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Sequence Analysis of Herbicide Target Genes in Herbicide-Tolerant Rice

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Sequence Analysis of Herbicide Target Genes in Herbicide-Tolerant Rice

An Honors Thesis submitted in partial fulfillment of the requirements of Honors Studies in
Biology

By

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Biology

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Abstract:

The tolerance of mutagenized rice (*Oryza sativa*) lines to selector herbicides was investigated and the sequence of *ALS* gene was analyzed in plants that survived treatment with 4x the label rate of imazethapyr herbicide. This was done to determine if insensitivity to imazethapyr is due to mutation(s) in the herbicide binding site. Seedlots previously treated with ethyl-methyl sulfonate were planted in the field and 3-leaf seedlings were treated with various herbicides to screen for herbicide-tolerant mutants. Seeds from survivors composed the rice lines tested in the current research. Seeds were planted in the greenhouse and 3-leaf seedlings were treated with either 4x the recommended rate of imazethapyr, 4x mesotrione, 2x topramezone, 1x fluridone, 1x fluazifop, or 1x clethodim with their respective recommended adjuvants. Rice injury was assessed visually 14 days after treatment, leaf tissue samples were collected from survivors, and processed for sequencing of herbicide target genes. RNA was extracted from frozen plant tissues and converted to cDNA. PCR primers were designed to amplify the target genes for mesotrione (*HPPD*) and imazethapyr (*ALS*), respectively. The expected PCR product was isolated via gel electrophoresis, purified, and sent off for sequencing. Attempts to amplify *HPPD* failed; therefore, only *ALS* was sequenced. *ALS* sequence analysis revealed a mutation, Ser653Asn. This is one of the *ALS* point mutations that confer high resistance to imidazolinone (i.e. imazethapyr) and broad cross resistance to other *ALS*-inhibitor herbicide families including sulfonylurea⁸.

Keywords: *ALS, acetolactate synthase, Oryza sativa, herbicide resistance*

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Table of Contents

Introduction.....	5
Materials and Methods.....	9
Results.....	14
Discussion.....	20
Appendix.....	23
Works Cited.....	24

Introduction

Rice (*Oryza sativa*) is a food staple to much of the world, making up over 50% of the world's diet²⁸. The United States is the third highest exporter global of rice, with rice being one of its most important agricultural commodities. Limited to a production region of only 1.1 million hectares, the United States manages to produce 7.12 million mt of rice a year, according to a 2016 study²⁵. Due to an increasing global population, there is a need for increased food production without increasing the planted area². Within the state of Arkansas, rice production reaches a production volume of over 4 million mt of rice a year, constituting 49% of the rice grown in the United States. This makes Arkansas the top producer of rice in the United States, with just under 1.3 million acres used for production²⁷. However, to produce such a high yield of rice, farmers must continually battle agricultural pests in an attempt to reduce crop losses and maintain high production levels. One such agricultural pest is weedy rice (*Oryza sativa*), historically known as red rice. Weedy rice (*Oryza sativa*) is a de-domesticated, weedy relative of rice²⁴ that has been an obstacle in the rice industry for the last century^{4, 26}, causing an estimated \$45 million in agricultural damages to the United States alone. Weedy rice is the same species as cultivated rice; therefore, it is a challenge to combat, as its physiology is similar to that of rice. This means that herbicides that kill weedy rice also kill, or severely injure, cultivated rice. Furthermore, weedy rice can generally adapt to environmental stress (i.e., water stress) better than cultivated rice. Weedy rice reduces rice productivity through competition for resources and reduces crop quality by seed contamination of the harvested rice grain²⁶. To reduce the impact this pest has on rice cultivation, herbicide-tolerant rice technology has been developed, and widely adopted by growers, so that certain herbicides can be applied to control weedy rice without killing domesticated rice.

The first commercially successful herbicide-tolerant rice technology was Clearfield® rice. This allows application of ALS-inhibitor imidazolinone herbicides (i.e. imazethapyr, imazamox), which are effective in controlling weedy rice. While ALS herbicides make up the highest number of herbicides on the market today, weeds with resistance to ALS herbicides make up the largest number of herbicide-resistant weeds, indicating that ALS herbicides are gradually becoming ineffective. ALS-inhibiting herbicides inhibit the first reaction step in the biosynthesis pathway of branched chain amino acids (valine, leucine, isoleucine) by inhibiting the activity of ALS, which catalyzes this reaction. The molecular structure of ALS-inhibiting herbicides allows bonding with certain amino acid residues at the ALS catalytic site, effectively blocking the entrance into the binding pocket and preventing the substrates from entering³¹. ALS herbicides can be absorbed through leaves or roots. This, ultimately, makes the herbicide effective against weeds prior to, or after, emergence. The plant dies from deficiency of these amino acids. Herbicide families such as imidazolinones, triazolopyrimidines, triazolinones, sulfonanilides, and pyrimidinyl benzoates are all examples of ALS-inhibiting herbicides²⁰.

