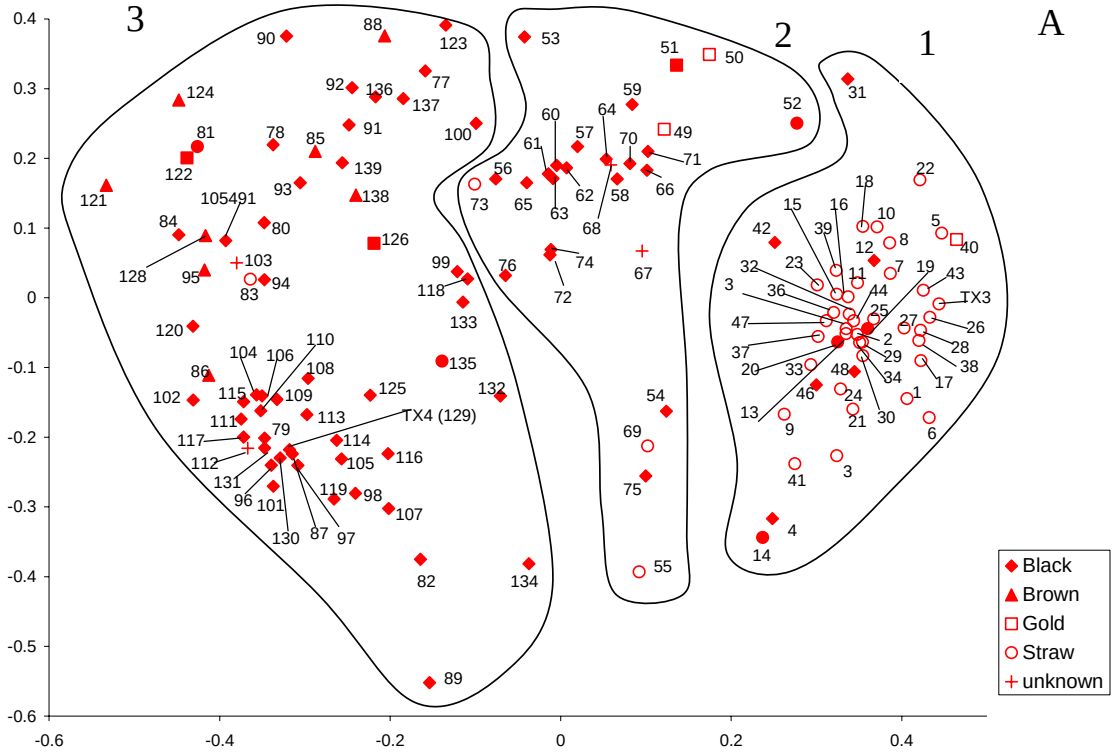


Figure 4-4. GD/MDS of microsatellite analysis for Texas red rice. A. Phenotypic distribution of samples. Cluster analysis reveals 3 groups which correspond to mainly straw-hulled samples (group 1), mainly black-hulled samples related to IRGC 105491 (group 3), and an intermediate group mainly consisting of black hulled samples (group 2). Sample labels are provided in Table 4-8.

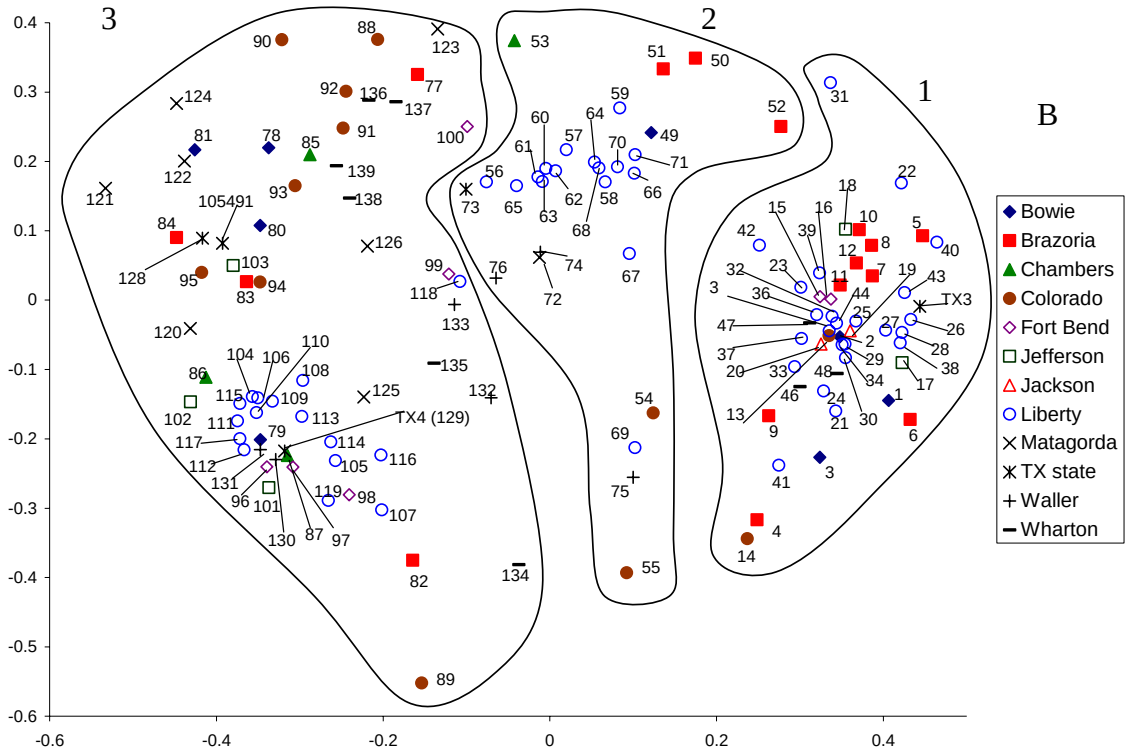


Figure 4-4 continued. GD/MDS of microsatellite analysis for Texas red rice. Figure B illustrates the collection location of different samples. Cluster analysis reveals 3 groups which correspond to mainly straw-hulled samples (group 1), mainly black-hulled samples related to IRGC 105491 (group 3), and an intermediate group mainly consisting of black hulled samples (group 2). Sample labels are provided in Table 4-8.

Table 4-6. Genetic distance data for Figure 4-4.

Overall GD	**0.4811**
85%	0.4090
Group 1	0.4240
Group 3	0.3767
Group 2	0.3648
1 vs. 2 and 3	0.6122
Black vs. Intermediate	0.4934

Table 4-7. Sample ID for Figure 4-4 and Figure 4-5.

Structure ID	Variety	Structure ID	Variety	Structure ID	Variety
1	Bowie Cnty #01-01	48	Wharton Cnty #24	94	Colorado Cnty #18
2	Bowie Cnty #01-06	49	Bowie Cnty #01-47	95	Colorado Cnty #22
3	Bowie Cnty #01-26	50	Brazoria Cnty #02	96	Fort Bend Cnty #01-02
4	Brazoria Cnty #03	51	Brazoria Cnty #07	97	Fort Bend Cnty #27-1
5	Brazoria Cnty #09	52	Brazoria Cnty #08	98	Fort Bend Cnty #27-3
6	Brazoria Cnty #10	53	Chambers Cnty #01-43	99	Fort Bend Cnty #28
7	Brazoria Cnty #11	54	Colorado Cnty #20	100	Fort Bend Cnty #28-2
8	Brazoria Cnty #12	55	Colorado Cnty #21	101	Jefferson Cnty #01-19
9	Brazoria Cnty #13	56	Liberty Cnty #01-03	102	Jefferson Cnty #01-44
10	Brazoria Cnty #14	57	Liberty Cnty #01-04	103	Jefferson Cnty #623
11	Brazoria Cnty #15	58	Liberty Cnty #01-05	104	Liberty Cnty #01-31
12	Brazoria Cnty #TX3	59	Liberty Cnty #01-07	105	Liberty Cnty #01-35
13	Colorado Cnty #01-14	60	Liberty Cnty #01-09	106	Liberty Cnty #01-37
14	Colorado Cnty #01-36	61	Liberty Cnty #01-12	107	Liberty Cnty #01-40
15	Fort Bend Cnty #01-13	62	Liberty Cnty #01-14	108	Liberty Cnty #01-44
16	Fort Bend Cnty #01-46	63	Liberty Cnty #01-15	109	Liberty Cnty #01-45
17	Jefferson Cnty #01-07	64	Liberty Cnty #01-20	110	Liberty Cnty #02-23
18	Jefferson Cnty #01-26	65	Liberty Cnty #01-24	111	Liberty Cnty #03-07
19	Jackson Cnty #01-01	66	Liberty Cnty #02-21	112	Liberty Cnty #03-16
20	Jackson Cnty #01-03	67	Liberty Cnty #03-12	113	Liberty Cnty #03-19
21	Liberty Cnty #01-13	68	Liberty Cnty #03-30	114	Liberty Cnty #06-01
22	Liberty Cnty #01-38	69	Liberty Cnty #04-12	115	Liberty Cnty #06-05
23	Liberty Cnty #01-46	70	Liberty Cnty #07-24	116	Liberty Cnty #06-41
24	Liberty Cnty #02-19	71	Liberty Cnty #15-25B	117	Liberty Cnty #07-23
25	Liberty Cnty #03-02	72	Matagorda Cnty #15	118	Liberty Cnty #08-37
26	Liberty Cnty #05-21	73	TX1	119	Liberty Cnty #12-20
27	Liberty Cnty #05-21	74	Waller Cnty #1	120	Matagorda Cnty #01-24
28	Liberty Cnty #06-19	75	Waller Cnty #2	121	Matagorda Cnty #01-27
29	Liberty Cnty #06-44	76	Waller Cnty #9	122	Matagorda Cnty #01-28
30	Liberty Cnty #07-22	77	Brazoria Cnty #01	123	Matagorda Cnty #01-30
31	Liberty Cnty #08-14B	78	Bowie Cnty #01-05	124	Matagorda Cnty #01-50
32	Liberty Cnty #08-30	79	Bowie Cnty #01-13	125	Matagorda Cnty #25
33	Liberty Cnty #09-27	80	Bowie Cnty #01-18	126	Matagorda Cnty #26
34	Liberty Cnty #10-16	81	Bowie Cnty #01-19	127	IRGC 105491
35	Liberty Cnty #15-26	82	Brazoria Cnty #04	128	TX2
36	Liberty Cnty #16-32	83	Brazoria Cnty #05	129	TX4
37	Liberty Cnty #17-06	84	Brazoria Cnty #06	130	Waller Cnty #10
38	Liberty Cnty #17-14	85	Chambers Cnty #01-10	131	Waller Cnty #3
39	Liberty Cnty #17-22	86	Chambers Cnty #01-27	132	Waller Cnty #4
40	Liberty Cnty #17-31	87	Chambers Cnty #01-34	133	Waller Cnty #5
41	Liberty Cnty #18-07	88	Colorado Cnty #01-21	134	Wharton Cnty #01-01
42	Liberty Cnty #18-15	89	Colorado Cnty #01-23	135	Wharton Cnty #12
43	Liberty Cnty #18-20	90	Colorado Cnty #01-27	136	Wharton Cnty #13
44	Liberty Cnty #18-23	91	Colorado Cnty #01-42	137	Wharton Cnty #14
45	TX3	92	Colorado Cnty #01-45	138	Wharton Cnty #17
46	Wharton Cnty #01-02	93	Colorado Cnty #01-50	139	Wharton Cnty #23
47	Wharton Cnty #01-43				

STRUCTURE analysis of the state-wide microsatellite data supports the MDS analysis (Figure 4-5, Table 4-7, Table 4-8). However, as in the Liberty County data, it suggests that the red rice samples actually consist of only two fundamental types (i.e. the proper population level of K=2). Samples 1-48 (primarily green) correspond to group 1 in Figure 4-4. Samples 77-139 (primarily red) correspond to group 3 in Figure 4-4. STRUCTURE reveals that the intermediate group in Figure 4-4 (group 2) can best be modeled as approximately equal mixtures of the other two groups, rather than as a separate group.

This result was initially surprising. Due to the nature of the GD/MDS analysis, one would expect that samples with intermediate composition would have been forced into the most closely related parental group or that they would have caused the two parental groups to fuse. Instead, based on the criteria used to from groups (within group GD less than 85% of overall GD and GD between the next closest group greater than the overall GD), they were identified as an independent group.

Examination of a haplotype chart (Figure 4-6) argues that the STRUCTURE analysis is correct. In this figure, samples are represented by rows and DNA marker data is shown as pairs of columns. Different alleles are indicated by different colors within a column. The samples are sorted according to the groups found by GD analysis. While the three groups detected by GD analysis are easily distinguishable, group 2 can readily be seen to consist of a mixture of alleles from the other two groups. However, the DNA marker types in group 2 are clearly not random mixtures of alleles from groups 1 and 3. The samples were collected from a wide range of locations (Figure 4-4 B), but most of them have very similar genotypes.

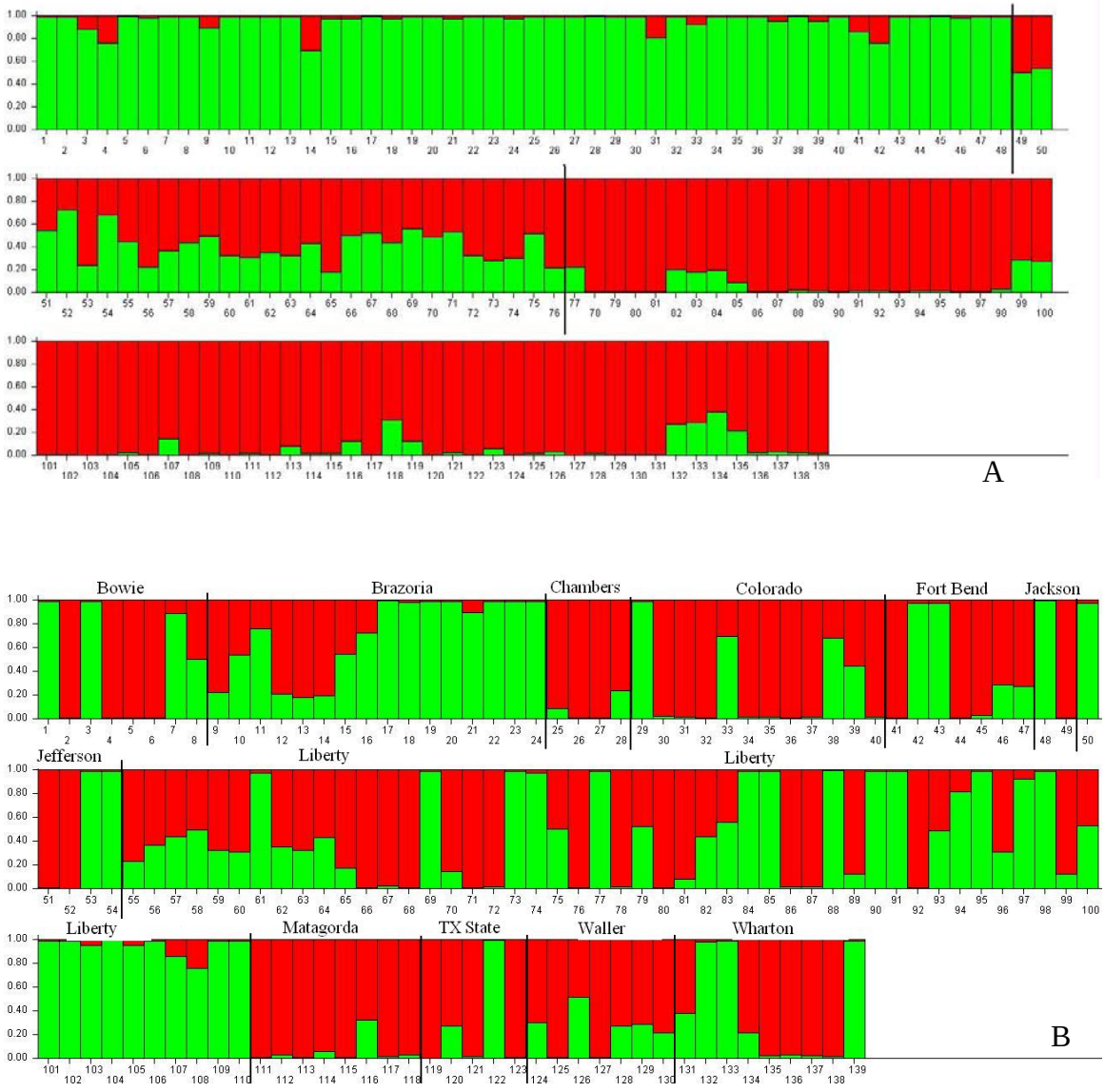


Figure 4-5. STRUCTURE analysis of Texas red rice samples. A. Samples are grouped based on MDS clusters and are in the order listed in Table 4-8. B. Samples grouped based on county. Vertical lines are used to divide the analysis into the groups identified by the MDS or to separate samples based on county of collection.

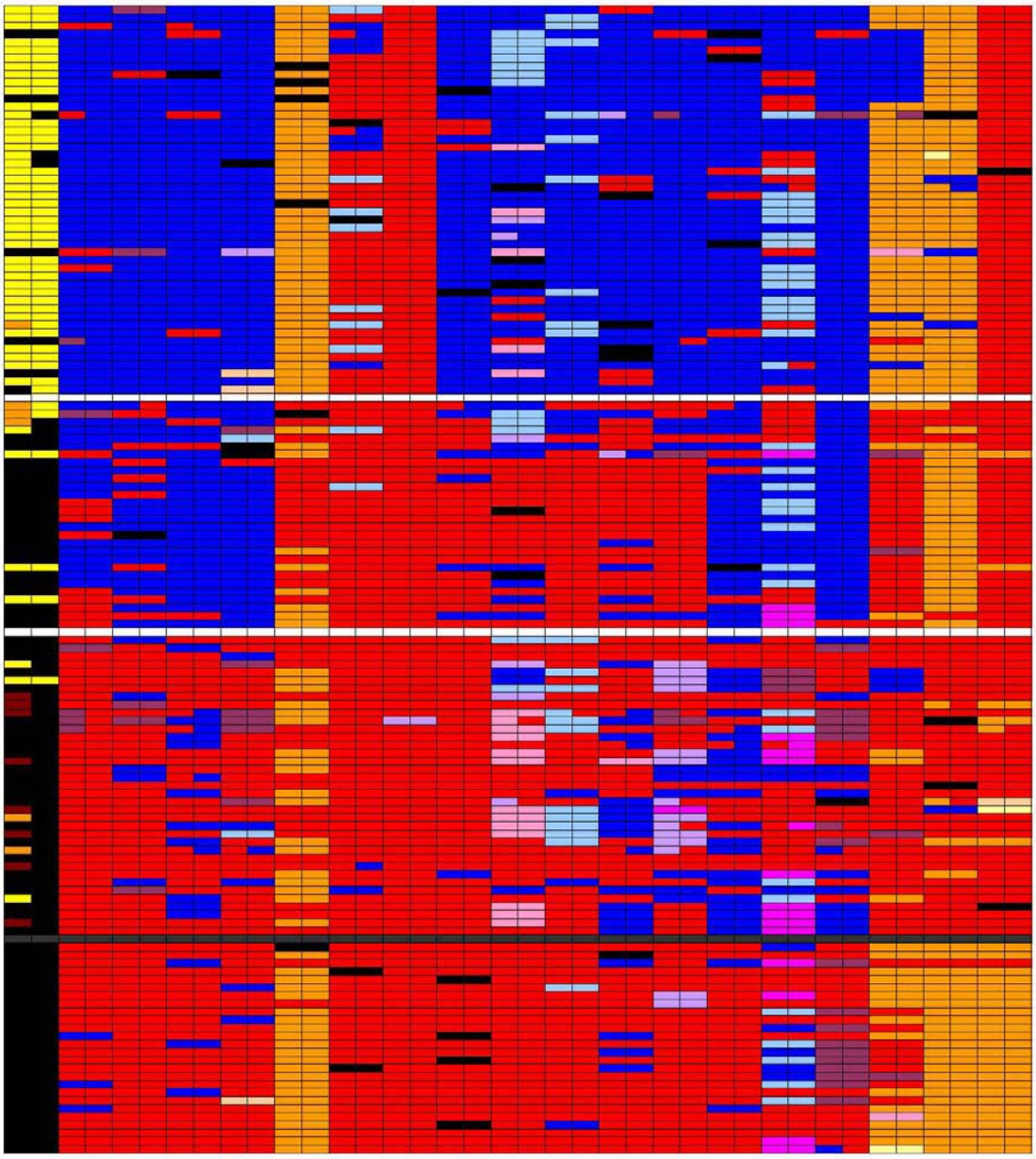


Figure 4-6. Haplotype chart for Texas red rice. Samples are grouped based on MDS clusters from top to bottom and groups are separated by a blank white line. Group one is on the top, group 2 in the middle and group 3 at the bottom. The solid black line in group three separates the two subgroups, with 3.1 at the top and 3.2 on the bottom. Colors are used to indicate alleles for a particular microsatellite locus. Data is shown for both alleles of the microsatellites in side by side columns. Hull color and presence of awns are indicated by the first two columns on the left of the figure, with black indicating black-hulls and awns and yellow representing straw-hulled and no awns. Samples are in the same order as in Table 4-7.

