ANNUAL REPORT
COMPREHENSIVE RESEARCH ON RICE
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PROJECT TITLE: Application of Molecular Marker-Assisted Selection to Rice Improvement

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OBJECTIVES AND EXPERIMENTS CONDUCTED BY LOCATION TO ACCOMPLISH OBJECTIVES:

The overall objective is to integrate molecular genetic approaches and conventional breeding methods to develop improved germplasm for the California rice industry. Primary emphasis will be placed on the development of molecular (DNA) markers that can be used to predict the presence or absence of a trait of interest (e.g. disease resistance, cold tolerance, grain quality) and the application of these markers via molecular marker-assisted selection to expedite the identification of useful germplasm and streamline the breeding of improved varieties.

1) Disease resistance
    1. Stem Rot: Our objective is to determine the genetic basis of resistance/tolerance to the stem rot pathogen *Sclerotium oryzae* and utilize that information to develop and implement tools for improving California rice varieties.

2) Cold tolerance
    1. Seedling Stage: Our objective is to determine the genetic basis for seedling cold tolerance exhibited by M-202 and use that information to develop germplasm with improved seedling cold tolerance for use in the RES breeding programs.

2. Booting Stage: Our objective is use the M-202/IR50 recombinant inbred line population to characterize tolerance to cold-induced blanking and identify genes that are involved in this tolerance.

SUMMARY OF 2008 RESEARCH (MAJOR ACCOMPLISHMENTS) BY OBJECTIVES:

1) Stem Rot:
    1. Assessment of parental lines of the R22400 mapping population: As of this report, testing of 87Y550 and 96Y480 breeding lines, which are the parents of the R22400 mapping population, is still underway using the paper disc method described in the 2007 annual report. The paper disc method has undergone very minor modification. Strips of filter paper are being used instead of discs. This facilitates easier and more rapid application of the fungal hyphae to the rice sheaths. In preliminary tests with younger/smaller seedlings, 87Y550 (field tolerant) and 96Y480 (field susceptible) did not exhibit any significantly different response to the stem rot fungus. Three different stem rot isolates (cc21, cc3, and cc36) were used. If additional tests produce similar results, assessment of the R22400 recombinant inbred line mapping population (150 RILs at the $F_{6:7}$ generation) will not be performed. As an alternative, these RILs may be field tested at the RES depending on their interest in this material.
    2. Additional mapping population development: A population derived from a cross between 87Y550 and S-102 was advanced two generations by single-seed descent. F_3 seeds from approximately 330 individual F_2 plants were planted and thinned to single F_3 plants per original F_2. F_4 seeds were harvested from individual F_3 plants. Lines were advanced an additional generation and F_5 seeds derived from the original F_2 plants have been harvested. Further advancement of these lines and bulking of F_7 or F_8 seeds will be performed to produce materials for stem rot evaluation. S-102 has consistently scored as very susceptible compared to 87Y550, the original tolerant long grain breeding line developed at the RES.
    3. Characterization of stem rot isolates: Two isolates, identified in 2007 as exhibiting different degrees of virulence (cc21>>>cc3) based on visual assessment of paper disc-inoculated plants, were used to examine the response of 87Y550 and 96Y480 (noted above). Preliminary assessment was consistent with the 2007 observation (i.e. cc21 appeared more virulent on both 87Y550 and 96Y480 than cc3). A third isolate, cc36, which was not tested in 2007, appeared to fall between cc21 and cc3 in virulence.

2) Cold tolerance
    1. Seedling Stage:

    Gene discovery: Work continued on the identification of the genes underlying the *qCTS12* and *qCTS4*-associated seedling cold tolerance. Emphasis was placed on *qCTS12* which confers tolerance of M-202 to constant exposure to low temperature (9°C). As described previously (2006 Annual Report for RB-3), two principal

candidates are the *OsGSTZ1* and *OsGSTZ2* genes. These genes encode proteins that are members of the zeta class of glutathione S-transferases (GSTs). GSTs are members of a large family of enzymes involved in detoxification of pesticides and aid in the reversal of reactive oxygen species and other oxidative damages.

To determine if either of these genes is responsible for the M-202 seedling cold tolerance conferred by *qCTS12*, DNA sequencing and gene expression analysis were undertaken. Most of the genomic sequence containing both genes has been sequenced from M-202 and IR50, as well as in 20 other accessions (see next section "*Germplasm characterization*"). Sequencing revealed 21 differences or polymorphisms between M-202 and IR50 including one 8 bp insertion (polymorphism # 1 shown in Figure 1) present in M202 but not in IR50 and 20 single nucleotide polymorphisms (SNPs; #2 through 21 in Figure 1). The majority of SNPs identified were located in introns of the genes. There were no SNPs located in the coding regions of *OsGSTZ1*, however, three SNPs were detected in the coding regions of *OsGSTZ2* (# 14, 15 and 21). SNP 14 is located in exon 3 and resulted in a synonymous change (i.e. no effect on amino acid). SNPs 15 and 21 were located in exons 6 and 10 and resulted in an isoleucine to valine and a lysine to asparagine change, respectively.

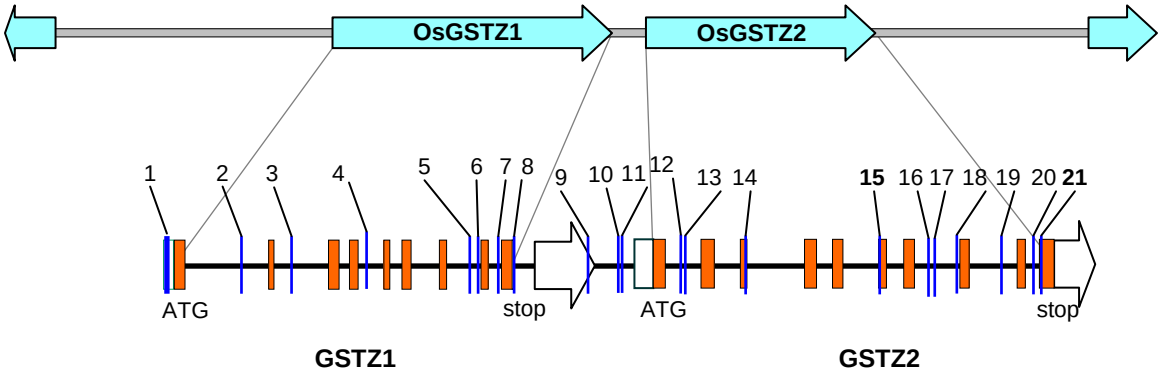

Figure 1. SNPs between M-202 and IR-50 in the *qCTS12* locus. Orange boxes represent exons and solid lines represent introns. Polymorphisms are indicated by numbers (15 and 21 are in bold to indicate a nucleotide change results in an amino acid change). Start and stop codons are indicated.

The SNP differences discovered between M-202 and IR50 in the coding sequence of *OsGSTZ2* suggest that this gene may be responsible for *qCTS12*-associated seedling cold tolerance. It is possible that these SNPs may result in differences in gene expression in IR50 and M-202. The close proximity of *OsGSTZ2* to *OsGSTZ1* suggests that the promoter sequence for *OsGSTZ2* may be within the *OsGSTZ1* gene. Although the SNPs located in *OsGSTZ1* do not alter its coding sequence, they may effect the expression of *OsGSTZ2*. Another possibility is that SNP 15 and 21, which lead to amino acid differences between IR50 and M-202 versions of *OsGSTZ2*, create an alteration in the structure of the enzyme, resulting in a lower specific activity.

Quantitative PCR analysis of the expression of the two genes in IR50 and M-202 before and during cold stress has been repeated multiple times. The data indicate that *OsGSTZ2* increases in expression by more than 6.5-fold in M-202 after 24 hours of growth at 9°C, whereas expression in IR50 increases less than 3-fold (Table 1). This suggests that IR50 may produce less of the *OsGSTZ2* protein under cold stress compared to M-202, which may also contribute to the sensitivity of IR50 at 9°C.

Table 1. Relative expression of *OsGSTZ* genes under constant 9°C

	OsGSTZ1			*OsGSTZ2*		
	Time Points (hours)					
Variety	0	6	24	0	6	24
M-202	1	0.33	0.167	1	1.09	6.69
IR50	1	0.41	0.294	1	2.11	2.93

* Baseline level of expression set at 1 (time point 0 hour). Other values represent the relative amounts of transcripts (i.e. gene expression).