Figure 4-6 continued. Markers from left to right are: OSM90, OSR16, RM102, RM110, RM143, RM146, RM152, RM153, RM162, RM166, RM201, RM241, RM20L, RM241, RM242, RM282, RM5, RSus1, RSus2, AND WAXY. Each marker is represented by two columns to provide data for both microsatellite alleles at a given locus.

Table 4-8. Summary of STRUCTURE results from Figure 4-5.

K	Ln P(D)	Var Ln P(D)	$\alpha 1$
1	-4061.1	22.5	
1	-4061.0	22.4	
1	-4061.1	22.5	
1	-4061.1	22.6	
2	-2913.5	94.1	0.1856
2	-2913.5	94.1	0.1850
2	-2913.5	94.0	0.1848
2	-2912.6	92.5	0.1852
3	-2605.8	132.3	0.1009
3	-2611.9	136.8	0.0915
3	-2608.9	130.5	0.0907
3	-2605.5	131.4	0.1007
4	-2311.5	140.8	0.0622
4	-2313.1	143.1	0.0619
4	-2312.5	142.6	0.0625
4	-2491.7	164.5	0.0840

One possible explanation for the origin of an intermediate group of red rice accessions would be recent crossing between groups. However, very few samples in GD/MDS group 2 of the state-wide samples are heterozygous for any of the alleles tested. Thus, this group is does not represent F1 hybrids. If it represented recent progeny of crosses between groups 1 and 3, one would expect a more random assortment of progeny. The similarity among the genotypes represented could reflect a particularly favorable allelic combination that provides a competitive advantage under Texas conditions. However, a more likely interpretation is that the differences between these three groups largely reflect founder effects and that there has been relatively little gene flow between groups.

As expected, there was considerably more genetic variation among the state-wide samples than those from a single county. This is particularly evident in group 3, the predominantly black-hulled group on the left side of Figure 4-4. The samples from Liberty County account for only a small portion of the diversity in this group. As illustrated in Figure 4-7, there are two main subgroups in group 3, with Colorado County # 01-23 representing an outlier. These two subgroups correspond to a group of red rice closely related to TX4 (subgroup 3.2), which consists mainly of the Liberty County samples, and a second group (subgroup 3.1) consisting mainly of samples from the other Texas Counties which is closely associated with IRGC 105491.

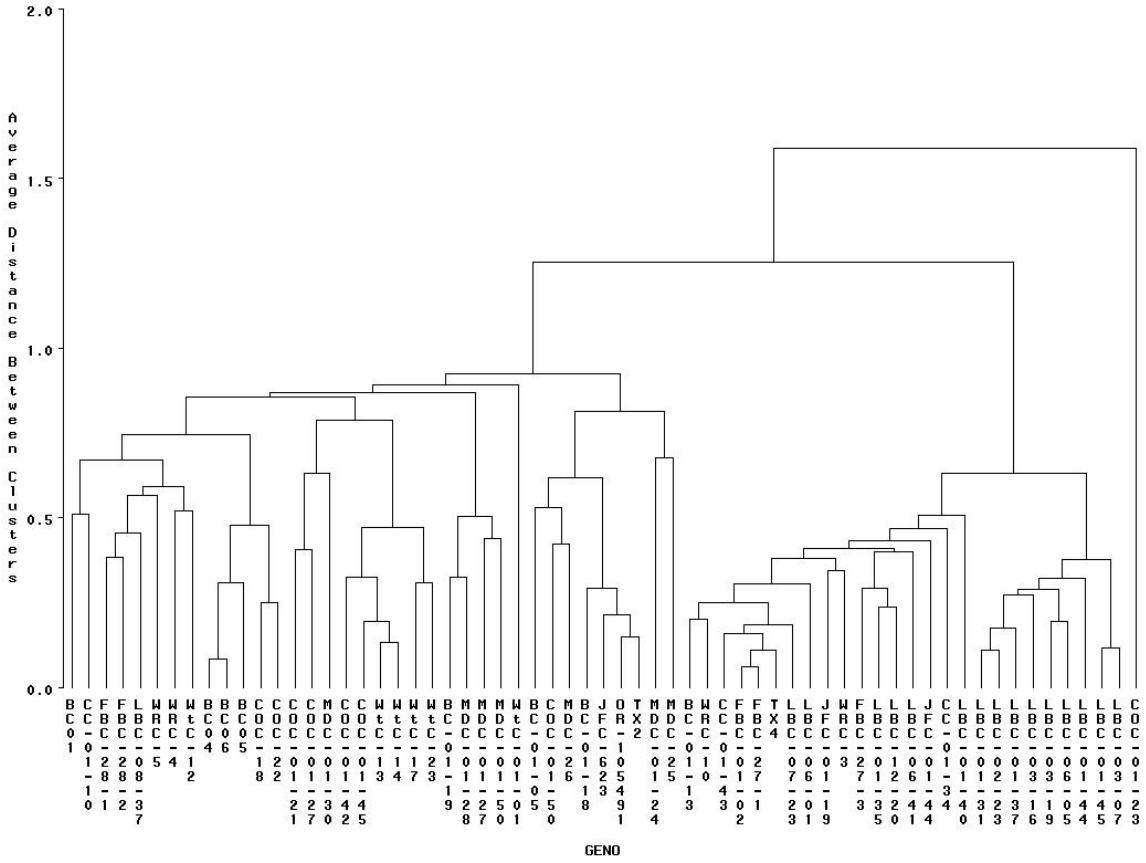


Figure 4-7. Hierarchical tree analysis of group 3 from Figure 4-4. The members of group 3 were subjected to hierarchal cluster analysis to illustrate the presence of two subgroups within group 3.

Other methods of analysis also support the presence of two distinct subgroups of red rice within group 3 and also reveal further details of the relationships. STRUCTURE analysis of group 3 revels that there was very little admixture between the two subgroups (Figure 4-8, Table 4-9, Table 4-10). Of the 63 samples in group 3 only one, Matagorda # 25, had a high degree of admixture (approximately 50%). A few others have a lower degree of mixture - Waller #4, Wharton #12, Colorado #18, Jefferson #623, Jefferson #01-24 and Colorado # 01-23 (the outlier identified in the tree analysis). The two subgroups can also be readily distinguished in the haplotype chart shown in Figure 4-6. The TX4 group (subgroup 3.2), which is below the solid black line in Figure 4-6, corresponds to the tight cluster of mainly Liberty County samples in the MDS analysis and has a much lower degree of allelic diversity than the other subgroup.

Plotting GPS coordinates of the samples that were analyzed with DNA markers illustrates the geographical distribution of the various groups and reveals some distinct trends (Figure 4-9). The most apparent was the distribution of subgroups 3.1 and 3.2 across the state. While both groups were found across the state, subgroup 3.1, which is related to IRGC 105491, was the predominant type of black-hulled red rice in the southwestern part of the rice producing region and in Bowie County in northeast Texas, The founding member of subgroup 3.2, TX4, was collected on the west side of Harris County near Katy, Texas. As might be expected, subgroup 3.2 was also found in nearby rice producing areas in Waller and Fort Bend Counties. However, this type of black black-hulled red rice was also found in Colorado County and on the east side of the Texas rice belt in Chambers, Liberty and Jefferson Counties as well as in Bowie County in northeast Texas. The wide distribution of subgroup 3.2 may be particularly significant since the founding member of this group, TX4, has substantial natural tolerance to the herbicide glufosinate (Noldin, 1999b).

Straw-hulled red rice was predominantly in Group 1 (Figure 4-6). This type of red rice was found in all counties examined except Chambers. While straw-hulled red rice was found in Waller County (Table 4-1), none of the Waller County straw-hulled samples were analyzed with DNA markers and thus it does not appear in Figure 4-9. Group 2, which appears to represent a natural mixture of groups 1 and 3, was present in six different counties including both the east and west side of the Texas rice belt and Bowie County in northeast Texas. However, no evidence was found for natural crossing between these types of red rice in Fort Bend, Wharton, Matagorda, and Jefferson Counties despite the close proximity of group 1 with subgroup 3.1 or subgroup 3.2.

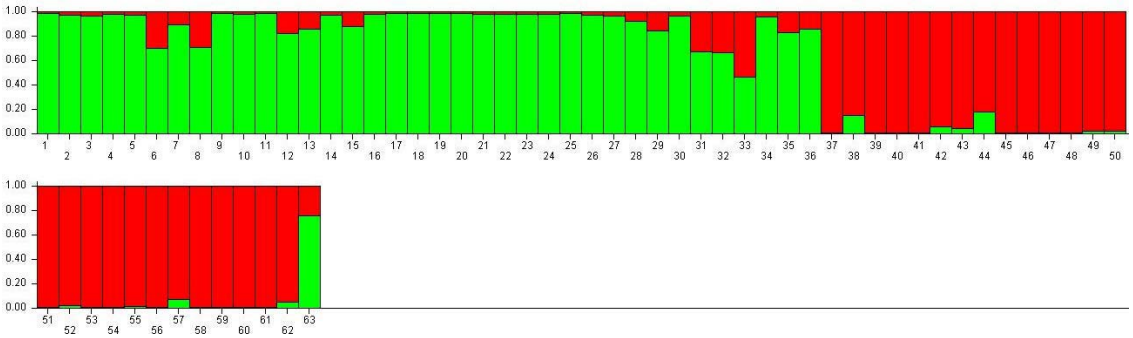


Figure 4-8. STRUCTURE analysis of MDS group 3. Samples are in the same order as Table 4-9 and are grouped based on the haplotype chart.

Table 4-9. Sample list for Figure 4-8.

Structure ID	Variety	Structure ID	Variety
1	BC01	33	Matagorda Cnty #25
2	Chambers Cnty #01-10	34	Matagorda Cnty #26
3	Fort Bend Cnty #28	35	IRGC 105491
4	Fort Bend Cnty #28-2	36	TX2
5	Liberty Cnty #08-37	37	Bowie Cnty #01-13
6	Waller Cnty #4	38	Chambers Cnty #01-27
7	Waller Cnty #5	39	Chambers Cnty #01-34
8	Wharton Cnty #12	40	Fort Bend Cnty #01-02
9	BC04	41	Fort Bend Cnty #27-1
10	BC05	42	Fort Bend Cnty #27-3
11	BC06	43	Jefferson Cnty #01-19
12	Colorado Cnty #18	44	Jefferson Cnty #01-44
13	Colorado Cnty #22	45	Liberty Cnty #01-31
14	Colorado Cnty #01-21	46	Liberty Cnty #01-35
15	Colorado Cnty #01-27	47	Liberty Cnty #01-37
16	Colorado Cnty #01-42	48	Liberty Cnty #01-40
17	Colorado Cnty #01-45	49	Liberty Cnty #01-44
18	Matagorda Cnty #01-30	50	Liberty Cnty #01-45
19	Wharton Cnty #13	51	Liberty Cnty #02-23
20	Wharton Cnty #14	52	Liberty Cnty #03-07
21	Wharton Cnty #17	53	Liberty Cnty #03-16
22	Wharton Cnty #23	54	Liberty Cnty #03-19
23	Bowie Cnty #01-19	55	Liberty Cnty #06-01
24	Matagorda Cnty #01-27	56	Liberty Cnty #06-05
25	Matagorda Cnty #01-28	57	Liberty Cnty #06-41
26	Matagorda Cnty #01-50	58	Liberty Cnty #07-23
27	Wharton Cnty #01-01	59	Liberty Cnty #12-20
28	Bowie Cnty #01-05	60	TX4 (Texas-4)
29	Bowie Cnty #01-18	61	Waller Cnty #10
30	Colorado Cnty #01-50	62	Waller Cnty #3
31	Jefferson Cnty #623	63	Colorado Cnty #01-23
32	Matagorda Cnty #01-24		

Table 4-10. Summary of STRUCTURE results for Figure 4-8.

K	Ln P(D)	Var Ln P(D)	α1
1	-1526.8	20.1	
1	-1526.8	20.1	
1	-1526.8	20.1	
1	-1526.8	20.2	
2	-1230.2	55.0	0.1134
2	-1230.1	54.9	0.1128
2	-1229.8	54.3	0.1147
2	-1230.5	55.4	0.1115
3	-1112.6	81.6	0.0851
3	-1106.1	79.4	0.0754
3	-1126.4	76.8	0.0878
3	-1106.3	79.5	0.0726
4	-1000.0	88.7	0.0730
4	-1010.8	89.4	0.0594
4	-1010.8	90.3	0.0617
4	-1001.7	91.4	0.0739

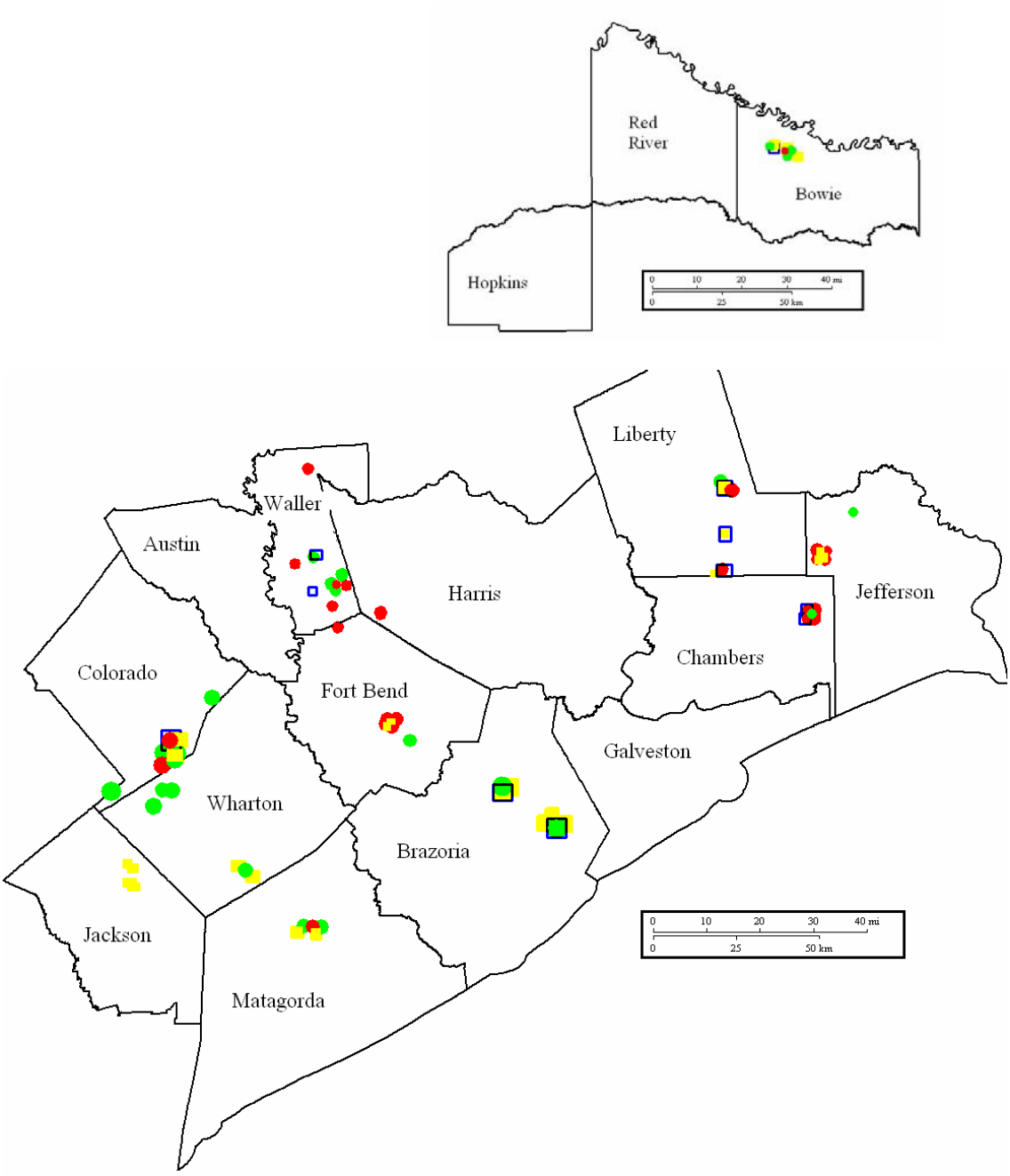


Figure 4-9. Geographic distribution of samples collected in Texas. The groups, to which samples belong based on MDS and STRUCTURE analysis, are indicated by the different colors. Yellow indicates group 1, blue open squares indicate group 2, green subgroup 3.1 and red subgroup 3.2. GPS coordinates were used to plot the location with the program MARPLOT.

Conclusion

The data presented here clearly demonstrates that red rice is not one single generic entity as previously thought, but instead consists of multiple species and subgroups with distinct geographical distributions. Most of the straw-hulled samples from across the rice production area of Texas were in group 1. This type of red rice is closely related to *Oryza sativa* ssp. *indica* in agreement with the traditional classification of red rice. However, black-hulled red rice that is closely related to IRGC 105496 and TX4 (subgroups 3.1 and 3.2 respectively) was also found to be widely distributed across the rice production area of Texas. As discussed in Chapter III, this red rice is most properly classified as *Oryza nivara*. A new type of black-hulled red rice that appears to represent an intermediate type of *Oryza sativa* ssp. *indica* and *Oryza nivara* was also found in six different counties. Whether this group represents progeny of crosses that occurred in Texas rice fields or it represents an independent group that was imported to Texas as a seed contaminant is unknown. Most of this type of red rice also had black hulls. While brown or gold-hulled red rice are abundant in several Texas counties, many of these were *Oryza nivara* rather than the apparent mixture of *Oryza nivara* and *Oryza sativa* ssp. *indica*.

As seen in Chapter II, a single field can contain multiple types of red rice, even within a single 9 m^2 collection area. However, both the distribution and diversity of red rice types differ substantially. The fields sampled in Jackson County, for example, contained only *Oryza sativa* ssp. *indica*-like straw-hulled red rice. The fields sampled in Chambers County, on the other hand, had *Oryza nivara* type red rice closely related to both IRGC 105491 and TX4 (subgroups 3.1 and 3.2) as well as the apparent *Oryza nivara*/*Oryza sativa* ssp. *indica* mixture (group 2), but did not contain any unmixed *Oryza sativa* ssp. *indica* (group 1).

The eastern part of the Texas rice belt, including Liberty County, has been in rice production for the past century; whereas the southwestern portion, including Brazoria County, has been in rice production only since World War II. It was expected that there would be a greater diversity among red rice samples from the areas which had been under cultivation for a longer period of time. Surprisingly, Liberty County had less diversity than did some of the other counties. This can be seen clearly for example, in group 3 of Figure 4.4 B, where most of the Liberty County samples are tightly clustered, but the Brazoria County samples are more dispersed. These samples also illustrate the difference in the type of black-hulled red rice that is the predominant type in the eastern versus the western part of the rice producing area of Texas. These differences may be due in part to cultural differences between the two areas. It may also represent the local history of importation of specific red rice ecotypes as contaminants in seed and its accidental movement from field to field. Regardless of the origin of the different types, it is clear that red rice is much more diverse than previously thought and that this diversity should be taken into consideration when investigating red rice control.