Additional gene expression studies are underway to examine the expression of other genes in the *qCTS12* locus to determine if any of these candidates exhibit different expression in M-202 and IR50 during low temperature exposure. The same type of analysis will also be conducted for genes at the *qCTS4* locus.

Germplasm characterization: In 2007, two sets of rice varieties from the USDA collection (each consisting of 96 entries with some overlap; total of 186 unique accessions, available on request) were identified using *qCTS4* (intermittent low temperature - leaf yellowing and seedling stunting) and *qCTS12* conditions (constant low temperature - leaf necrosis and seedling wilting). Tolerance (resistance) and sensitivity (susceptibility) to low temperature conditions were assessed visually. Each set consists of 48 varieties rated the most tolerant and 48 varieties rated the most sensitive with M-202 and IR50 as the tolerant and sensitive checks. This year 20 varieties (10 most tolerant and 10 most sensitive) from the *qCTS12* set were examined (Table 2). Reaction of these varieties to constant 9°C was consistent with previous observations from 2007. In addition, the regions of the *OsGSTZ* genes that contained different DNA sequences were analyzed in each of these accessions (Table 3). None of the sequence differences shown in Table 3 exhibit a complete correlation with tolerance or sensitivity although it should be noted that there may be some correlation (e.g. all the tolerant lines contain the M-202 sequence at polymorphism #15). Also, although the *qCTS12* gene (or genes) contributes a large part of the tolerance of M-202 to constant low temperature exposure, there are other loci (genes) involved in the expression of this trait.

Assessment of cold tolerance: In addition to molecular analysis of the tolerant and sensitive rice germplasm, these materials were also examined in conjunction with efforts to identify different assessment or phenotyping methods. During 2008, three methods (or metrics) were examined to complement or replace the visual rating method used for assessing cold tolerance under *qCTS12* and *qCTS4* conditions. These

phenotyping methods included 1) measurement of relative growth rate, 2) assessment of photosynthetic capacity by measuring photodamage, and 3) assessment of cell membrane damage through electrolyte leakage measurements.

Table 2. Varieties selected for characterization of *qCTS12* seedling cold tolerance

PI Number	Accession Name	Country of Origin	*qCTS12* Score	Rating
391859	Gambiaka Sebela	Mali	1.7	R
439107	CH 153-138	Chile	1.9	R
391756	Sukananadi B	Indonesia	2	R
400708	GPNO 23386	Taiwan	2	R
215917	Tainan 5	Taiwan	2.1	R
401431	Ching Yueh 1	China	2.1	R
154563	Taino 33	Taiwan	2.2	R
602637	WAB462-10-3-1	Cote D'Ivoire	2.2	R
392656	Kahago ex Mwabagale 1/146	Tanzania	2.2	R
615210	Shangyu 394	China	2.2	R
373346	Kaluwee	Sri Lanka	9	S
431086	Sadri Dum Siah	Iran	9	S
414712	H 5	Sri Lanka	9	S
220758	Urang Urangan 89	Indonesia	8.9	S
389360	Wong Chim	Honk Kong	8.8	S
403703	Makalioka 752	Madagascar	8.7	S
420241	Juma 58	Dom. Republic	8.7	S
615033	4595	China	8.7	S
615222	Zhongbai No4	China	8.7	S
373536	ARC 10633	India	8.6	S

* *qCTS12* scores for M-202 and IR50 are 3.0 and 8.1, respectively.

Rating: R= resistant (tolerant), S = susceptible (sensitive)

Relative growth rate involves comparing the difference in growth of tolerant and sensitive rice seedlings at low and normal temperatures. Measurements of seedling height (from soil surface to tip of longest leaf) and leaf area (sheath and blade) were taken/calculated. No significant differences between M-202 and IR50 were observed during the time frame examined (data not shown) and this method was not used to examine the other germplasm.