One issue in particular that needs to be addressed in future work is the distribution of red rice that is naturally tolerant to herbicides that scheduled for use in red rice control. The red rice ecotype TX4 was originally isolated in western Harris County near Katy, TX (Noldin et al. 1999a). As originally noted by Noldin et al. (1999b) and discussed further in Chapter V, this red rice ecotype has substantial natural tolerance to the herbicide glufosinate that is scheduled for use with the herbicide resistant Libery-Link^{TM} commercial rice varieties. Thus, an important question addressed during this study was to determine if TX4 was a rare ecotype that is limited to only one small area or whether it was abundant and wide spread. Of the approximately 250 samples analyzed 23 samples were found to be identical to TX4 with the DNA markers tested.

These were present in six different counties (Chambers, Colorado, Fort Bend, Jefferson, Liberty and Matagorda). Most other counties, including Bowie County in northeast Texas contained samples very closely related to TX4 (subgroup 3.2). Whether any of these other samples also have substantial levels of natural herbicide tolerance is currently under investigation. These ongoing investigations also include other subgroup of *Oryza nivara* (the IRGC 105491-like subgroup 3.1) since it is also widely distributed and has a high degree of allelic diversity.

CHAPTER V

CHARACTERIZATION OF NATURALLY OCCURRING GLUFOSINATE TOLERANCE IN RED RICE

Introduction

Herbicide resistance genes provide new opportunities for the control of weeds in cultivated crops. They are particularly advantageous in crops with closely related weeds that can not be controlled by conventional methods without damaging the crop. However, herbicide resistance genes also have limitations including public distrust of GM foods (Marchant and Marchant 1999) and the possibility of the creation of herbicide resistant weeds.

Despite these concerns, there has been a great deal of interest in using herbicide resistant rice varieties as a way to control the weed red rice in production fields. As discussed in previous chapters, red rice competes for light and nutrients and also contaminates the commercial crop, lowering its quality. However, since red rice is very closely related to cultivated rice it is difficult, if not impossible, to control by conventional means.

The CLEARFIELD^{TM} herbicide resistant rice currently in use in the US is non-GM. This herbicide resistant rice was created by selecting for resistance to imazethapyr, an inhibitor of acetolactate synthetase (ALS). Approximately 200,000 acres of CLEARFIELD^{TM} rice are currently in production (http://www.clearfieldsystem.com). While this rice does not have the

stigma of having the GM label, it still has the very real problem of herbicide resistant red rice developing.

One of the best methods of reducing the development of herbicide resistant weeds is to use two or more herbicide resistance genes with different mechanisms of action (Gealy et al. 2003). Thus, it would be useful to develop rice varieties with additional herbicide resistance genes that could be used as part of an integrated red rice management techniques in conjunction with the ALS inhibitor resistant CLEARFIELD™ varieties.

Liberty-Link™ herbicide resistant rice has been developed by the Bayer Corporation. These rice varieties are part of a series of Liberty-Link™ GM crops that contain the bacterial bialaphos resistance gene (BAR). This gene provides resistance to the glutamine synthetase inhibitor, glufosinate (phosphinothricin or PPT) which is the active ingredient in the commercial herbicide, Liberty™. The product of BAR, phosphinothricin N-acetyltransferase, detoxifies this herbicide by catalyzing the acetylation of the amino group (Droge-Laser et al. 1994). The BAR gene has been used in numerous GM crops, including cotton, corn and soybeans both as a source of herbicide resistance and is also used as a selectable marker during plant transformation (Giri and Laxmi 2000). Liberty-Link™ rice is currently in advanced field trials and has been approved for US markets. However, its widespread release is uncertain.

In addition to issues involved in public distrust of GM foods, another key issue for Liberty-Link™ herbicide resistant rice is that some ecotypes of red rice may already have a significant degree of natural tolerance to glufosinate. A naturally glufosinate red rice ecotype, TX4 was first identified in 1999 (Noldin et al. 1999a, 1999b). In the original study, 19 red rice ecotypes

collected from Arkansas, Louisiana, Mississippi and Texas were screened under greenhouse conditions. All of the ecotypes with the exception of TX4 were effectively controlled (>93% "control") with a single application of 0.56 kg ai ha^{-1} glufosinate. However, only 46% control was obtained with TX4.

Since the original study, the level of glufosinate tolerance in TX4 has been investigated by several groups and has been the subject of controversy. Some researchers have claimed that TX4 does not have enough tolerance to be a significant issue for weed control, while others have reported that TX4 has significant tolerance to glufosinate

It is important to note than in several of these studies, evaluation of the degree of glufosinate tolerance in TX4 was not the primary objective. In the studies by Wheeler et al. (2000 and 2001), for example, the primary objective was to investigate the transfer of the BAR gene from transgenic rice to red rice. Three transgenic rice varieties containing the BAR gene were surrounded by red rice ecotypes that matured at different rates. One of the red rice ecotypes used was TX4. The red rice plants in these experiments were allowed to undergo a normal reproduction cycle in the field and 100 seed from each plant was then grown in a greenhouse and assayed for glufosinate tolerance at the 1-3 leaf stage.

In both studies, offspring from the TX4 plants showed significantly higher amounts of tolerance than did any of the other red rice ecotypes tested; with levels of 12-27% resistance with a rate of 0.42 kg/ha. For comparison, note that the maximum herbicide rate on the proposed label for the use of glufosinate in rice is 0.45 kg ha^{-1} (J.M. Chandler, personal communication). With a rate of 2.24 kg ha^{-1}, TX4 still had 8.1% survivors (Wheeler et al. 2001).

These studies attributed the high levels of tolerance to outcrossing with the transgenic BAR rice cultivars; even though the Wheeler et al. (2001) did not find the BAR gene in any of the surviving TX4 progeny. The fact that none of the TX4 progeny contained the BAR gene is not surprising given that TX4 typically matures later than commercial rice varieties (Noldin et al 1999) and was thus unlikely to sexually cross with the BAR-containing test varieties used in this study. Rhetoric from other papers from this group would also indicate that the chance of outcrossing is extremely low, less than 0.5% (e.g. Gealy et al. 2003). Perhaps because the primary objective of this work was to ascertain the amount of outcrossing between transgenic rice and red rice, rather than to examine TX4, the TX4 data was essentially ignored.

A 1998 publication from the same group claims that TX4 is not a problem since none of the glufosinate treated plants survived long enough to produce seed (Gealy and Black, 1998). The reason for this is not likely due to the glufosinate treatment since TX4 produced 40% as much dry weight as untreated controls, but instead due to the authors burning the field before TX4 had gone to seed. In other studies from this group, the amount of TX4 surviving herbicide application has ranged from 0-44% (Gealy and Black 1998; Gealy et al. 2000). Very few of these studies involving TX4 have been published in peer-reviewed journals. While a number of studies on herbicide tolerance in red rice and effectiveness of glufosinate in transgenic BAR cultivars have been published in peer-reviewed journals (Sankula et al. 1997a; Sankula et al. 1997b; Zhang et al. 2003), they have not included TX4.

Direct comparison between the various studies that have been published on TX4 is difficult. As detailed in Chapter I, environmental conditions can have a dramatic effect on the effectiveness of the glufosinate. However, the environmental conditions in most of the studies are not described.

Plants were also treated at different growth stages, different herbicide rates were used, and different parameters were measured.

The primary objective of the current study was to determine whether TX4 actually has a biologically a meaningful level of tolerance to glufosinate. This is a key issue for two reasons. First, TX4 and related red rice might survive herbicide treatment, making weed control less effective. Even if the proportion of herbicide resistant rice was low initially, it would likely rapidly increase in response to selection pressure unless appropriate actions were taken.

Secondly, there is also a possibility that red rice which survived herbicide treatment might sexually cross with commercial Liberty-Link™ rice varieties. Since red rice falls to the ground when mature and can remain dormant in the soil for many years, this could result in fields essentially permanently contaminated with GM red rice that contains the bacterial BAR gene.

At noted above, the TX4 sample collected by Noldin (1999a) matures later than most commercial rice varieties and is thus unlikely to cross with Liberty-Link™ rice varieties. However, as indicated in Chapter IV, 23 red rice samples that are identical with TX4 with DNA markers tested were found among red rice samples from across Texas that matured at the same time as predominant commercial rice varieties. Hybridization levels of more than 50% between commercial rice varieties and some ecotypes of red rice have been documented in field trails (Langevin et al. 1990). It was also found that crosses could be easily made between TX4 and commercial rice varieties and gave good seed set (A. McClung, personal communication).

Materials and Methods

Field Experiments

Field experiments were conducted both for determination of herbicide resistance and to increase important seed sources. The test plots were managed in the same way as commercial rice grown in south east Texas. An early pre-emerge herbicide application was typically made with 0.3 lb of clomazone, 0.3 lb of quinclorac and 1.5 lb propanil per acre After two days, the plot was flushed. Three weeks later, the plot was sprayed again with 2 quarts of molinate/propanil and 1.6 oz of Londax™ per acre. After 30 lbs/acre of 46-0-0 fertilizer was applied, the plots were flooded and remained in this way until the completion of the study. Upon completion, plants were collected for further use or the plots were destroyed. All field experiments were carried out with the assistance of Brian Ottis, members of Dr Chandler's lab, and the staff of the Texas A&M Agricultural Experiment Station in Beaumont, Texas.

Greenhouse Experiments

Seeds were planted in rice mix soil (equal parts peat moss and vermiculite supplemented with micronutrients) at a constant depth of 1 inch. Three to four seeds were planted per chamber in six-pack plant growth chambers with six six-packs per flat. The flats were watered with a 1% solution of a 20-20-20 NPK fertilizer and not watered again until the plants had emerged. After germination, flats were thinned to 1 seedling per chamber and were watered as needed to maintain moist soil. Plants were fertilized with 20-20-20 NPK, urea, or Osmocote as needed. Herbicide experiments were conducted in the late spring, summer and early fall to most closely

approximate the conditions in the field. Temperature was maintained at 30-32C in the day and 26-28C at night with 14 hours of dark per 24 hour period.

Plants were grown to various growth stages as discussed below and then treated with Liberty™ herbicide in a spray chamber with a volume of 187 L/ha through a flat fan spray nozzle. Herbicide was applied in two passes to achieve a total of of 0.62 kg ai ha^{-1} (1.25 x the label rate). Visual ratings of chlorosis, necrosis, leaf damage, and occurrence of new growth were taken weekly, with 10 indicating complete plant death and 0 indicating no injury when compared to untreated control plants. The final visual rating was taken at 4-6 weeks and plants were either destroyed or transferred to larger pots and allowed to set seed.

Growth Chamber Experiments

Growth chamber experiments were conducted as above, except plants were grown in a controlled environment growth chamber. Temperature and humidity were controlled at 27C and 80% humidity. Light levels were approximately 500 $\mu E/m^2/sec$ with a 16 hour day. Flats were rotated frequently to reduce any effect of position in the chamber.

Tissue Culture

The tissue culture based screen of herbicide resistance was based on the method of Toldi et al. (2001). Seeds were dehulled and surface sterilized with 70% ethanol for three minutes and then sterilized under vacuum in a solution of 50% commercial bleach for 30 minutes. Sterilized seeds were transferred to a laminar flow hood and washed five times with sterile water and then sealed

in sterile Petri dishes. The seeds were germinated at 27C (16hr light/8 hr dark) and the storage tissue of the seed removed from the embryo just after the root emerged with the use of a dissecting microscope.

The excised embryos were then placed on hormone free MS media (pH 5.8 with MS macro and micro elements, MS vitamins, 30g/L sucrose) and placed back in the incubator. Seeds were removed from the MS media when the coleoptiles had reached at least 5mm in length, turned green, and 1-2 roots had formed. These seedlings were placed in selection media, MS media (pH 5.8) with 0-10mg/L (active L isomer) filter sterilized glufosinate. Seedlings were returned to the 27C (16 hr light/8hr dark) incubator and remained under observation for at least four weeks.

Seedlings were monitored for phenotypic expression of herbicide resistance or susceptibility weekly. Color, degree of chlorosis or necrosis, relative size, number of leaves, degree of tillering or micro-shooting, and amount of root growth were recorded and a digital image of each plate was taken weekly. Examples were removed at each week and side by side comparison photos for all treatments were taken. For some experiments, a portion of the samples were also used to obtain fresh and dry weight at each stage. After 4-6 weeks samples were destroyed, used for fresh/dry weight, or transferred to the Magnta^{TM} GA7 vessels for eventual transfer to greenhouse.

Samples transferred to Magenta^{TM} vessels were grown on N6 media (N6 elements and vitamins, 30g/L sucrose, 2.5g/L Gelrite pH 5.8) without or with glufosinate (2mg/L) until they had reached sufficient size to be transferred to the greenhouse. Plants were removed from the media and transferred to rice mix. Plants were watered and fertilized with a 1% solution of 20-20-20 NPK

fertilizer and protected under a plastic humidity-dome until established. Once established, plants were treated as discussed above. A portion of the plants were used for herbicide resistance analysis. Those that survived herbicide treatment, as well as some untreated controls, were allowed to produce seed. These seed were used as in Toldi et al. (2001) to test for glufosinate tolerance in the progeny

Tetrazolium Red and Evan's Blue were used to test for viability and cell death in seeds and plant tissue. Samples were incubated with 0.5% tetrazolium red and/or 0.5% Evans Blue at 37C for 3-12 hours, excess dye was removed with a 70% ethanol wash and then samples were examined under the microscope. (Naredo et al. 1998; Ayala et al. 2002; Chen et al. 2004)

Results and Discussion

TX4 Herbicide Resistance- Field

Field studies are the least controlled method of evaluating herbicide tolerance, but provide data that is the most directly relevant to commercial practice. Thus, a preliminary field study was performed during the summer of 2000. In this experiment the collection of red rice ecotypes collected by Noldin (1999a) was sprayed twice with 0.62 kg ai ha^{-1} glufosinate. The first herbicide application was at the three to four leaf stage and the second was 10 days later, as proposed for commercial practice. As shown in Figure 5-1, more than 50% of the TX4 survived, while all of the other ecotypes tested were effectively controlled. It should be noted that this level of herbicide is 1.25X the maximum that will be allowed under the proposed label for the use of glufosinate in rice.

This study was planted later than commercial rice fields and the seedlings were thus exposed to higher temperatures and more intense light after herbicide application than would be typical in actual production. Higher temperature and light levels would be expected to increase the rate of both photosynthesis and photorespiration, and therefore increase the demand on the nitrogen assimilation pathway (see Chapter I). These conditions would be expected to make seedlings very susceptible to glufosinate. This expectation was met for all of the red rice ecotypes tested, except TX4. These results are consistent with the observation of Noldin et al. (1999b) and with the observations of most other investigators that TX4 is highly glufosinate tolerant under typical conditions.

This study was repeated in 2001. In 2001, essentially all of the red rice sprayed with glufosinate died, including TX4. However, it is difficult to interpret this data since the fields were submerged for several days after herbicide application due to massive flooding. Subsequent greenhouse experiments were undertaken to try to investigate the effects of complete submersion after herbicide treatment as a possible mechanism of red rice control. However in all cases, a large proportion of TX4 survived. Consequently, the reason that almost all of the TX4 was killed in the 2001 field experiments remains unknown.

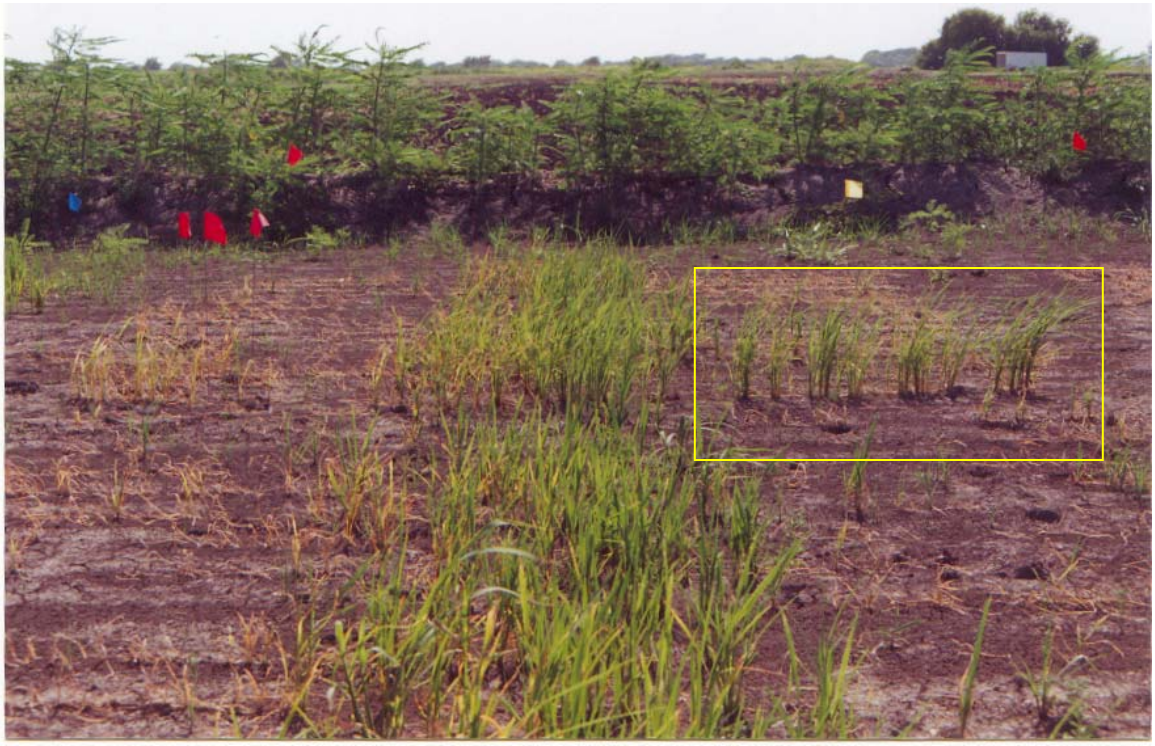


Figure 5-1. Surviving TX4 from 2000 field study. Plants were treated with two applications of 0.62kg ai ha-1 glufosinate 10 days apart, starting at the 3-4 leaf stage. Plants in the middle part of the plot are untreated controls. Surviving TX4 is indicated by the white box.

TX4 Herbicide Resistance- Greenhouse and Growth Chamber

The standard practice in weed science is to treat plants with herbicide and then to score the number of plants killed, or "controlled" 2-4 weeks later. A score of 100% indicates that the plants are completely dead, while a score of 0% indicates that the herbicide had no effect on the plant when compared to control, or untreated, plants (Sankula et al. 1997a, 1997b; Noldin et al. 1999b). The investigator then uses his or her own personal judgment to determine the cut-off

score at which plants are considered to be “controlled” or to have survived the herbicide treatment.

There are several points in this process that could lead to differences between one report and another. The most obvious is the personal interpretation of the scoring. One person’s cut-off may be a score of 40% while another person’s cut-off might be 50%. In the current study, two individuals independently scored the plants and any discrepancies between the scoring were addressed in an attempt to decrease the subjectivity.

The amount of time that has elapsed between treatment and scoring can also affect the scores that individual plants receive. Typical experiments evaluate the number of plants “controlled” after 1-2 weeks of application. This is particularly an issue for TX4 since it typically shows moderate to severe damage after herbicide application. However, unlike other red rice ecotypes, damaged TX4 often survives, resprouts, and will go on to produce seed (Figure 5-2).

To avoid the subjective nature of visual screens some authors have measured the rate of photosynthesis, transpiration, and relative chlorophyll content (Gealy and Black 1998). While this does provide a more accurate measure of the physiological state of the plant, the parameters were only measured for 120 hours. This is well short of the time at which TX4 begins to recover from the detrimental effects of the herbicide (Noldin et al. 1999b).

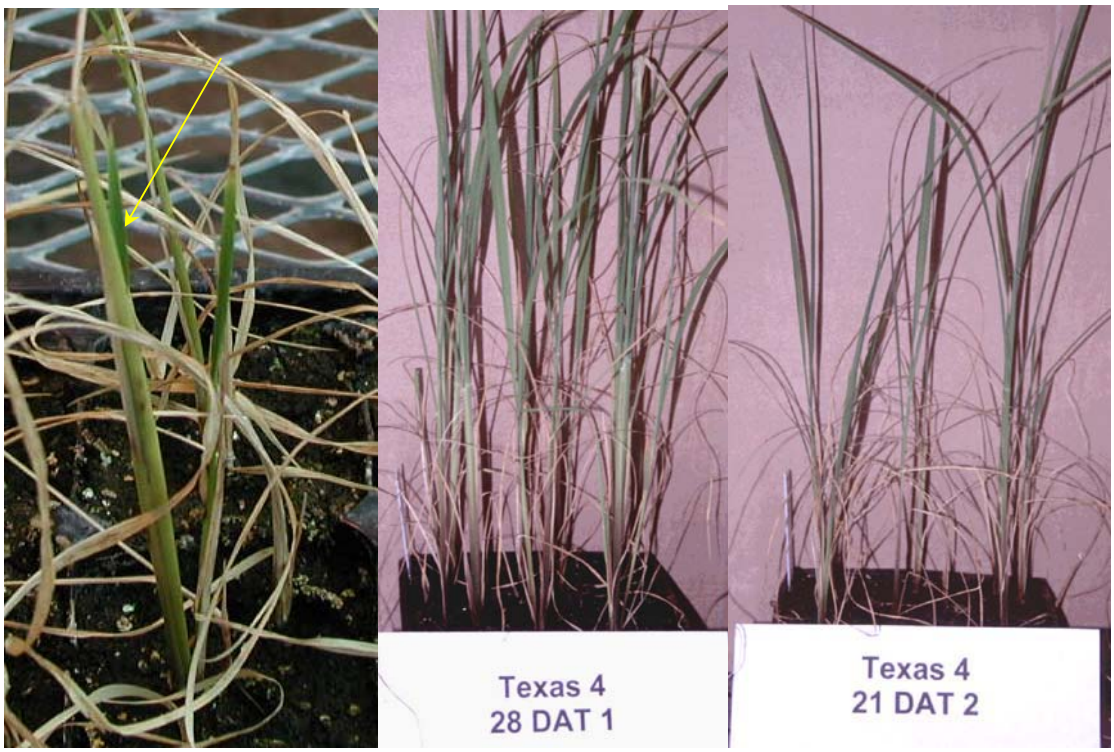


Figure 5-2. Glufosinate treated TX4. Plants treated with of 0.62 kg ai/ ha glufosinate. A. Representative plant 2 days after treatment (DAT) showing typical damage after glufosinate treatment. Notice extensive leaf burn, with new leaf emerging from stem (indicated by arrow). B. TX4 4 weeks after a single application. C. TX4, 3 weeks after the second herbicide application (10 days after the first). The plant from part A would be expected to grow and produce plants those shown in B and C. Note that the chlorite leaves at the base of the plants in B and C, would correspond to the chlorotic leaves in part A.

Since TX4 will often recover from initial damage from glufosinate, traditional visual screens for herbicide damage typically yield the paradoxical result that the amount of "control" decreases over time. However, the most important issue for red rice control is not the degree of initial damage after herbicide treatment. Rather, it is whether the plants actually die or whether they can recover, resprout and go on to set seed. With this in mind, plants were scored for potential viability. This was typically done 4 weeks after treatment to allow surviving plants a chance to recover and to begin to resprout. On this scale, 0 = dead and 10 indicates that the plants look like untreated controls.

In much of this work, plants were sprayed with a two application of 0.62 kg ai ha^{-1}. This corresponds to the maximum number of herbicide applications that will be allowed under the proposed label for use of glufosinate in rice and 1.25 X the maximum rate that will be allowed (0.45 kg ai ha^{-1}). Even under these stringent conditions, 50-100 % of TX4 routinely survived. An example of typical results is shown in Figure 5-3. Development is usually delayed, but surviving plants produced large amounts of seed. In some greenhouse studies, we have found a few (1%) survivors even after treating TX4 with glufosinate at 2.24 kg ai ha^{-1} (5X the proposed maximum label rate).

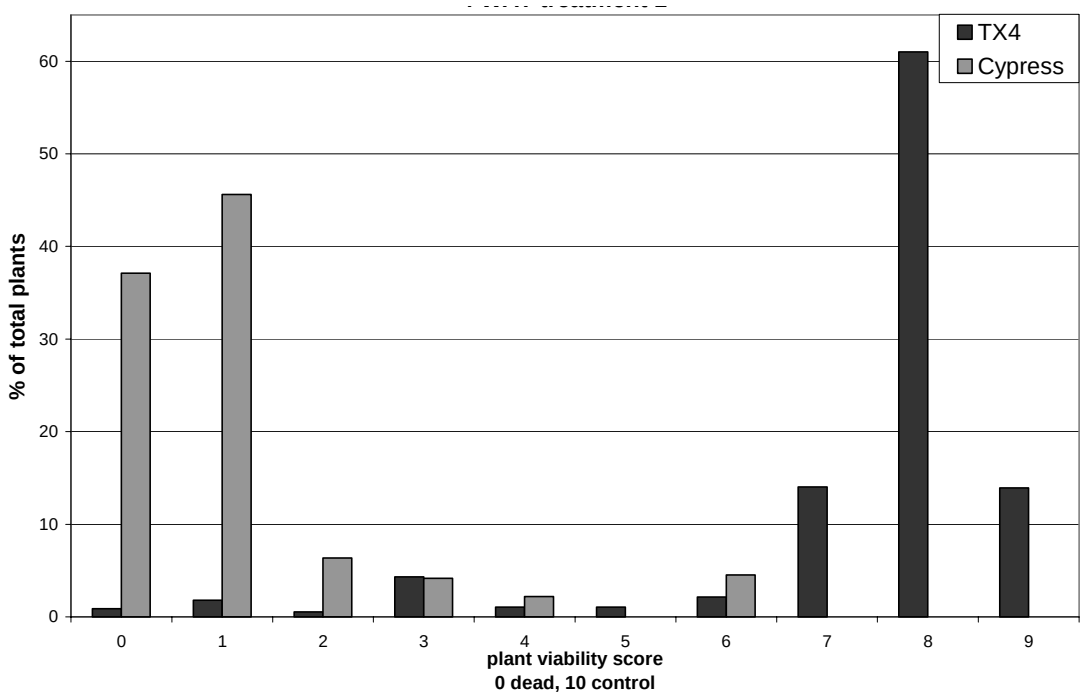


Figure 5-3. Typical greenhouse survival ratings for Cypress and TX4 after two applications of 0.62 kg ai ha^{-1} glufosinate. Plants were sprayed initially at the 3-4 leaf stage and sprayed again 10 days later. The plants were scored 4 weeks after the first herbicide application. A score of 0 indicates a dead plant and a score of 10 indistinguishable from untreated controls.

The typical results shown in Figure 5-3 are consistent with survival of TX4 under similar conditions in the 2000 field study described above. However, it is important to note that there was a large variation. Within the experiment depicted above the percentage of plants with a score of 8 varied from 51% to 73% in the four different replications of 50 plants each. Other experiments, under what were expected to be comparable conditions, gave completely different distribution of plant scores. In some experiments the differences between the two were less

extreme than in Figure 5-3 (Figure 5-4A), although the majority of TX4 will survive and the majority of Cypress will not. Figure 5-4B represents a dual problem, not only has TX4 decreased in tolerance, but Cypress has increased. The results of these studies are quite perplexing. In retrospect some of the variation can be attributed to factors such as old light bulbs (decreased light levels) and vents not completely open. The variation in the different studies does reveal the complexity of the glufosinate interaction with the plant system, and illustrates that there are many factors that we do not understand.

Variability has been the greatest challenge faced in the work with TX4 and glufosinate. As we moved from preliminary observations to replicated studies, it became obvious that there was substantial variation in the amount of leaf damage and even in survival among different replications of the same sample.

Initially it was thought that this was simply due to experimental error in herbicide application. However, variable results were also seen when herbicide was applied using a carefully calibrated spray chamber in the Department of Soil and Crop Sciences. Some of the variability was found to be due to the growth stage (Table 5-1). As discussed in Chapter VI, this difference in glufosinate tolerance may be related to the differences in GS1 isoforms found in seedlings at the 1-2 vs. 3-4 leaf stage.

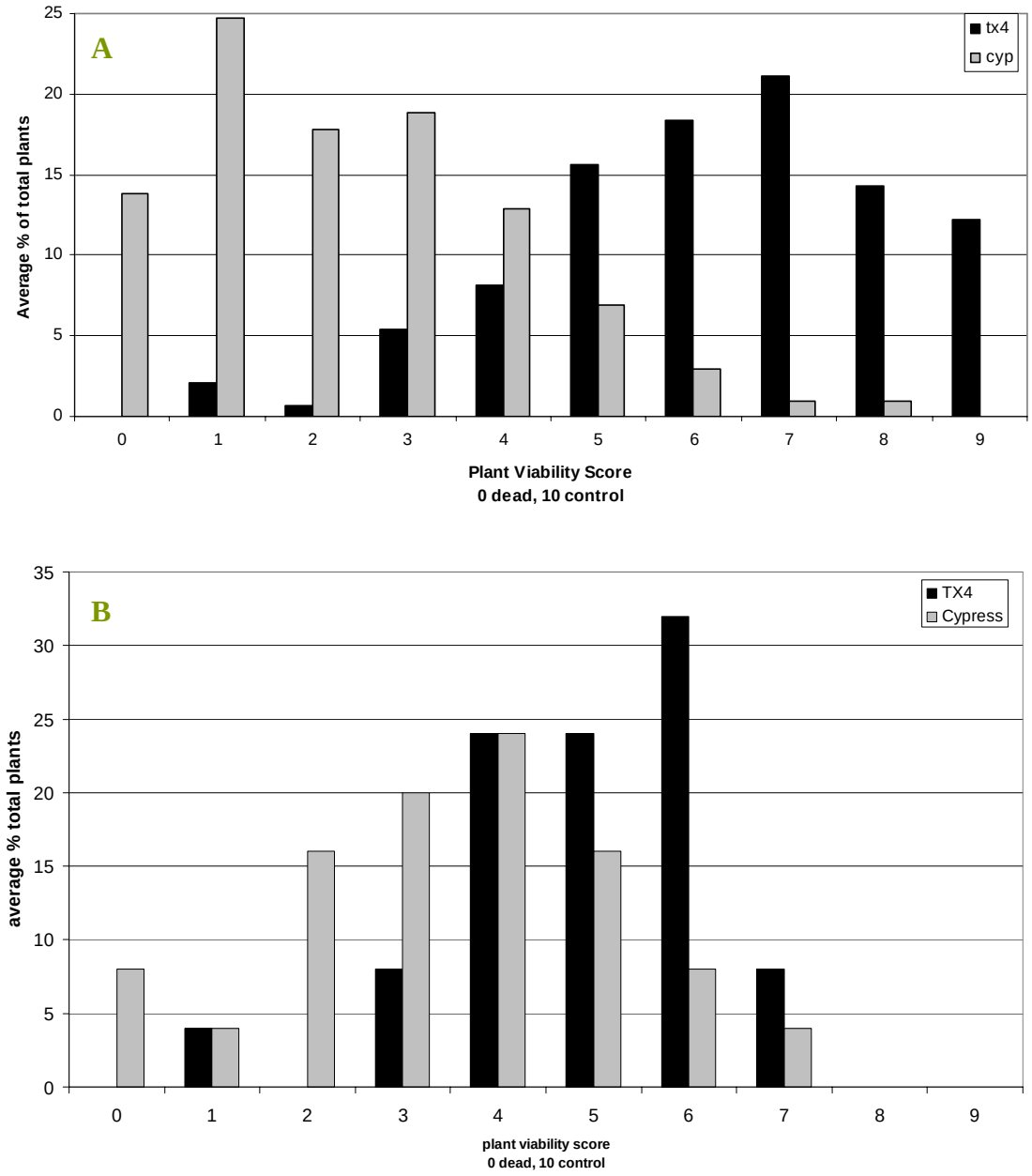


Figure 5-4. Variation in results for glufosinate treatment of TX4 and Cypress. A. Less extreme results than Figure 5-3. B. Results to the opposite extreme for Cypress viability and TX4 susceptibility. Glufosinate treatments were the same as in Figure 5-3.

Table 5-1 Growth stage dependent differential response to glufosinate.

Sample	Average score
TX4 1.5 leaf	6.88
TX4 3 leaf	5.00

In an attempt to control variability experimental procedures were vigorously standardized, including planting seedling at precisely the same depth and rotating flats in the greenhouse and growth chamber to minimize position effects. This reduced, but did not eliminate variability.

Other factors that may affect response to herbicide include the environmental conditions during before and after herbicide application. As discussed in Chapter I, glufosinate failed to control a normally sensitive form of red rice in a Liberty-Link™ field in 1998 whereas the next year the same type of red rice was efficiently controlled. Analysis of the light levels and temperature for the two different years indicates a strong correlation between lack of control with low temperature and light levels (Steele 2000).

To determine whether this environmental effect could be replicated in the greenhouse, TX4 and the cultivar Jefferson were grown side-by-side with some plants protected by a shade cloth. As expected, plants exposed to lower light levels showed much less damage in response to glufosinate symptoms than those exposed to higher light levels Figure 5-5. This light effect may also explain some of the variability in the greenhouse as well as in growth chamber studies since light bulbs lose intensity over a relatively short period of time.

Figure 5-5 A

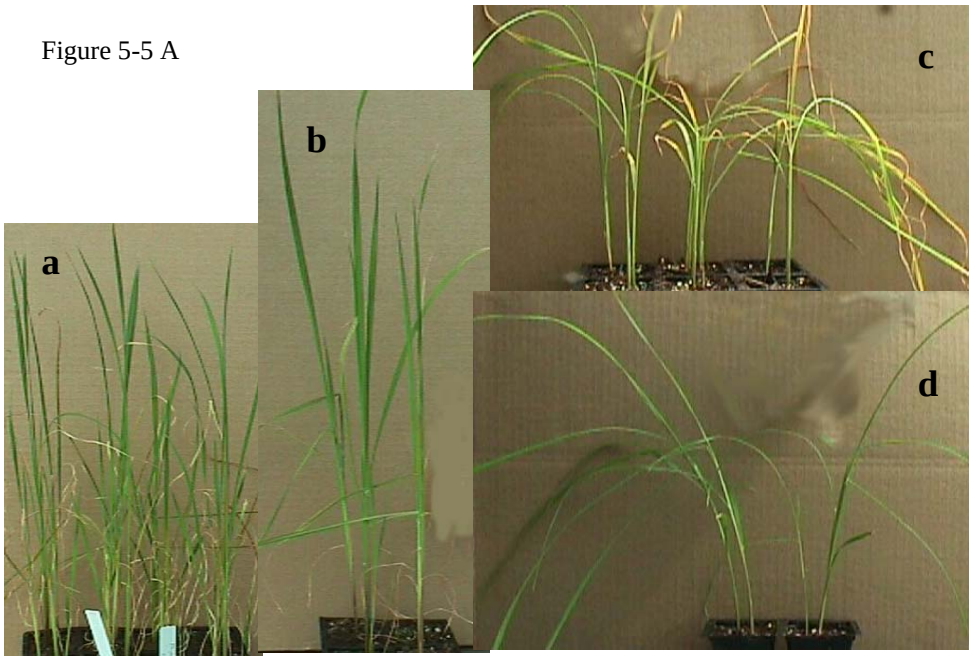


Figure 5-5 B

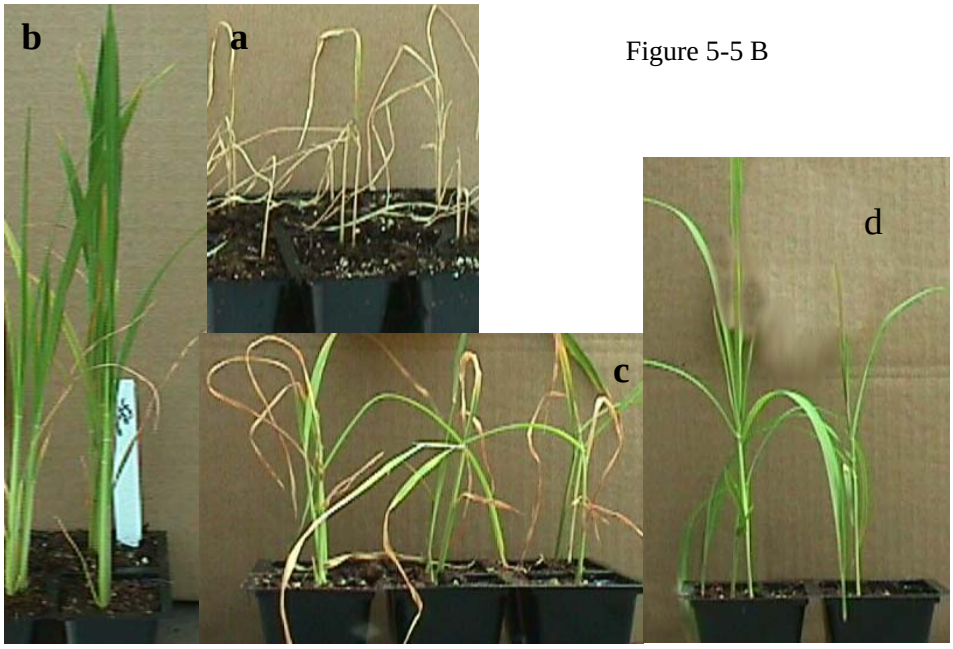


Figure 5-5. TX4 and Jefferson low and high light PPT treated and controls. A. TX4 B. Jefferson. Plants were grown side-by-side in the greenhouse with low light plants under a shade cloth. Plants were grown to 3-4 leaf and treated with 0.62 kg ai ha-1 glufosinate in spray chamber. Pictures are 4WAT (Weeks after Treatment) a. High light treated. b. High light control (not treated). c. Low light treated. d. Low light control.

Even after the rigorous standardization of greenhouse and growth chamber experiments substantial variation was still observed. Among the possible causes for this variation is the fact that plants grown in the greenhouse and growth chamber had to be transported across campus to another facility to be sprayed. A short exposure to stressful conditions may have been enough to alter a plants response. Something as simple as completely opening vents in a growth chamber, as opposed to leaving them half open, was to be enough to push the plants into photorespiratory conditions that favored abnormally high levels of survival even in sensitive varieties (data not shown).

Glufosinate Tolerance in Tissue Culture

In this assay system, Cypress exhibited only 10% survival at 2 mg/L PPT (Figure 5-6 and Figure 5-7). However, TX4 showed much higher levels of glufosinate tolerance than cultivars or other red rice ecotypes. It showed vigorous growth up to 4 mg/l glufosinate and survived to over 8 mg/L (Figure 5-6 and Figure 5-8). Other cultivars such as the Chinese *Oryza sativa* cultivar TP309 had intermediate levels of tolerance (Figure 5-6).