Cold stress is known to affect photosynthetic capacity of plants, especially under high light conditions. Photosynthetic capacity is reduced under cold stress due to accumulation of photodamage. During chilling stress, damage can occur at photosystem I or II (PSI, PSII) due to inhibition of photosynthetic capacity, reduced

Table 3. Single nucleotide polymorphisms sequenced from M-202, IR50, and 20 accessions

		Single nucleotide polymorphisms*																		
Line	*qCTS12*	2	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
391859	R	t	a	c	g	c	t	t	c	t	a	t	a	a	g	t	a	t	a	t
439107	R	t	a	c	g	c	t	t	c	t	a	t	a	a	g	t	a	t	a	t
400708	R	t	a	c	a	t	-	t	c	t	a	a	g	a	g	c	g	c	a	a
391756	R	t	a	c	g	c	t	t	c	t	t	t	a	a	g	t	a	t	a	t
401431	R	-	a	c	g	c	t	t	c	t	a	t	a	a	g	t	a	t	a	t
215917	R	-	a	c	g	c	t	t	c	t	a	t	a	a	g	t	a	t	a	t
615210	R	-	a	c	g	c	t	t	c	t	a	t	a	a	g	t	a	t	a	t
154563	R	t	a	c	g	c	t	t	c	t	a	t	a	a	g	t	a	t	a	t
392656	R	t	a	c	g	c	t	t	c	t	a	t	a	a	g	t	a	t	a	t
602637	R	t	a	c	a	t	-	t	c	t	t	a	g	a	g	c	g	c	a	a
M202	R	t	a	c	g	c	t	t	c	t	a	t	a	a	g	t	a	t	a	t
IR50	S	-	t	t	a	t	-	g	t	c	t	a	g	g	t	c	g	c	c	a
373346	S	t	a	c	g	c	t	t	c	t	a	t	a	a	g	t	a	t	a	t
414712	S	t	a	c	a	t	-	t	c	t	t	a	g	a	g	c	g	c	a	a
431086	S	-	t	t	a	t	-	g	t	c	t	a	g	g	t	c	g	c	c	a
220758	S	-	t	t	a	t	-	g	t	c	t	a	g	g	t	c	g	c	c	a
389360	S	-	a	c	a	t	-	t	c	t	t	a	g	a	g	c	g	c	a	a
615033	S	-	a	c	a	t	-	t	c	t	t	a	g	a	g	c	g	c	a	a
403703	S	t	t	t	a	t	-	g	t	c	t	a	g	g	t	c	g	c	c	a
420241	S	t	t	t	a	t	-	g	t	c	t	a	g	g	t	c	g	c	c	a
615222	S	t	a	c	a	t	-	t	c	t	t	a	g	a	g	c	g	c	a	a
373536	S	t	a	c	a	t	-	t	c	t	t	a	g	a	g	c	g	c	a	a

* Numbers are as indicated on Figure 1. Data for #1 and #3 polymorphisms not available.

repair of photodamage or antioxidant activity. Interestingly, in chilling resistant plants, the site for photodamage upon chilling appears to be located at PSII, whereas in chilling sensitive plants, PSI is the main site for photoinhibition. Photodamage is measured using a fluorometer as the ratio Fv/Fm (Fm = maximum fluorescence, Fv = Fm – Fo, where Fo = minimum fluorescence), which decreases due to photoinhibition and damage. Fluorometer measurements were taken for all accessions every day for 4 days. On the first day, plants growing at 25°C were measured and then moved to constant 9°C conditions. Measurements were taken every 24 hours at 9°C for the next two days. On the third day plants were returned to 25°C for a final measurement. Of the 20 rice accessions tested, a steady increase in photodamage (decrease in Fv/Fm ratio) was seen for each day at 9°C followed by a recovery at 25°C (Figure 2). Under the conditions of the experiment, no significant differences in photodamage were observed between resistant and susceptible lines. It is likely that changing the

conditions (e.g. using *qCTS4* conditions) and/or when measurements are taken will impact the utility of this method for distinguishing between accessions.

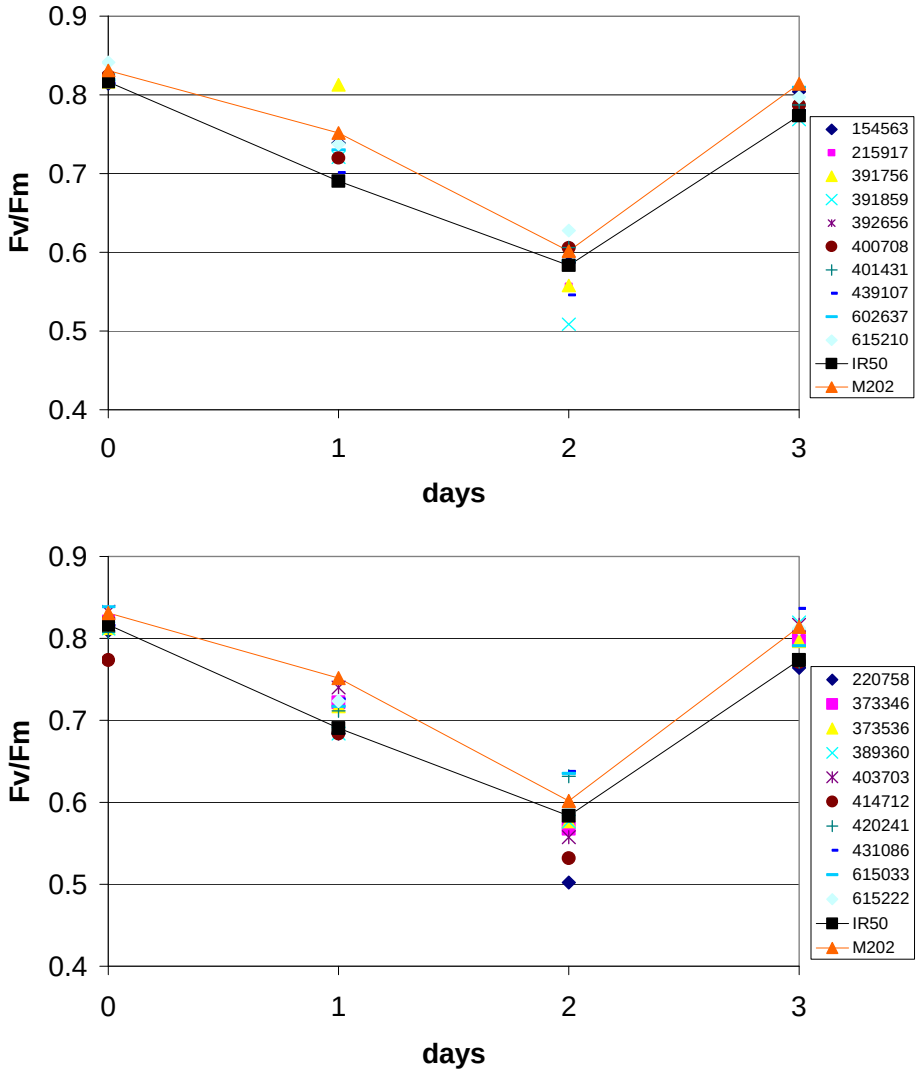

Figure 2. Photodamage (Fv/Fm) of rice accessions exposed to constant 9°C. Upper graph compares resistant germplasm and lower graph compares susceptible germplasm to checks M-202 and IR50.

Measuring solute leakage from plant tissue is a long-standing method for estimating membrane permeability in relation to environmental stresses, growth and development, and genotypic variation. Damaged membranes will slowly 'leak' electrolytes into the surrounding medium, which can then be detected by an electrical conductivity meter. Electrolyte leakage (EL) measurements aid in determining the degree to which a plant membrane is damaged due to chilling and may be used as a quantitative indicator for tolerance or susceptibility. EL measurements were taken from leaf samples prior to exposure to 9°C and after 2 days of treatment. On average,

the 10 sensitive accessions had > 3.5-fold higher EL measurement than the 10 resistant accessions (Figure 3).