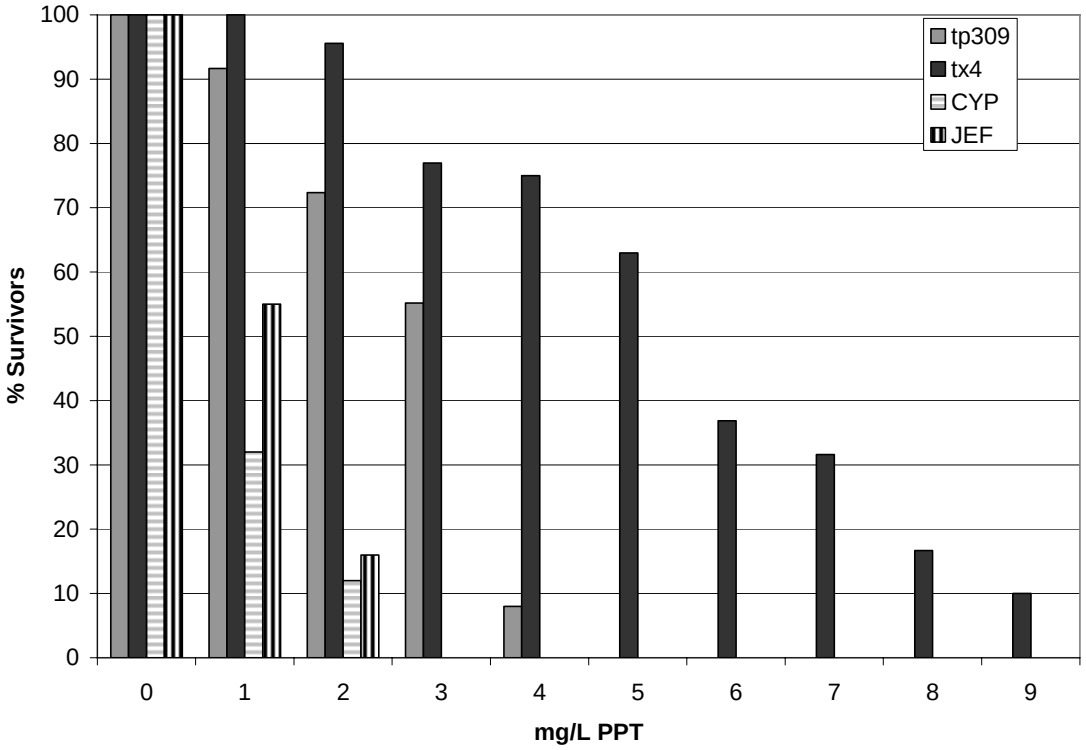


Figure 5-6. Tissue culture PPT survival curve. Plants were selected with PPT as described in material and methods. Percent survivors at each level of PPT are indicated. TP309 in solid gray, TX in solid black and Cypress in dashed gray. Each column represents the average of approximately 50 plants. In an effort to overcome the variability seen in greenhouse and growth chamber experiments and the logistic problems of field studies, a tissue culture assay was developed for glufosinate tolerance. To avoid potential complications with seed reserves, germinating embryos were excised from the residual seed and then cultured in on MS media without hormones.

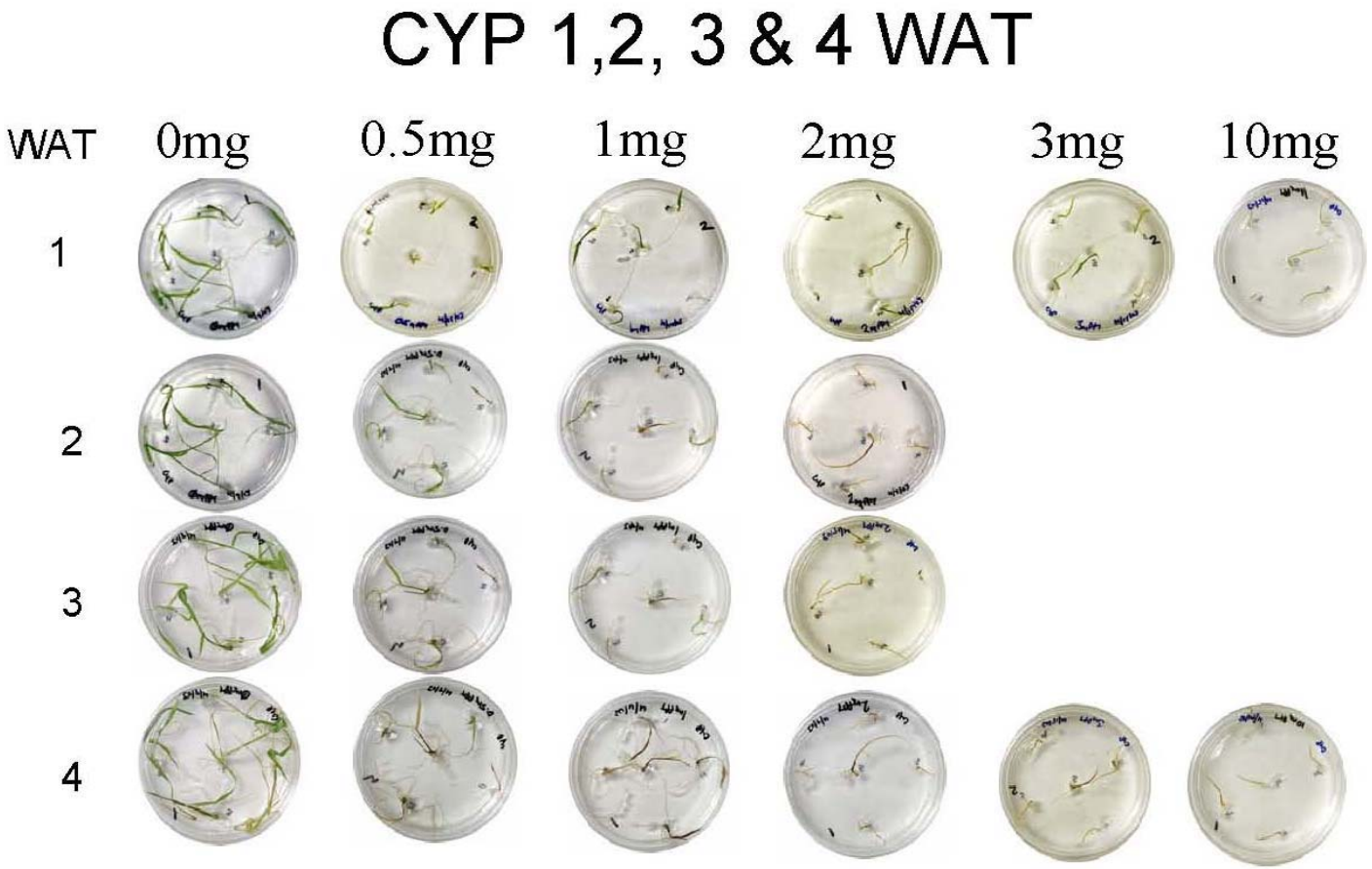


Figure 5-7. Dose response of Cypress to glufosinate in tissue culture. Media was supplemented with the concentration of glufosinate indicated. Photograph was taken after 6 weeks in culture.

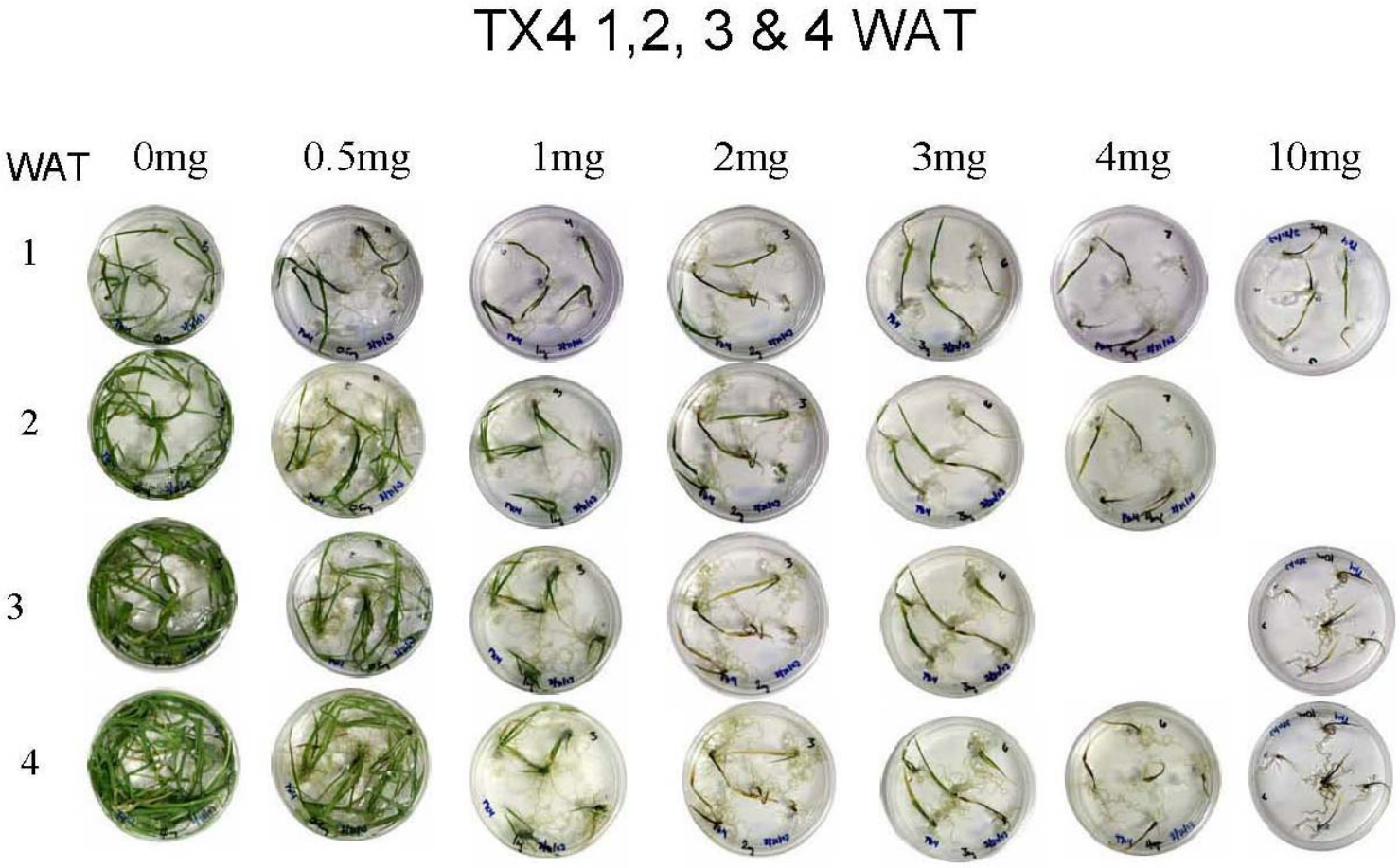


Figure 5-8. Dose response of TX4 to glufosinate in tissue culture. Media was supplemented with the concentration of glufosinate indicated. Photograph was taken after 6 weeks in culture.

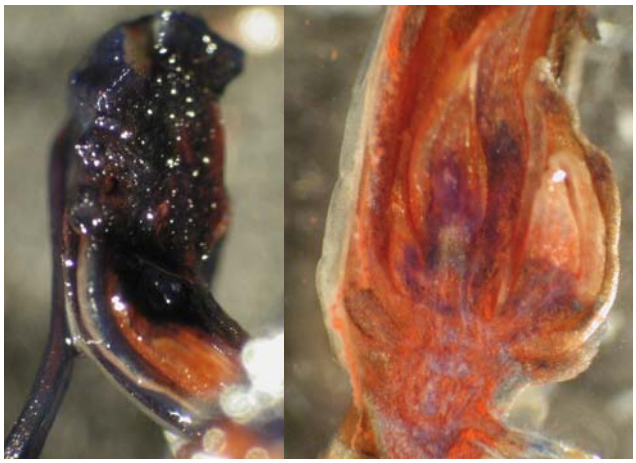
Other red rice and cultivars were also tested for glufosinate tolerance (Table 5-2). These samples showed a range of tolerance, although none exhibited the same high levels of tolerance seen in TX4. Most notable were the red rice ecotypes MS2, MS3 and LA2, which all have survivors at or above 3mg/L PPT. The variety MS4, which had been reported to have an intermediate level of glufosinate tolerance between typical cultivars and TX4 (Gealy et al. 2000) also had an intermediate level of tolerance in the tissue culture assay, with 20% survival at 4 mg/L glufosinate.

As has been noted earlier, TX4 is damaged by the application of herbicide, but the plants are able to recover from the severe chlorosis and necrosis and produce new leaves (Figure 5-2). To directly investigate the extent of meristem damage in TX4 versus other sensitive varieties we utilized the dyes tetrazolium red and Evan's blue. The tetrazolium red dye stains living issue a bright red, while the Evan's blue dye binds only to dead tissue, coloring that tissue a dark blue (Ayala et al. 2002; Chen et al. 2004). In TX4, when a majority of the leaf tissue was dead (stained blue) the meristem remained alive (stained red). In contrast, the meristem of Cypress was dead, as illustrated in Figure 5-9.

The tissue culture assay provided the most rigorously controlled conditions. Although the tissue culture assay was consistently more reproducible than any of the other methods, it still demonstrated some levels of variability. An example of this variability can bee seen in Figure 5-10. Seeds from the same source, prepared and germinated together, and plated on media from the same batch sometimes have a uniform, but vastly different response to the herbicide treatment in the two separate plates. This variability was effectively reduced by the large number of plants (several thousand) and the large number of replications assayed.

Table 5-2. Percent survival of different types of red rice from Noldin et al. 1999a on tissue culture based PPT screen. ND indicates no data available, usually from contaminated plants.

mg/L PPT	MS 1	MS 2	MS 3	MS 4	MS 5	MS 6	LA 1	LA 2	LA 5	AR 1	AR 4	TX 3	TX 4	CY P
0	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1	89	89	94	83	12	11	76	100	100	57	93	100	100	32
2	11	90	34	55	0	9	5	100	56	0	20	0	82	12
3	ND	73	18	32	0	9	0	50	0	0	0	0	62	0
4	0	10	19	23	0	0	0	ND	0	0	0	ND	48	0
5	0	0	10	ND	ND	0	0	ND	0	ND	ND	0	51	0
6	0	3	0	ND	ND	0	0	0	0	0	ND	0	22	0
8	0	0	0	ND	ND	0	0	0	0	ND	ND	0	12	0
10	0	0	0	ND	ND	0	0	0	0	0	ND	0	0	0



A. Cypress B. TX4

Figure 5-9. Viability stain of TX4 and Cypress 4 weeks after treatment. The dark blue stain on the Cypress meristem indicates that the cells were dead, while the red stain for TX4 indicates the meristem cells were viable at the time of staining.

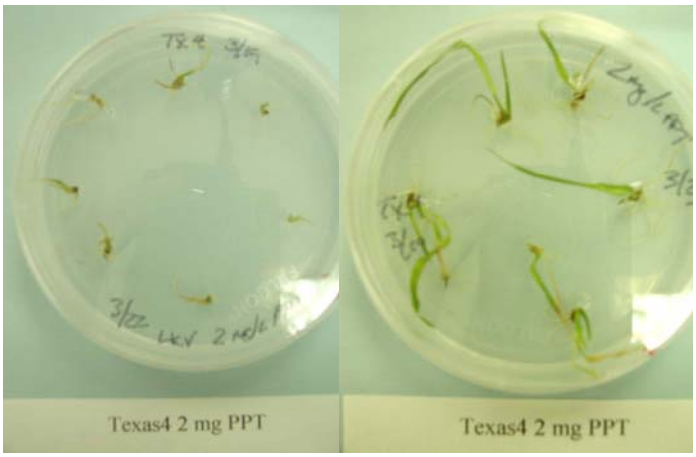


Figure 5-10. Variability in tissue culture selection. TX4 plants from the same seed source, seed prep and plated on media from the same batch. A. Low growth. B. High growth and viability.

Relationship to the Work of Toldi et al. (2000)

The tissue culture method used here was similar to a system developed by Toldi et al. (2000). In their study, Toldi found that a narrow concentration range of glufosinate (2-3mg/L glufosinate) would induce the formation of multiple shoots on up to 60% of the plantlets. All varieties in the current study also produced microshoots, or stems originating from the meristem at the base of the plant at sub-lethal levels of glufosinate (Figure 5-11). TX4 was noted to produce significantly more microshoots over a wider range of PPT concentrations than the cultivars (Table 5-3).

Figure 5-11. Microshooting TX4. After 5 weeks of selection on 3 mg/L PPT the plantlet was transferred to a Magenta box and maintained on 2mg/L PPT for an additional 4 weeks. Each shoot from the above microshoot could be removed and transferred to fresh media to grow, resulting in over 20 plants derived from a single plant.

Table 5-3. Percent microshoot induction for TX4 and TP309. Plants were selected on PPT and induction of microshooting was measured at 4 weeks after treatment began.

	0.0 mg/L	**1.0 mg/L**	**2.0 mg/L**	**3.0 mg/L**	**4.0 mg/L**	**5.0 mg/L**
TP309	0	55	19	3	0	0
TX4	0	23	17	34	5	10

The most surprising result of Toldi et al. (20000) was that this procedure could induce glufosinate tolerance in otherwise sensitive plants. When Toldi et al. grew up several hundred microshoots derived from seedlings grown on sub-lethal concentrations of glufosinate and transferred them to pots in the greenhouse, 78% survived two applications of a glufosinate-based herbicide. The same concentration of herbicide killed all of the control tissue cultured as well as all the greenhouse grown plants not previously exposed to the herbicide.

The results of Toldi et al. 2000 have not been cited in other published work. Thus we were initially skeptical. However, in agreement with Toldi et al. (2000), microshoots from “sensitive” cultivars such as Cypress gave rise to seedlings that were glufosinate tolerant. Not only did most of these plants survive what would normally be a lethal does of glufosinate, they went on to produce seed.

The results of Toldi et al. (2000), which were confirmed in this study, may explain why a second application of herbicide does not effectively kill plants which managed to survive the first application. The poorly understood mechanism for induced tolerance in some of the samples may also explain the variability seen in greenhouse, field, and growth chamber studies. Whether TX4

is generally constitutive for the same tolerance that can be induced in other cultivars by sub-lethal doses of glufosinate is unknown. However, it is an attractive possibility.

Conclusion

It was initially hoped that TX4 might serve as a non-GM source of glufosinate resistance that could be breed into cultivated rice, analogous to the non-GM ALS inhibitors that have been introduced into CLEARFIELD™ rice varieties. However, this appears unlikely. The meristem of TX4 is usually not killed by a dose of glufosinate sufficient to control most red rice accessions, but seedlings typically sustained severe leaf damage and thus development was substantially delayed. The variable degree of tolerance observed also suggest that glufosinate tolerant commercial rice varieties based on TX4 would not be sufficiently robust and reliable to be commercially viable.

On the other hand, TX4 does often appear to have sufficient tolerance to complicate the use of Liberty-Link™ rice varieties as a method of weed control. Even though the field plots in 2000 were treated with Liberty the maximum number of times allowed under the proposed label for the use of this herbicide in rice (twice) with 1.25 the maximum allowed rate, more than 50% of the TX4 survived. These results are in general agreement with the original observation of glufosinate tolerance in TX4 by Noldin et al. (1999b) and with most other published work on this red rice accession.

Recent work from Asia also indicates that some cultivars have significant tolerance to glufosinate (Hsu and Kao 2004) These investigator found that *Oryza sativa* ssp. *japonica* cultivar Tainung 67 (TNG67) was resistant to 10mM glufosinate. Twelve day old plants with three leaves showed no signs of chlorosis seven days after treatment. In contrast, the cultivar Taichung Native 1

(TN1) demonstrated high levels of chlorosis and necrosis. A decrease in transpiration rate, chlorophyll and protein content, and an increase in NH_4^+ content were associated with glufosinate damage in TN1, whereas they were not adversely affected by glufosinate treatment in TNG67.

TX4 matures later than most commercial rice varieties (Noldin et al. 1999a) and the developmental delay induced by glufosinate treatment is likely to further delay flowering. Thus TX4 is unlikely to cross with Liberty-Link™ rice varieties during the main crop cycle. However, it should be noted that some commercial rice in the US produce a second, or ratoon, crop by resprouting after the plants are harvested the first time. Whether ratoon crop production should be allowed for Liberty should be carefully considered since it may allow TX4-like red rice that has recovered from an initial application of herbicide the opportunity to cross with the BAR containing commercial rice.

The surprising observation of Toldi et al. (2000) that exposure to a sub-lethal doses of glufosinate can induce tolerance in normally sensitive varieties was confirmed in these studies. It also suggests potential problems in the deployment of Liberty-Link™ rice. Tolerance was expressed only transiently in the progeny of these plants (Toldi et al. 2000), but it may be sufficient to allow the resulting the next generation of seedlings to also survive glufsoinate treatment. Particularly since herbicide is often applied using aircraft, one would expect that a certain proportion of red rice would be exposed to sub-lethal doses of herbicide on a routine basis.

CHAPTER VI

CHARACTERIZATION OF GLUTAMINE SYNTHETASE

Introduction

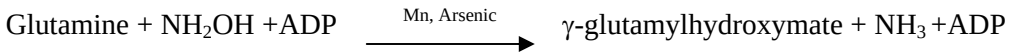
TX4 has a higher level of tolerance to glufosinate than rice cultivars and any of the other rice ecotypes tested. However, the mechanism(s) responsible for this tolerance are as of yet unknown. As discussed in Chapter I, glufosinate is an irreversible inhibitor of glutamine synthetase, a primary enzyme in nitrogen metabolism. Therefore, it was reasonable to imagine that TX4's tolerance is a result of an altered form or altered regulation of glutamine synthetase.

To further investigate the mechanism of tolerance, the different forms of GS from TX4 and Cypress were purified and examined. Before discussing the results, it is important to realize that there are two different types of assays that are generally used to examine glutamine synthetase. The physiologically relevant activity can be directly measured by assaying the conversion of glutamate to glutamine. Since this conversion requires the hydrolysis of ATP to ADP and inorganic phosphate, the physiological activity can also be measured by the assaying the release of inorganic phosphate with malachite green (Forlini 2000).

$$\text{Glutamate} + NH_4 + \text{ATP} \xrightarrow{Mg} \text{Glutamine} + \text{ADP} + P_i$$

This assay is physiologically relevant, but suffers from high background since many enzymes release inorganic phosphate. Consequently, it is much more common to assay GS using the

"transferase assay" which measures a related, non-physiologically relevant, activity of glutamine synthetase. In this assay the formation of L-γ-glutamylhydroxamate from glutamine and hydroxylamine is measured in the presence of manganese and arsenic by spectroscopic methods (Forlani 2000). This assay is much more specific for GS and is not distorted by other phosphate producing enzymes.



Unfortunately, these assays do not necessarily give the same results (Forlani 2000). For example, a single amino acid mutation has been found in pea glutamine synthetase that confers resistance to L-methionine sulfoximine, an active site inhibitor very closely related to glufosinate (Clemente and Marquez 1999b). This mutation completely abolishes the transferase activity. However, it has very little effect on the biosynthetic activity when the enzyme is assayed with ammonia in excess.

For this study the transferase assay was employed for the purification of GS since it is easier and more specific. A biosynthetic assay based on direct measurement of glutamine by HPLC based amino acid analysis was utilized for the inhibition analysis.

Materials and Methods

Glutamine Synthetase Purification

Purification and characterization of the glutamine synthetase isozymes were based on the methods of Hirel and Gadal (1980) and Iyer et al. (1981). Fifty grams of tissue from greenhouse grown plants or five grams of tissue culture plants were frozen with liquid nitrogen and ground in a Waring^{TM} blender in 10 volumes of ice cold buffer A (40mM Tris-HCl, 10mM glutamine, 1mM EDTA, 5mM MgCl2, 10 mM 2-mercaptoethanol, pH 7.6). The homogenate was filtered through 4 layers of cheese cloth and centrifuged for 20 minutes at 15,000g and 4C. The supernatant was then used for ammonium sulfate precipitation. Soluble proteins fractionating between 30-50% saturation were collected by centrifugation at 15,000g for 20 minutes at 4C. The final pellet was suspended in 25mL buffer A and dialyzed for 12 hours versus buffer A. After the initial freezing in liquid nitrogen, all processes were carried out at 4C.

The ammonium sulfate fraction was batch loaded onto DEAE-Sephacel (2x20cm) equilibrated with buffer B (5mM Tris-HCL, 1mM EDTA, 5 mM MgCl2, 10 mM 2-mercaptoethanol, pH 8.0) by incubating 25mL of the dialyzed ammonium sulfate 30-50% batch with enough DEAE Sephacel slurry for a final column volume of 75ml. The column was washed with 300ml of buffer B and then eluted with a linear gradient of 0-0.5M NaCl dissolved in 200ml buffer B. Four mL fractions were collected. Fractions with activity were pooled for further analysis. The pooled fractions were applied to a Sephacryl S-300 column (100x2.5cm) equilibrated with buffer C (50mM Tris-HCl, 1mM $MgCl_2$, pH 7.6). Two mL fractions were collected. Fractions with activity were pooled and layered on hydroxyapatite column (10x1cm) equilibrated with buffer D

(10mM K-phosphate pH7). The proteins were eluted with linear gradient of 0.1-0.3M phosphate buffer (total volume 100mL) and 1mL fractions were collected.

Protein concentrations were determined spectroscopically by absorbance at 280 nm and by the Bradford method (Bradford 1976). Discontinuous SDS-PAGE (4% stacking 12% separating) with Coomasie blue staining was used for protein visualization (Forlani 2000).

Glutamine Synthetase Activity Assays

Biosynthetic Assay

The biosynthetic assay mixture included 100 mM Tris-HCl (pH 7.0), 50 mM NH_4Cl, 50 mM $MgCl_2$, 100 mM glutamate, 7.5 mM ATP (Clemente et al. 1999a) in a final volume of one mL. After 15 minutes at 37C, the reactions were stopped by the addition of 0.6 mL 1N HCl. The activity was measured by the release of inorganic phosphate from ATP by the malachite green method (Baykov et al. 1988) or by the amount of glutamine produced in the reaction measured by HPLC amino acid detection (Martin et al. 1982; Marques et al. 1989).

Transferase Assay

The transferase reaction assay (Shapiro & Stadman 1977) contained: 50 mM glutamine (pH 7.0), 40 mM K-arsenate (pH 7.0 with KOH), 5 mM NaADP (pH 7.0 with NaOH), 25 mM hydroxylamine-HCl, 4 mM $MgCl_2$, 25 mM imidazole-HCl buffer (pH 7.0). The reaction mixture was incubated with enzyme in a total volume of 1 mL for 15-30 minutes at 37C. The reaction was stopped by addition of 2 ml of 10% $FeCl_3$, 5% trichloracetic acid, 6.67% HCl. Activity was determined by the formation of γ-glutamyl-hydroxymate measured at 540 nm and quantified by

comparison to a standard curve. The transferase assay was used to measure activity during purification.

HPLC based amino acid analysis was carried out by Mrs. Jenny Johnson in the Protein Chemistry Lab in the Department of Biochemistry & Biophysics at Texas A&M University.

Results and Discussion

The purification scheme was designed both to purify GS and to separate the different forms. Like most other plants, rice contains a single form of GS2, but multiple forms of GS1 (Hirel and Gadal 1980; Iyer et al. 1981). In addition to root and leaf forms of GS1, a second GS1 in leaves of young (2 leaf) plants has recently been reported (Ishiyama et al. 2004b). Published reports indicate that the cytosolic GS, GS1, elutes from DEAE Sephacel at a concentration of 0.075-0.15 M NaCl, whereas the chloroplastic form, GS2, elutes at 0.15-0.3 M (Hirel and Gadal 1980; Iyer et al. 1981).

The purification profiles obtained generally correspond to those observed by Hirel and Gadal (1980) with one major exception. Most of the GS activity did not bind the DEAE Sephacel during the batch loading procedure and eluted from the column in the wash portion of the profile (Figure 6-1). However, when the unbound fractions were pooled and re-applied to the same amount of DEAE Sephacel, the GS activity bound and could be eluted at salt concentrations similar to those observed by Hirel and Gadal (1980).

It was very surprising that most of the GS activity did not bind to the first DEAE Sephacel column. However, similar results were seen with new DEAE Sephacel and the phenomenon was consistent for multiple experiments with samples harvested at different growth stages and for TX4, Cypress, and other rice types tested.

It should be noted that Hirel and Gadal (1980) used isolated chloroplasts and etiolated leaves for investigation of GS1 and GS2 rather than whole seedlings and that Iyer et al. 1981 ran the ammonium sulfate cut through a size exclusion column before applying the extract to DEAE Sephacel. However, the amount of tissue used was less than that used in Hirel and Gadal (50g vs. 200g), the column volume is greater (75mL vs. 63mL) and the amount of protein applied to the column is less (approximately 400mg vs. 1,400 mg). Thus, it would seem that column overloading was not an issue. Similar result were seen when the ratio of DEAE Sephacel to extract was increased (data not shown).

It is also possible that the lack of binding to the initial DEAE Sephacel column was due to residual ammonium sulfate. However, the ammonium sulfate fraction was dialyzed for more than 12 hours against 100 volumes of the loading buffer before being mixed with DEAE Sephacel. Other experiments were conducted with the ammonium sulfate fraction fractionated through a size exclusion column (Sepharose 4B, per Iyer et al. 1981). Even after gel filtrations a large portion of activity is eluted in the wash portion of the column, indicating that ammonium sulfate contamination was unlikely to be the cause of the phenomenon (for example refer to Figure 6-7).

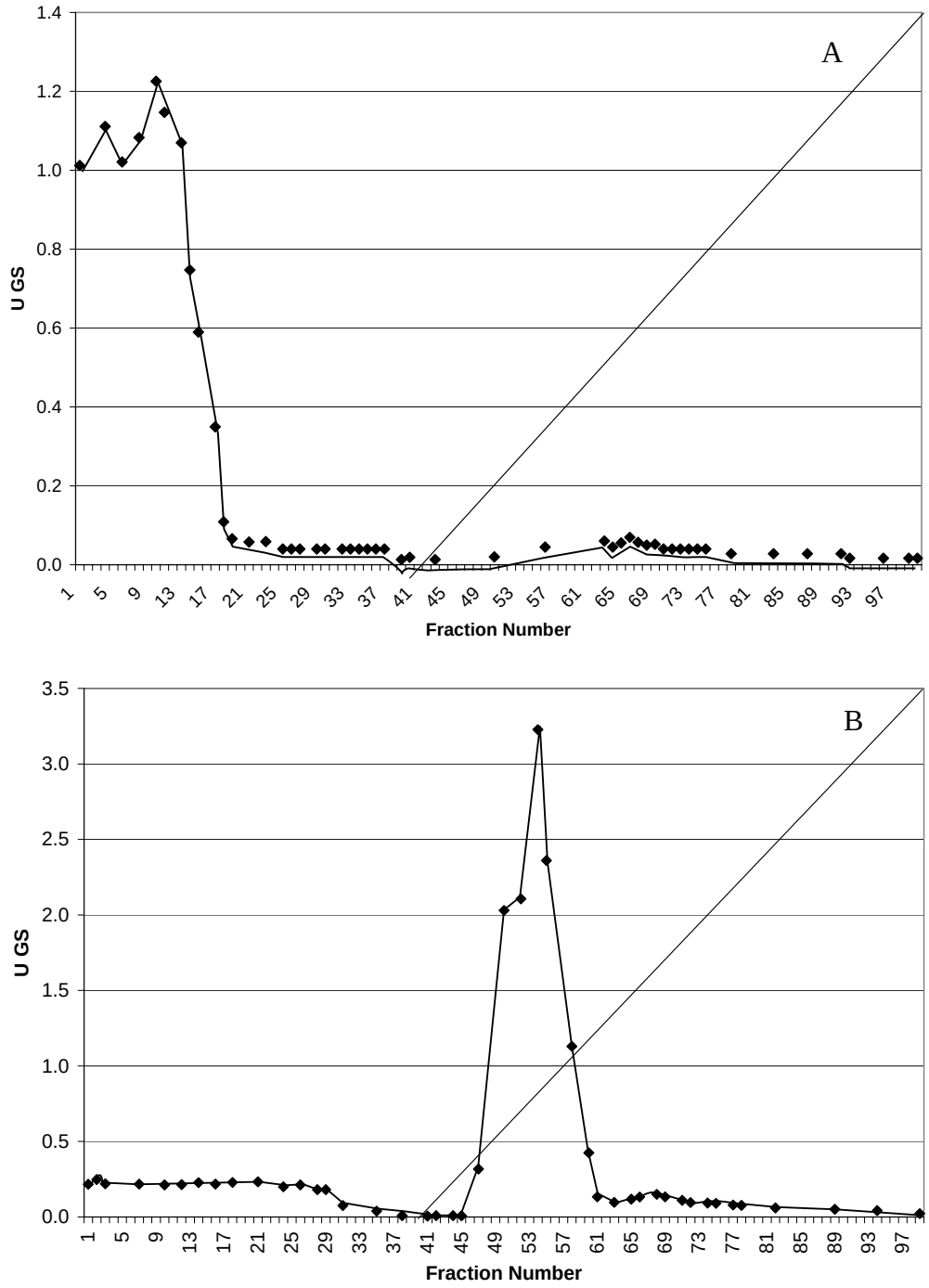


Figure 6-1. Elution profile of 1-2 leaf TX4 GS from DEAE Sephacel. When a dialyzed 30-50% ammonium sulfate fraction was batch loaded onto DEAE Sephacel, most of the GS1 activity was in the wash fraction (panel A). However, when the unbound samples were re-applied to a second DEAE Sephacel column, most of the activity bound and could be eluted with a 0-0.5M NaCl gradient (panel B).

Other possibilities include competition from other proteins, post-translational modification, and proteolysis occurring in the extract. Both forms of GS are phosphoproteins, with GS1 activity increasing with phosphorylation and GS2 requiring phosphorylation of serine residues for activity. Additionally, both forms have been shown to associate with 14-3-3 proteins in vivo. 14-3-3 binding has been shown to increase the activity of GS1 and has been suggested to be required for GS2 activity (Comparot et al. 2003). Phosphorylation and 14-3-3 binding increases the stability of GS1, but may decrease the stability and increase the breakdown of GS2 (Finnemann and Schjoerring 2000; Riedel et al. 2001). The enzymes required for phosphorylation and dephosphorylation, as well as 14-3-3 proteins are included in the 30-50% ammonium sulfate cut used in GS purification, therefore it is not unreasonable that the phosphorylation state of the different GS isoforms and a possible interaction with proteins such as 14-3-3s could be responsible for the lack of binding in the first DEAE Sephacel column (Finnemann and Schjoerring 2000). Unfortunately, the actual reason that most of the GS did not bind the first DEAE Sephacel column remains unknown. However, since the phenomenon was consistent across several experiments and between growth stages and different types of rice, the analysis was continued.

Developmental Differences

As discussed in Chapter I, it has been well documented that GS1 and GS2 are present in different ratios during different developmental stages (Masclaux et al. 2000; Habash et al. 2000; Morey et al. 2002). In soybean there are 3 classes of GS1, with each class having two isoforms. The different forms of each class are differentially regulated during development (Cock et al. 1991; Morey et al. 2002). In the cereal grains such as rice and wheat, GS1 is the main isoform in very

young plants, with GS2 becoming dominant with the development of green tissue. As the plant matures and produces grain, the GS ratio shifts to favor GS1. This is due to the initiation of senescence, which favors GS1 for the remobilization of glutamine (Habash et al. 2001; Kamachi et al. 1991). The levels of GS1 also increase during grain development; GS1 is the primary form in the rachis and the only form present in the developing and mature grains (Zhang et al. 2000).

Cypress shows growth stage related differences in the purification profiles (Figure 6-2). When profiles from 3-4 leaf plants and mature plants are compared to the profiles from 1-2 leaf profiles several differences were apparent. The first DEAE Sephacel column shows little difference for the Cypress growth stages. However, the second DEAE Sephacel reveals that there are 2 main peaks. The 3-4 leaf activity elutes first, with the 1-2 leaf activity eluting last. A similar amount of tissue from mature plants contains less GS1 activity than either of the earlier stages and has a distinct chromatographic profile.

TX4 again shows few differences in the first DEAE Sephacel profile (Figure 6-3) except that the mature peak elutes first and the wash peak for the 1-2 leaf extract has lower activity than the others. The second DEAE Sephacel column better illustrates the growth stage differences in the profiles. The 1-2 leaf extract has the highest level of activity and elutes from the column at a lower NaCl concentration than the other two. The mature extract has two small peaks that elute under the peaks of the 1-2 and 3-4 profiles.

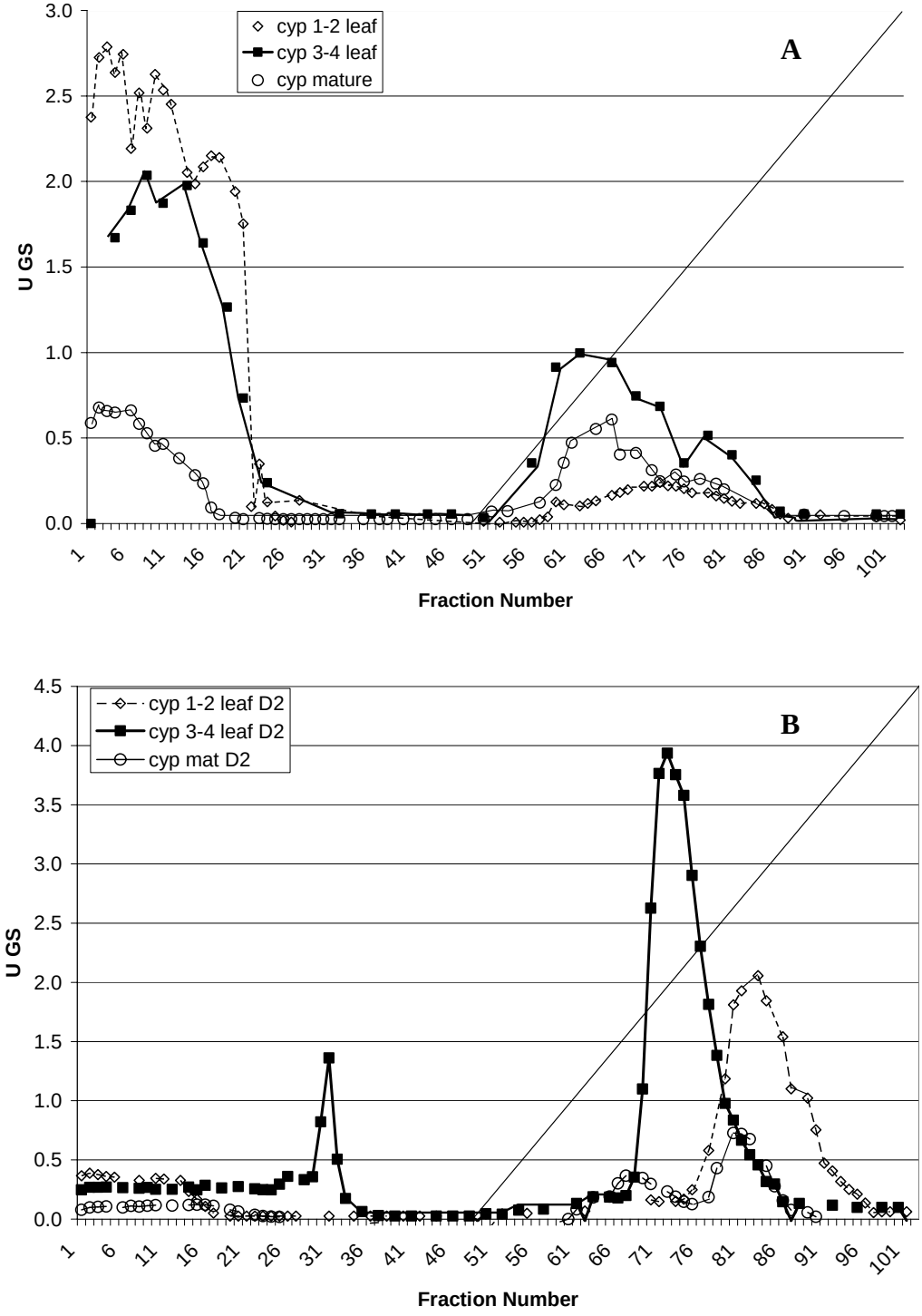


Figure 6-2. Chromatography of GS from Cypress at different growth stags on DEAE Sephacel. A. First DEAE Sephacel. B. Second DEAE Sephacel. NaCl gradient is indicated by the diagonal line, beginning at fraction 50 with 0.0 M NaCl and ending with 0.5 M NaCl.