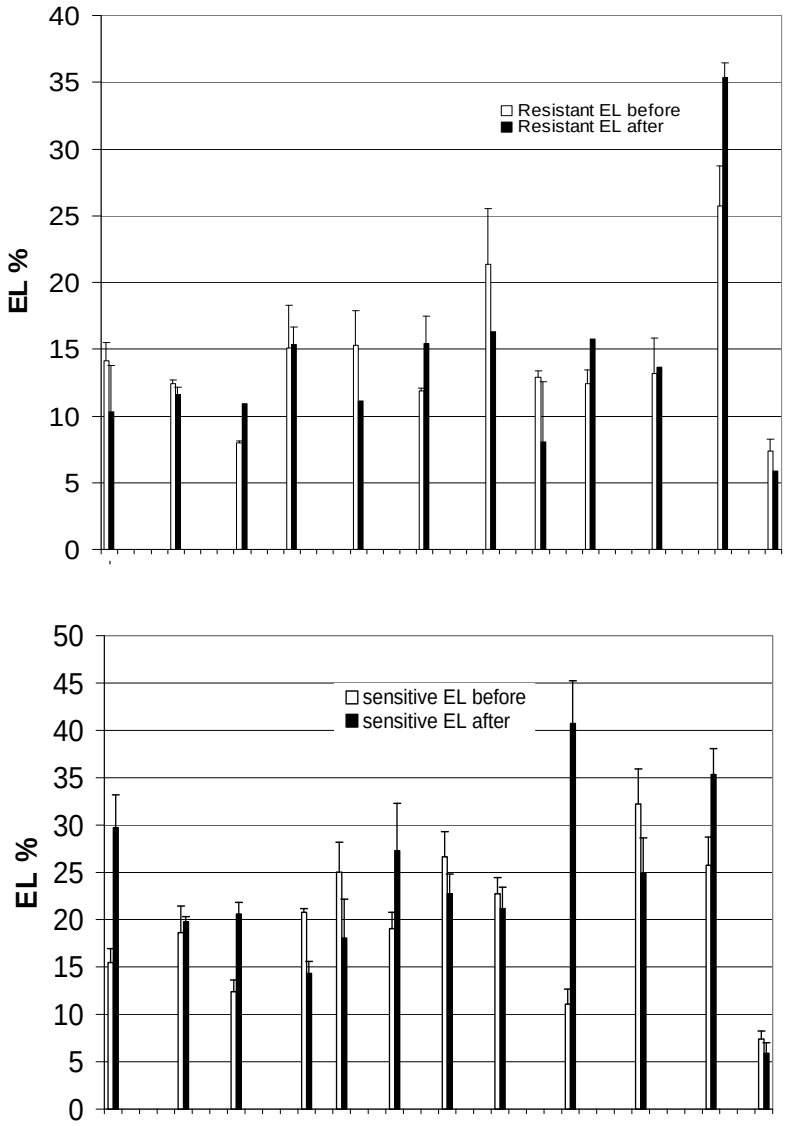

Figure 3. Electrolyte leakage (%) of rice accessions before (open bars) and after (solid bars) exposure to 9°C. Resistant accessions shown in upper graph and susceptible accessions in lower graph. Accessions are as listed in legend of Figure 2 (from left to right in this figure, i.e. M-202 is farthest right in both graphs). Note that Y-axes of graphs are not represented to scale.

Of the methods of assessment examined, electrolyte leakage (EL) measurement is the most promising with regard to screening large numbers of rice accessions. Although all of the resistant varieties were scored visual more tolerant than M-202, the EL results suggest that at least for this trait and the experimental conditions, M-

202 appears to be the least affected by low temperature. Further studies to refine both the EL and photodamage assays are planned.

Germplasm amplification and development: In preparation for future experiments, the two sets of germplasm identified for *qCTS12* and *qCTS4* characterization were grown for seed purification/amplification. Work was also continued on the development of near-isogenic lines (NILs) containing *qCTS4* and *qCTS12* regions from M-202 in the genetic background of IR50. The goal of this effort is to develop IR50 NILs containing *qCTS4* only, *qCTS12* only, and both loci from M-202. BC_4F_4 seeds from 14 lines developed by Dr. V. Andaya were planted for seed (amplification and advancement) and DNA analysis. All these lines were derived from backcrossing a recombinant inbred line (MI-71), which contains both *qCTS12* and *qCTS4* from M-202, with IR50. Advanced backcross lines were selected using DNA markers for *qCTS4*. Marker analysis of the 14 lines grown this year indicates that some of these lines also contain *qCTS12*. This was confirmed by testing BC_4F_5 seedlings from these BC_4F_4 lines under *qCTS12* assay conditions (the BC_4F_5 seeds were harvested this year from individual BC_4F_4). These lines will be further backcrossed to IR50 and marker-assisted selection will be used to obtain *qCTS12* only lines. Genotyping of individual BC_4F_4 plants using about 120 microsatellite DNA markers revealed that 2 to 8% of the markers were of the M-202 parent. Additional backcrossing and/or marker-assisted selection will be performed to develop the desired NILs for field testing.