The differences in the DEAE Sephacel profile of GS in 1-2 leaf vs. 3-4 leaf seedlings may be related to the fact that 1-2 leaf seedlings were still utilizing seed storage tissue, whereas it had largely been exhausted by the 3-4 leaf stage (data not shown). However, the possibility that these apparent stage-specific differences are an artifact or due to the same unknown factor that caused differential binding to the first and second DEAE Sephacel column can not be ruled out.

Differences in the profiles that are directly related to growth stage also reveal the main differences between TX4 and Cypress (Figures 6-2 and 6-3). Analysis of the elution profiles for the re-run fractions on DEAE Sephacel show several distinct differences between TX4 and Cypress. In the first DEAE Sephacel the profiles are similar in shape and NaCl concentration with the exception that the mature peaks elute first in TX4, as opposed to similar to the other peaks in Cypress, and the 3-4 leaf profile has a very small extra peak in TX4 that was not present in Cypress. In the second DEAE Sephacel purification profile the 1-2 leaf and 3-4 leaf peaks are reversed in terms of NaCl elution concentration in TX4 versus Cypress.

To further examine the GS1 isoforms in Cypress and TX4, pooled fractions from both the first and second DEAE Sephacel columns were further purified by chromotography on Sephacryl S-300 and then fractionated on hydroxyapatite. The Sephacryl S-300 column profiles were similar for all the experiments. An example is shown in Figure 6-4. Hydroxyapatite (HA) was then used to further separate the GS isoforms.

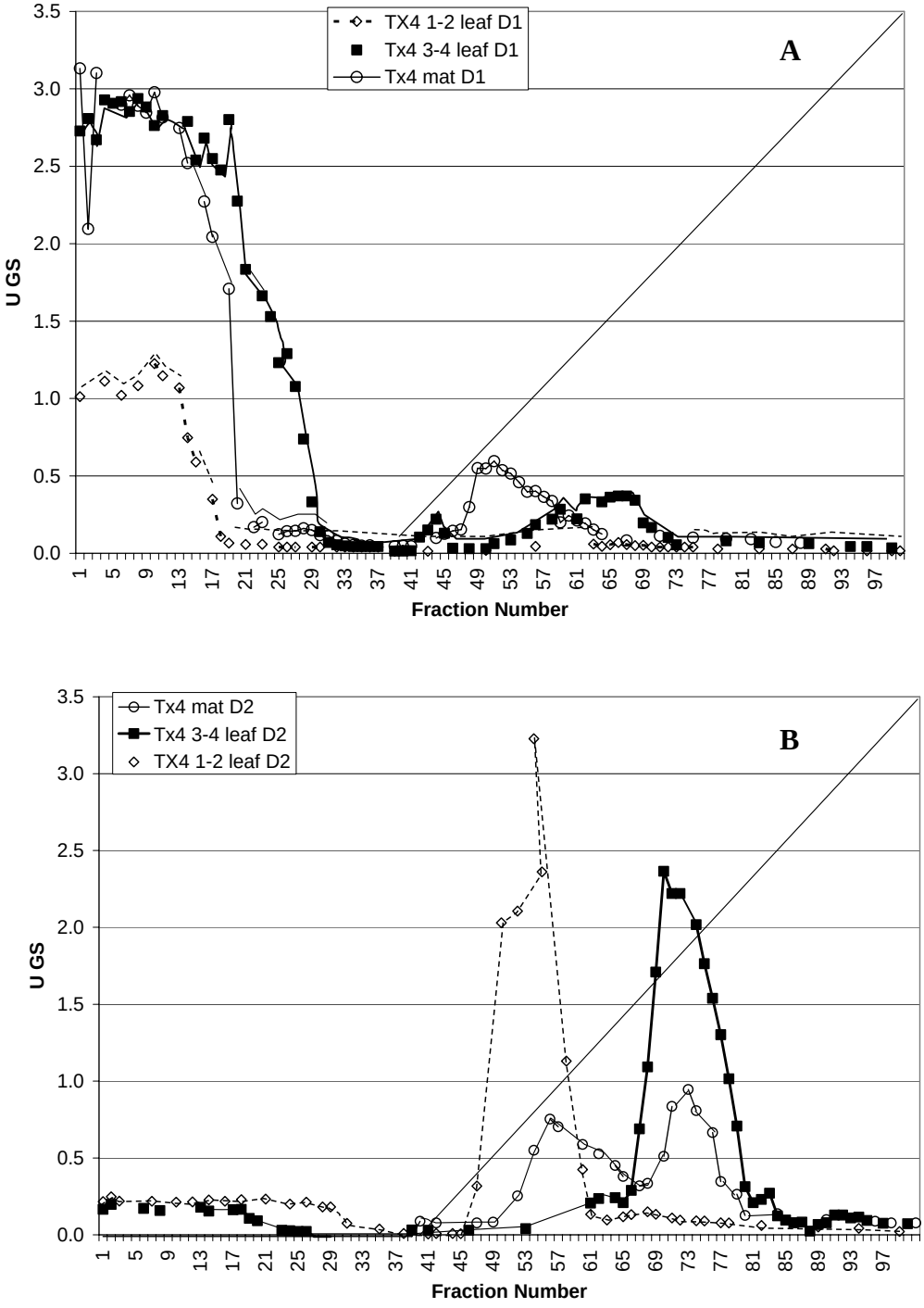


Figure 6-3. DEAE Sephacel profiles of TX4 comparable to the Cypress ones in Figure 6-2. A. First DEAE Sephacel. B. Second DEAE Sephacel.

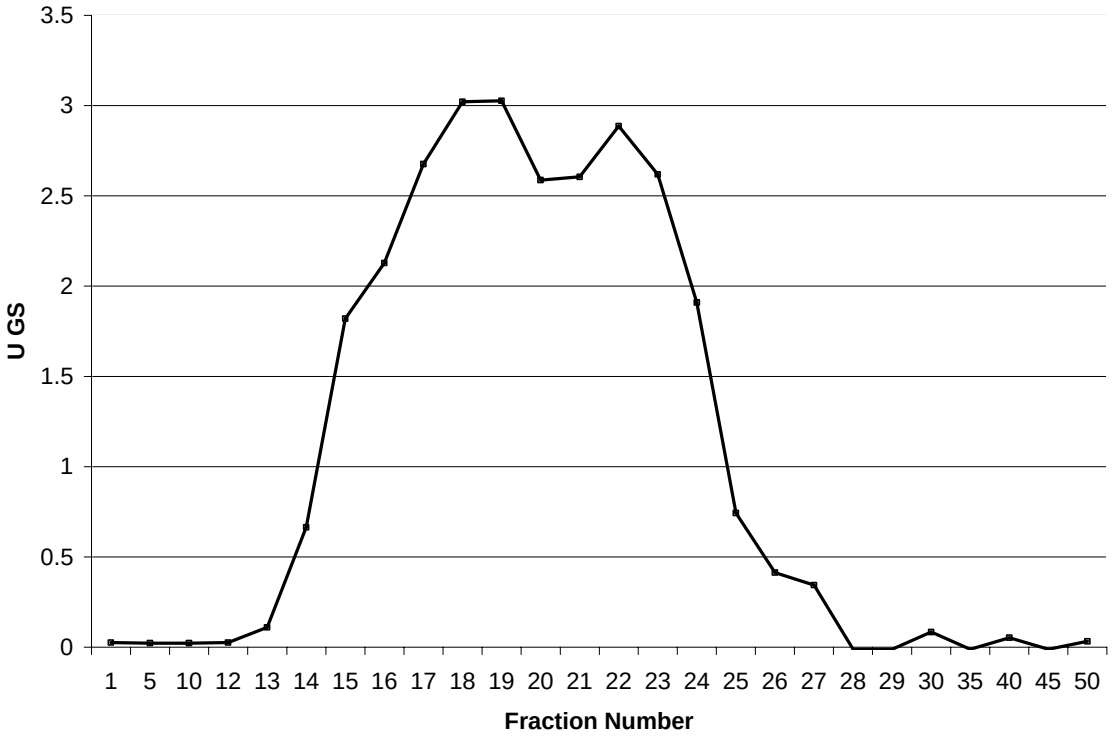


Figure 6.4. Example of a typical Sephacryl S-300 column. TX4 Second DEAE Sephacel pooled NaCl fractions. Sephacryl S-300 has a globular exclusion limit of 10^4- 10^6 Mr.

Hydroxyapatite chromatography also reveals differences in the profiles from the different growth stages for Cypress (Figure 6-5). Again, the peaks from the different growth stages were distinct. The 1-2 leaf stage peaks are similar for the HA of the first and second DEAE Sephacel columns, with the exception that the activity is much greater in the second column. The 3-4 leaf stage peaks are also similar for both profiles. The major difference between the two is the mature profile, which has three peaks in the second HA but only one in the first.

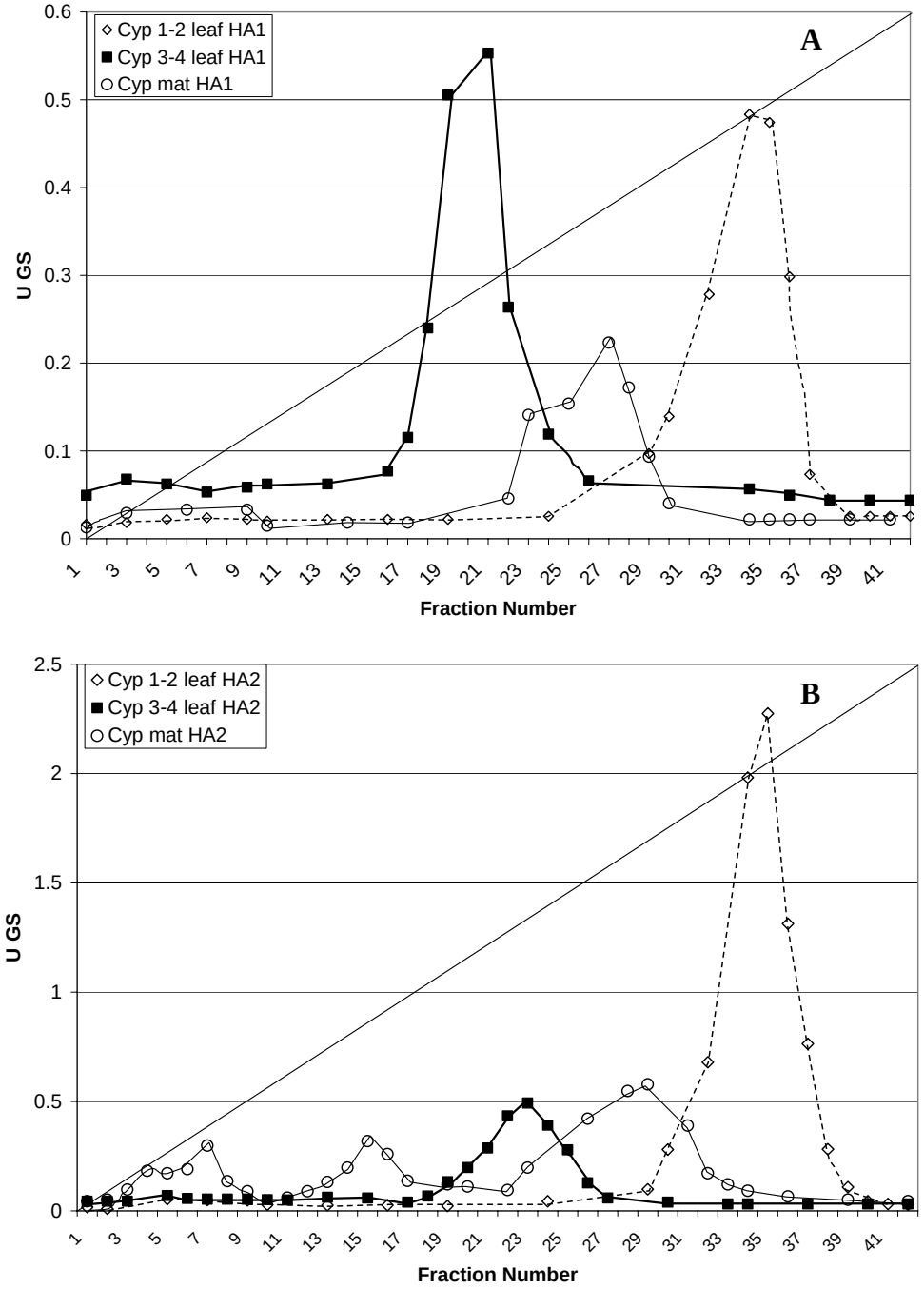


Figure 6-5. Hydroxyapatite profiles for Cypress first DEAE Sephacel (A) and second DEAE Sephacel (B). Fractions from respective DEAE Sephacel columns were pooled and loaded onto HA columns. After washing Activity was eluted with 0.0-0.3 M phosphate buffer (indicated by the diagonal line with 0.0M on the bottom and 0.3M on the top.). The different growth stages are indicated by different symbols.

TX4 also has striking differences in the HA profiles that are related to the growth stage of the samples (Figure 6-6). The most noteable difference was that no activity could be recovered when the activity from the first peak of the first DEAE Sephacel column in the 3-4 leaf extract is loaded onto the HA column. This is seen again in the HA of the second DEAE Sephacel column, when the activity from the 3-4 leaf DEAE Sephacel peak is not recovered. This is in contrast to the Cypress profiles where the activity from the 3-4 leaf plants is recovered in all instances. Interestingly, Noldin noted that there were differences in the control between 2 and 3 leaf red rice, with more plants surviving the application of 0.28 kg ha^{-1} at the 2 leaf stage than at the 3 leaf (Noldin et al. 1999b).

At first glance, the hydroxyapatite profile of the first DEAE Sephacel seems to be similar for TX4 and Cypress, with the exception that the TX4 mature tissue peak has three times more activity than does Cypress. However, when the two DEAE Sephacel peaks from 3-4 leaf TX4 and Cypress were separated, the first peak does not elute from the column for TX4 but does for Cypress. This could be due to the low activity of the first peak. However, when the TX4 3-4 leaf peak from the second DEAE Sephacel column was loaded onto hydroxyapatite none of the activity was recovered from the column, suggesting that the TX4 protein had either been lost or that it was very strongly bound to the column.

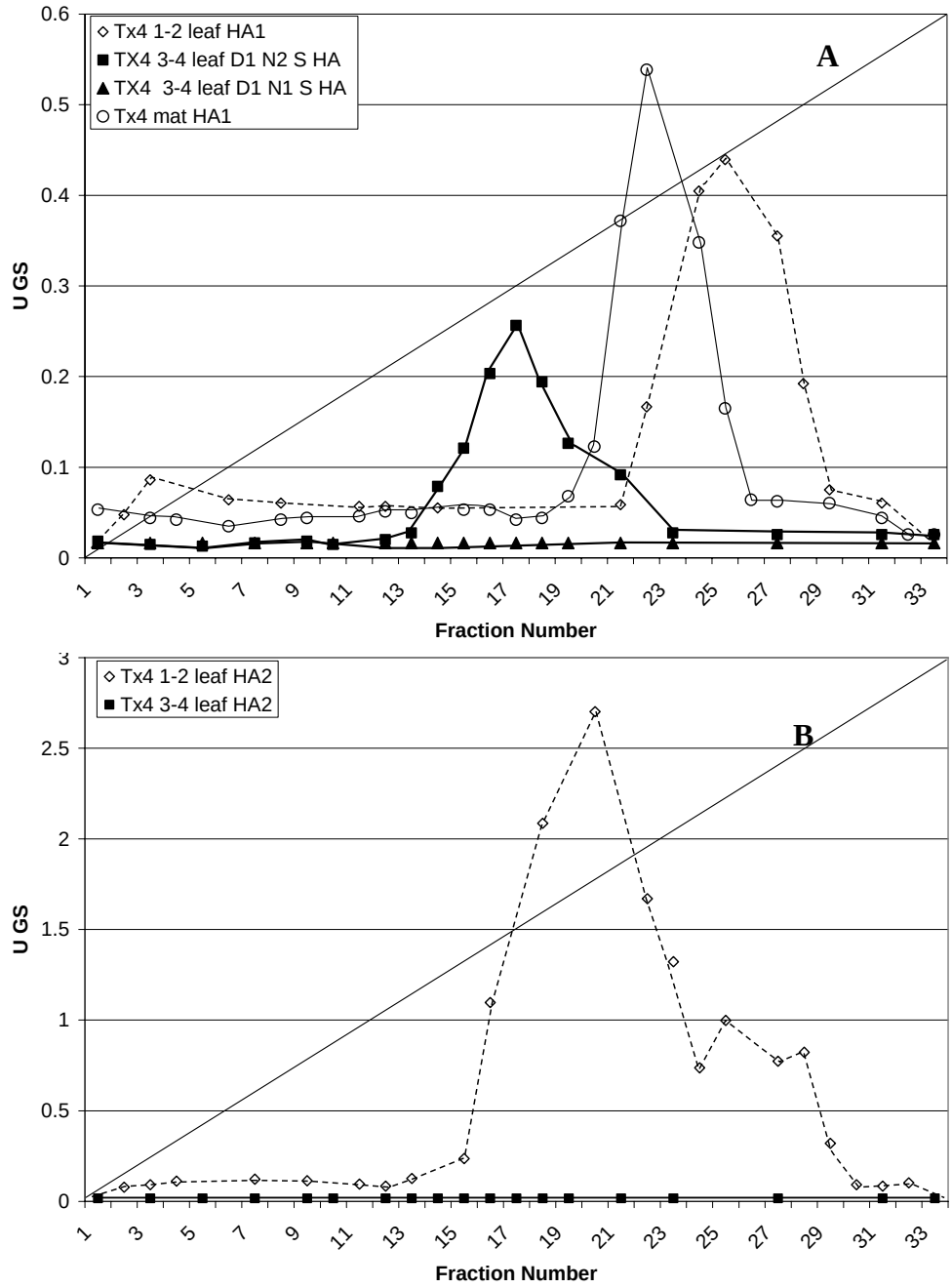


Figure 6-6. Hydroxyapatite profile for TX4 first and second DEAE Sephacel columns. A. First DEAE Sephacel column. Activity from the two peaks from the NaCl elution of the first DEAE Sephacel at the 3-4 leaf stage were separated and run through the S-300 and HA columns independently. D1 N1 indicates the first DEAE Sephacel peak, and D1 N2 indicates the second DEAE Sephacel peak, both from the first DEAE Sephacel column. B. HA of the second DEAE Sephacel column. Mature tissue was not included. Phosphate gradient begins with 0.0 M and ends with 0.3 M as indicated by the diagonal line.

The results of chromatography on both DEAE Sephacel and HA suggest that there are differences in the GS from Cypress or TX4. Some of these differences appear to be related to the growth stage of the plant. These differences could be due to different isoforms of the enzymes or it could be due to modification of the different enzymes. As would be expected for a key metabolic enzyme, GS is subject to numerous control mechanisms. One of these is post-translational modification. For example, GS1 is regulated post-translationaly by reversible phosphorylation (Finnemann and Schjoerring 2000). The enzymes are modified differentially to maintain the homeostatic control of nitrogen metabolism. As reviewed in Chapter I, there are multiple isoforms of GS which have different expression and regulation. These different isoforms can form into different hetero-octomers which would be expected to have different properties than the homo-octamers (Forde and Cullimore 1989). These differences could be related to the observed differences in growth stage. However, it is not known whether all of the differences in chromatographic behaviors observed actually reflect differences in vivo or whether some of them post-translational modifications or proteolysis that occurred after the tissue was harvested and during fractionation.