2. Booting Stage:

Mapping population assessment: Data were collected on panicles harvested from three replications of about 480 MI recombinant inbred lines (F_{10} generation; derived from the cross M-202/IR50) that were planted in the UC Davis rice nursery in 2007. Measurements of panicle length, exsertion (M-202 – non-exserted, IR50 – exserted), and total weight of seeds of 10 panicles from each line/rep (~1440) were taken. Data on fertility have not been recorded. Heading date data from the Davis test and a test at Biggs were recorded in 2007. These lines have previously been used in our seedling cold tolerance research and have been extensively characterized with DNA markers. Following collection of fertility data from Davis test and tests conducted at Davis and Biggs in 2006, MI lines for cold blanking studies will be selected.

3) Additional 2008 research activities (RF-1 related)

1. Generation advance:
During the 2008 growing season, approximately 5,000 lines (mapping populations, mutant populations, germplasm accessions) were grown for advancement or seed amplification/purification in the greenhouse. In addition, 7,200 rows (including 809 marker rows) were planted in the UC Davis rice research facility.

Mutant population: The varieties S-102 and Terso were subjected to mutagenesis in 2007. Seeds of S-102 were subjected to gamma irradiation (200, 205, and 300 Gray treatments) and chemical mutagenesis (ethyl methanesulfonate – EMS, sodium azide – Az, and sodium azide plus methyl nitrosourea – AzMNU). Terso seeds were subjected to AzMNU mutagenic treatment only. Treated seeds were planted in the

field (gamma irradiation) and/or greenhouse (chemical). Seeds from the resulting plants (M_2 seeds) were collected in 2007. This year M_2 seeds from S-102 (all treatments except EMS which yielded very few fertile plants) and Terso were sown in the greenhouse and field.

Table 4. Mutant lines planted in 2008

Variety	Treatment	Greenhouse	Field
S-102	Az	100	480
S-102	AzMNU	100	1957
S-102	200 Grays	100	320
S-102	250 Grays	100	385
S-102	300 Grays	374	2309
Terso	AzMNU	2675	none

These mutant populations are being screened for useful traits (e.g. glabrous S-102, early maturing and semidwarf Terso). A total of 83 of the Terso lines planted exhibited segregation for early flowering (this represents a partial screen of the population as a large percentage of the lines were planted late and flowered somewhat synchronously due to shorter days).

Mapping population: Three recombinant inbred line mapping populations were also advanced in the field this season: Cypress/Lagrue (423 lines planted, 305 harvested), Lemont/L202 (185 lines planted, still being processed), L202/Lemont (216 lines planted, still being processed).

PUBLICATIONS OR REPORTS:
None at this time.

CONCISE GENERAL SUMMARY OF CURRENT YEAR'S RESULTS:

In 2008, research continued on the genetic analysis of stem rot and seedling cold tolerance with the ultimate goal of identifying and developing DNA markers for use in breeding efforts. The analysis of stem rot tolerance continues to be hampered by difficulties in phenotyping (i.e. scoring) this complex trait. Mapping populations have been and are being developed using parental rice lines that exhibit differential responses to stem rot fungus. However, the ability to accurately score subtle differences in lines derived from crossing these parents remains questionable. Significant advances have been made in the genetic analysis of seedling cold tolerance with emphasis on the *qCTS12*-associated tolerance. Characterization of candidate *qCTS12* genes continues and electrolyte leakage measurements have been identified as a possible alternative phenotyping method to that of visual rating which is more subjective. Development of mutant and mapping populations for genetic studies and identification of useful germplasm continues. Assessment of these populations has been initiated and will continue in 2009.