Sensitivity to Glufosinate

As discussed in Chapter I, there are precedents for substantial variation in the glufosinate sensitivities among the different GS isoforms in plants. For example, the mesophyll GS (cytosolic) in maize was 50% inhibited at 2.0 μM glufosinate, while the bundle sheath cell GS (chloroplastic) was 50% inhibited at 30 μM. Glufosinate tolerance has also been seen in plant cell lines selected on glutamine synthetase inhibitors. In most cases this appears to be due to

higher levels of GS although novel forms of GS have also been reported (Yamaya et al. 1990; Avilia et al. 1998; Perez-Garcia et al. 1998).

Glufosinate resistant forms of glutamine synthetase can also be created in the laboratory. Replacement of Asp51 by Glu (D51E) in *Anabena azollae* glutamine synthetase resulted in a high resistance to glufosinate by decreasing the enzymes phosphorylation ability (Crespo et al. 1999). A more pertinent report comes from Clemente and Marquez (1999a). When these investigators used site directed mutagenesis to change Glu-297 of *Phaselous vulgaris* GS1 to Ala (E297A), the biosynthetic activity of resulting enzyme was only 30% inhibited at 5 mM MSX (a structural analog of PPT) compared to 100% inhibition for the wild type. Even with 20mM MSX the mutant was only inhibited by 60%.

To test whether any of the GS1forms in TX4 or Cypress was glufosinate tolerant, the transferase activity in fractions from DEAE Sephacel purification were compared in the presence or absence of 100 μM glufosinate (Figure 6-7). The activity of the profiles for TX4 and Cypress are clearly not completely inhibited with 100 μM glufosinate. While the results from this analysis were not very promising, it should be noted that these numbers were obtained using the transferase assay.

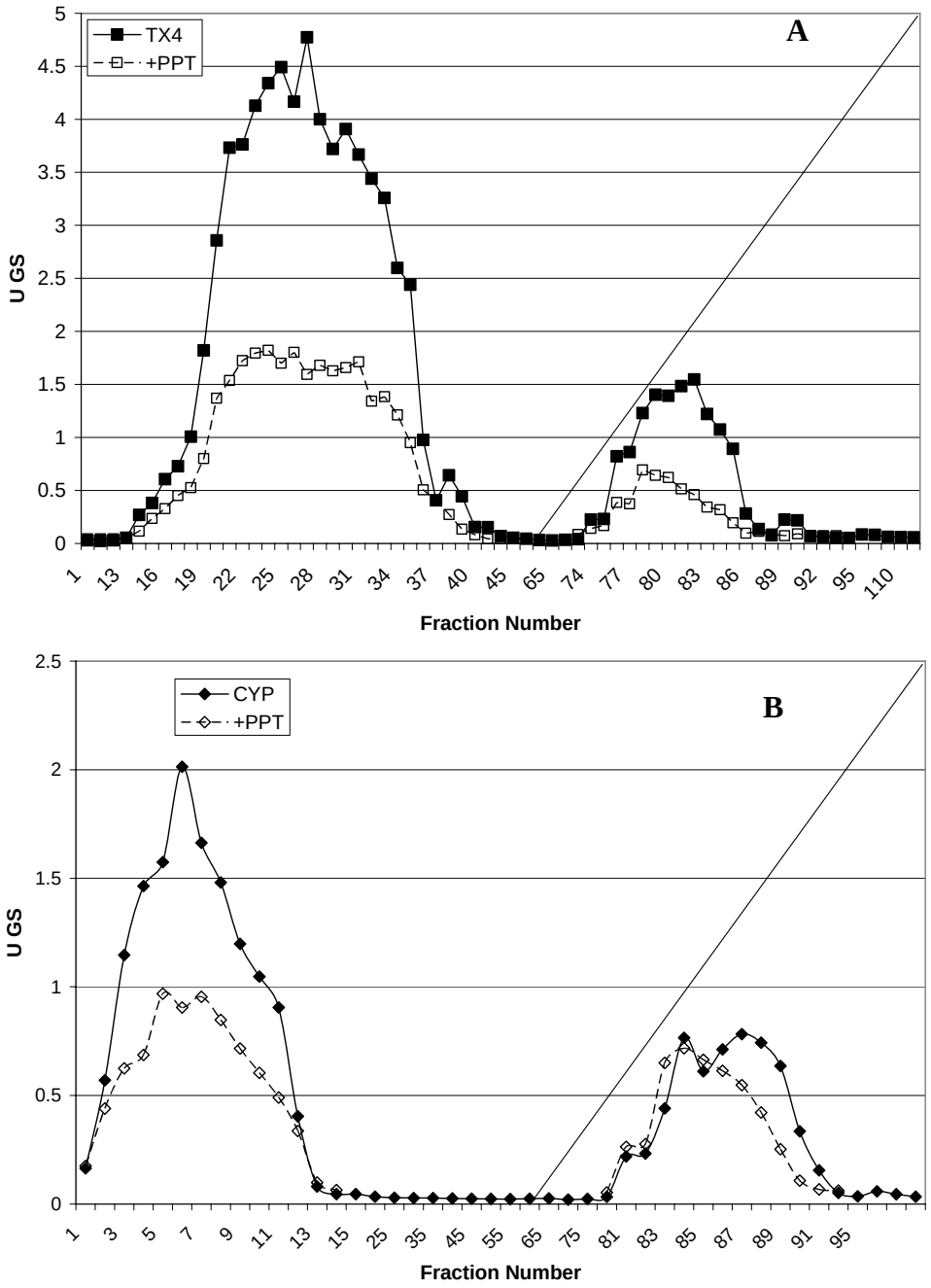


Figure 6-7. DEAE Sephacel activity and PPT inhibition profile for TX4 (A) and Cypress (B). Representative DEAE Sephacel profiles with PPT inhibition profiles. These figure represent extract that was purified first on Sepharose 4B and then on DEAE Sephacel per Iyer et al. 1981. Note that as in the batch DEAE Sephacel a large amount of activity was lost in the wash phase of the purification. The activity in the wash phase is more sensitive to PPT than that in the NaCl part for both TX4 and Cypress. NaCl gradient is indicated by the diagonal line with 0.0 M NaCl beginning at fraction 60 and 0.5 M at the end of the profile.

As discussed previously, this assay may not provide biologically relevant information. For example, Clemente and Marquez (1999b) obtained strikingly different results when they assayed the (E297A) mutant in *Phaseolus vulgaris* with the biosynthetic and transferase assays. The physiologically relevant biosynthetic assay showed greatly increased MSX tolerance and a similar level of total activity when compared to wild-type. However, the non-physiological transferase assay indicated a 70 fold loss of activity, but similar levels of MSX inhibition to the biosynthetic assay.

The biosynthetic activity is typically assayed by measuring inorganic phosphate release using Malachite green to assay inorganic phosphate was hindered by the non-specific nature of the assay (Forlini 2000). Since many enzymes release inorganic phosphate, the results can be misleading. To circumvent this problem, the biosynthetic assay was combined with HPLC based amino acid analysis to directly measure the production of glutamine in the reaction. Biosynthetic and transferase analysis of the inhibition of the proteins in the 30-50% $(NH_4)_2SO_4$ fraction showed differences between the extracts of tissue from the roots and leaves of plants grown in the greenhouse and those grown in tissue culture (Table 6-1).

Unfortunately, as has been the case with glufosinate inhibition all along, the values were not reproducible. For the transferase assay one experiment showed that TX4 from the greenhouse was significantly less inhibited than the comparable Cypress at 100 μM PPT, the second experiment gave opposite results. For the biosynthetic assay the first experiment on extract from greenhouse grown TX4 leafs showed 96% inhibition with 500 μM PPT, while Cypress was completely inhibited. In another experiment Cypress was actually less inhibited than the TX4 samples.

Table 6-1. Percent inhibition for transferase and biosynthetic assays .The 30-50% ammonium sulfate cut was subject to transferase (A) and biosynthetic (B) assay for inhibition after dialysis. The two experiments are from independent preparations of the same tissue source preformed 3 days apart. Biosynthetic activity was determined by HPLC analysis.

A	**Greenhouse**		**Tissue Culture**					**Greenhouse**		**Tissue Culture**	
			0 mg PPT		**1 mg PPT**					**0 mg PPT**	
	TX4	**TX4**	**TX4**	**TX4**	**TX4**	**TX4**		**CYP**	**CYP**	**CYP**	**CYP**
μM PPT	**Root**	**Leaf**	**Root**	**Leaf**	**Root**	**Leaf**		**Root**	**Leaf**	**Root**	**Leaf**
10	0.00	0.00	9.21	18.34	5.57	1.16		3.24		0.00	1.95
50	3.12	0.00	25.32	34.53	47.70	36.65		9.58	15.94	0.00	5.38
100	19.90	4.14	46.63	45.37	58.35	47.76		33.67		43.00	57.19
500	22.60	46.76	74.17	75.63	79.42	71.81		51.71	68.99	67.50	75.54
1000			87.89	86.37	79.66	78.61				71.83	86.90
10	0.00	0.47			0.00	3.66		1.11	2.36	2.33	0.00
50	4.75	1.41			7.99	11.35		6.13	5.29	4.67	9.26
100	59.01	20.19			39.58	57.45		3.90	38.92		
500	74.92	34.04			64.58	72.22		49.58	75.59	65.50	82.94
1000		59.62			74.13	77.42		64.76		75.33	83.43

B	**Greenhouse**		**Tissue Culture**					**Greenhouse**		**Tissue Culture**	
			0 mg PPT		**1 mg PPT**					**0 mg PPT**	
	TX4	**TX4**	**TX4**	**TX4**	**TX4**	**TX4**		**CYP**	**CYP**	**CYP**	**CYP**
μM PPT	**Root**	**Leaf**	**Root**	**Leaf**	**Root**	**Leaf**		**Root**	**Leaf**	**Root**	**Leaf**
50	50.28	64.46	21.90	82.05	77.85	85.06		51.02	33.31	22.85	4.50
500	89.12	93.42	87.99	97.96	99.35	96.19		89.80	87.90	96.71	98.72
50		72.21							76.21		
500		96.29							100.00		

Variability has been the greatest challenge faced in the work with TX4 and glufosinate. As we moved from preliminary observations to replicated studies, it became obvious that there was substantial variation in the amount of leaf damage and in survival even among different replications of the same sample. These experiments have shown that part of the variability may be related to the growth stage of the plants. It is also possible that environmental conditions and other factors on the metabolic status to the plant, or translocation of the herbicide may have a role in the variability of glufosinate response.

Conclusion

Although exact mechanism of glufosinate resistance in TX4 remains undetermined, the data presented here suggests that plants in the field are undergoing a key metabolic transition at the 2-4 leaf stage when the herbicide is typically applied. The 1-2 leaf extracts showed reproducible differences in their purification properties when compared to the 3-4 leaf and mature extracts for both TX4 and Cypress. It is during this same time period that the plants are shifting off seed reserves and becoming self-sufficient. It is possible that the successful control of plants is related to the application of herbicide during this key period. Plants that are still on seed reserves may be more resistant than the plants at a slightly later stage, resulting in better control, which may explain the results of Noldin et al. (1999b) where more plants survived the application of 0.28 kg ha^{-1} at the 2 leaf than at the 3 leaf stage. This observation also fits with observations in the tissue culture experiments where greater variation of sensitivity was seen when the plants were left on the seed during germination and selection.

CHAPTER VII

SUMMARY AND CONCLUSIONS

The original objective of this research was to identify the genes responsible for glufosinate tolerance in TX4 and using them to develop a non-GM alternative to Liberty-Link™ rice. However, TX4 does not appear have a sufficient degree of resistance to create robust glufosinate tolerant commercial rice varieties. In particular, while the meristem frequently survived and the plants were able to resprout, TX4 typically was severely damaged by glufosinate and suffered developmental delay. Furthermore, the degree of glufosinate tolerance in TX4 was variable. Part of the variability was apparently due to difference in environmental conditions, but variability remained a significant issue even after rigorously standardizing experimental conditions and the use of a tissue culture system.

While TX4 does not appear to have sufficient glufosinate tolerance for production of robust non-GM herbicide resistant commercial rice, it does to have sufficient tolerance to complicate the use of rice varieties which contain the bacterial BAR gene. It is likely that TX4 and similar types of red rice present in fields where glufosinate resistant rice is used will not be efficiently controlled by glufosinate. The amount of such red rice would increase in response to selection pressure. Since TX4 can readily cross sexually with commercial rice varieties, it is also possible that the bacterial BAR gene could move into TX4-type red rice. Because red rice shatters and can remain dormant in the soil for many years, this might result in fields that are essentially permanently contaminated with red rice that contains both the resistance mechanism in TX4 as well as the bacterial BAR gene.

In agreement with previous work by Steel *et al.* (2000), substantial herbicide tolerance was sometimes also seen in "sensitive" varieties, particularly with cool temperatures and lower light levels. Furthermore, in agreement with previous work by Toldi *et al.* (2001), it was found that tolerance could be induced in normally sensitive rice varieties by culture on sublethal concentrations of glufosinate. These mechanisms likely contributed to the variability observed in the studies reported here. More importantly, they are also likely to also contribute to periodic failure of red rice control by glufosinate under conditions of actual agricultural practice using glufosinate resistant rice varieties and contribute to the potential movement of the BAR gene into red rice.

Glutamine synthetase, the target enzyme for glufosinate, is a complex enzyme with multiple isoforms and with an array of complex interactions. While the mechanism of resistance in TX4 was not elucidated, the work presented here raises numerous questions concerning basic nitrogen metabolism and the regulation of glutamine synthetase over the life of a plant. One factor in particular that merits comment was the dramatic transition in the glutamine synthetase isozyme profile over the 2-4 leaf stage during which glufosinate is typically applied to rice seedlings. Such differences in isozyme profile are not surprising since two leaf seedlings are typically still utilizing nitrogen reserves from the seed while these have been exhausted by the time plants have reached the four leaf stage. These changes in glutamine synthetase composition and/or regulation may be responsible for part of the variation seen in glufosinate tolerance under field conditions.

During the early part of this study red rice samples, including TX4, and IRGC *Oryza rufipogon* 105491 were included in a routine microsatellite analysis that was being conducted for another

project in the Park laboratory. The surprising results from this experiment led to the work on rice taxonomy and genetic relationships which are discussed in Chapters II, III and IV.

Traditionally, all of the red rice present in commercial fields in the United States has been considered to be in the same genus and species as commercial rice, *Oryza sativa*. Consistent with this view, most of the red rice in commercial fields was found to be closely related to Asian rice varieties in the *indica* subspecies of *Oryza sativa*. Other varieties, such as MS5, were found to be closely related to the commercial rice varieties grown in the United States which are in the *japonica* subspecies of *Oryza sativa*. Most surprising was the discovery that a large proportion of the red rice ecotypes with black hulls were sufficiently divergent from either the *indica* or *japonica* subspecies of *Oryza sativa* to be considered a separate species. Importantly, this included TX4. As discussed in Chapter II, the proper classification of these samples was unclear since they grouped both with samples that have been classified as *Oryza nivara* and those which have been classified as *Oryza rufipogon*.

As discussed in Chapter III, this distinction is very important since *Oryza rufipogon* is subject to stringent regulation under the Federal Noxious Weed Act. *Oryza nivara*, on the other hand, is completely unregulated. Part of the confusion regarding *Oryza rufipogon* is that this term is used in several different ways. It is sometimes used broadly to refer to all Asian red rice. Other workers reserve the term *Oryza rufipogon* for rhizomatous perennial red rice and classify the annual types as *Oryza nivara*. Under the Federal Noxious Weed Act, however, *Oryza rufipogon* is defined as an annual species (Plant Protection Act (PPA).
www.aphis.usda.gov/ppq/weeds/weedhome.html.

To help resolve this issue, microsatellite, as well as transposable element based, markers were used to examine all of the available *Oryza rufipogon* from the US National Small Grains Collection (NSGC). Since the samples from the NSGC are annual types, samples of perennial *Oryza rufipogon* were also obtained from Asia and from a small area of infestation in the Florida Everglades. The data was then analyzed using both genetic distance and model-based statistical methods.

This analysis demonstrated that all of the red rice samples collected from US commercial fields could be readily distinguished from the perennial rhizomatous *Oryza rufipogon* from Florida described by Vandiver et al. (1992) and from all of the perennial *Oryza rufipogon* samples from China provided by Dr. Snow. The perennial rufipogon samples from Vietnam were more similar, but could still be distinguished from all of the red rice in US commercial fields both microsatellite markers and the SINE and MITE markers that have been used previously to resolve the annual species *Oryza nivara* from the perennial species *Oryza rufipogon*. These results were not surprising since all of the red rice samples from US commercial fields are annual species and none have rhizomes. Thus, none of the red rice samples from US commercial fields fit within strict definition of *Oryza rufipogon* based on either morphological characteristics or DNA marker data and they are more properly classified as *Oryza nivara*.

The Federal Noxious Weed Act attempts to make a distinction between annual *Oryza rufipogon*, which is regulated, and annual *Oryza nivara*, which is not regulated. This distinction is supported by the fact that a number of annual plants in the US National Small Grains Collection are classified as *Oryza rufipogon* while others annuals in the collection are classified as *Oryza nivara* However, in all of the DNA marker analyses, the annual samples that have been classified

as *Oryza nivara* and the annual "*Oryza rufipogon*" from the NSGC were found to be indistinguishable from the annual *Oryza sativa* and red rice samples. These results indicate that the distinction between *Oryza rufipogon* and *Oryza nivara* accessions in the NSGC is not scientifically valid and are in agreement with taxonomist who explicitly state the terms "annual *Oryza rufipogon* and *Oryza nivara* are synonymous (e.g. Cheng *et al.* 2003). Thus, not only does none of the red rice in US commercial fields fit under the strict definition of *Oryza rufipogon* as a perennial rhizomatous species, none of it can be validly distinguished from *Oryza nivara* and/or *Oryza sativa*; neither of which is regulated under the Federal Noxious Weed Act.

It should be emphasized that confusion surrounding the classification of samples from the US National Small Grains Collection is not indication of lack of due diligence. There are several factors that could contribute to misclassification. One of the key factors could be seedbanks' bias towards collecting samples that have a high seed set. True perennial *Oryza rufipogon* typically has a low seed set. When the perennial types do set seed, there is a much higher degree of outcrossing than in the annual species. This, combined with the possibility of contamination and outcrossing during seed increase, could have resulted in the NSGC samples no longer being true to type. Regardless of the source of the error for the NSGC samples, it is imperative that the classification of these samples be resolved due to their "standards" status. Classification of red rice as *Oryza rufipogon* based solely on these samples would have far reaching effects for the US rice industry.

Chapter III also introduced a new method of statistical analysis that had previously not been utilized in rice genetic research. The model based statistical analysis program STRUCTURE was utilized to analyze the molecular marker data, and in all instances complemented the distance

based statistical analysis used previously. One of the advantages of the STRUCTURE based analysis is the ability to deal with admixture between populations.

Analysis of red rice samples collected from across the rice producing areas in Texas that is presented in Chapter IV reveals that the red rice population in Texas is quite diverse. It also revealed that black hulled red rice can be divided into three groups using STRUCTURE. *Oryza nivara* can be separated into two subgroups, one more similar to IRGC 105491 and another group very similar to TX4. A third group of black hulled red rice appears to represent a non-random mix of the *Oryza sativa* ssp. *indica* and *Oryza nivara* types. All three types are widely distributed across the rice producing areas of Texas. Most were found in almost every county, and all three can be found in an area as small as nine square meters. There are general trends in the geographical distribution of the different types of red rice. For example, there were more red rice samples closely related to IRGC105491 compared to those close related to TX4 in the southwestern part of the Texas rice belt. Some of theses differences may reflect differences in cultural practice and soil type. However, it may also reflect the local history of accidental introduction of specific types of red rice into individual farms and their movement between fields.

Of particular interest is the distribution of samples that are identical to the glufosinate resistant red rice TX4. Of the 240 samples analyzed, 23 were found to be identical to TX4. These samples were found in six of the eleven counties samples, and samples belonging to the same subgroup as TX4 were found in all but one county. The wide spread occurrence of TX4 types could have significant implications for the release of glufosinate resistant commercial varieties if these other TX4 ecotypes also prove to have glufosinate tolerance.

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