Two mechanisms have been proposed for ALS herbicide resistance. Through collaboration with the Washington University in Missouri, the Burgos lab has verified that outcrossing with herbicide-resistant rice and selective pressure are the two mechanisms selecting for herbicide resistance. A common weed control practice is to plant herbicide-resistant crops to control weeds that would otherwise not be controlled when planting herbicide-sensitive crops. However, when working with weedy rice, a challenge presents itself. Being of the same species allows cross-pollination between the domestic rice and weedy rice. This can ultimately result in gene flow from crop to weed, endowing weedy rice with herbicide resistance. The other mechanism is through herbicide-driven selective pressure. Weedy rice that can tolerate the

deleterious effect of an herbicide are selected for, allowing for that specific variant of weedy rice to proliferate and continue to evolve a hardier resistance to herbicides³². These variations can stem from alternate enzyme formations that can have a variety of point mutations in amino acid sequence, overexpression of the *ALS* gene, or enhanced rates of herbicide metabolism¹⁴. Multiple examples of mutations in the *ALS* gene have been documented, with many conferring broad-spectrum resistances to ALS-inhibiting herbicides. Seven known amino acid mutation loci have been documented among ALS-resistant weed species thus far: Ala 122, Pro 197, Ala 205, Asn 376, Arg 377, Trp 574, Ser 653, and Gly 654¹⁵.

In the instance of Ala122, multiple mutations have been reported, where Ala122 has been substituted for Tyr¹⁴, Asn, Val, and Thr²² and Ser²¹. In all cases, the mutations have conferred some type of resistance to herbicides. In specific, Tyr and Asn substitutions result in resistance to ALS inhibitors^{14, 22}. Pro197 has eleven documented mutations where Pro197 is substituted for Tyr³⁴, Ile⁷, Glu¹⁷, Asn¹⁸, Arg, Ala, Gln, Leu, Ser³³ and Thr¹⁰. All of these mutations induced ALS resistance. One substitution mutation in Ala205 has been documented, where Ala was substituted for Phe, conferring ALS-inhibitor resistance³. In a similar case, one mutation has been reported for Asn376, where substitution has occurred, replacing Asn with Glu, giving ALS-inhibitor resistance once again¹⁶. Arg377His is a reported mutation that, similar to previously mentioned mutations, also gives resistance to ALS-inhibitors¹¹. Four mutations have been reported in Trp574, where Met, Leu¹⁹, Gly⁹ and Arg¹² also give ALS-inhibitor resistance to plants carrying the mutations. At Ser653 in the *ALS* gene, three mutations have been reported where Ile³⁰, Thr¹, and Asn¹³, where all three have been linked to ALS-inhibitor resistance. Documented mutations have also occurred at Gly654, where Glu, Tyr, and Asn confer ALS-inhibitor resistance⁵.

In rice production, developing herbicide-tolerant rice cultivars still has value because of the lack of alternative herbicides to control weedy rice. All other weed species in rice can be controlled by various combinations and sequences of herbicides from 15 mode-of-action groups²⁰. The evolution of weed resistance to herbicides is primarily due to the intensive, sustained use of the same herbicide mode of action. For example, the evolution of ALS-resistant weedy rice is the inevitable consequence of planting Clearfield rice[®] for over 20 years. If, during this period, there had been other herbicide-tolerant rice options, the weedy rice resistance evolution could have been avoided. Mutation breeding is a common approach in generating non-transgenic, herbicide-tolerant crops. This research was part of a project that aimed to generate new herbicide-tolerant rice lines by mutagenesis. Selecting for *HPPD*-tolerant mutants with fluridone, mesotrione, and topramezone resistance would usher in a novel generation of herbicide-tolerant rice cultivars that broadens the weed control spectrum of grass and broadleaf weeds. Selecting mutants with ACCase inhibitors quizalofop and clethodim would diversify options for selective grass weed control. With respect to ALS herbicides, the aim was to find possible double mutants, or a novel mutation that would exhibit very high herbicide tolerance and, therefore, excellent crop safety in any environment.

The objectives of this research were to: 1) confirm herbicide tolerance of various mutagen-treated rice lines, 2) determine if mutation(s) in the target site endowed tolerance to any of the *HPPD*-inhibitor herbicides, and 3) determine if mutation(s) in the target site endowed high tolerance to the ALS-inhibitor imazethapyr.

Materials and Methods

This experiment tested mutagen-treated rice seedlots. These seeds were from a batch that had been treated with ethyl-methyl sulfonate (EMS). The seeds were planted in the field at the Southeast Research Center, Rohwer, Arkansas and seedlings were then sprayed with various herbicides including fluridone, mesotrione, topramezone, quizalofop, clethodim, and imazethapyr. The survivors that produced seeds were harvested and planted again in the next season for another round of selection with the same herbicides to purify the lot and produce herbicide-tolerant lines. The herbicide-tolerant rice lines used in this experiment are lines that survived HPPD-inhibitor herbicides (mesotrione 1x, mesotrione 4x, fluridone 1x, and topramezone 4x), one line that survived ACCase inhibitor quizalofop 1x and, one line that survived the ALS-inhibitor herbicide imazethapyr 4x. These seedlots were planted in the greenhouse at the Shult Agricultural Research and Extension Center (SAREC) and sprayed with their corresponding herbicide selectors (Table 1).

The seeds were planted in trays filled with potting soil at ten seeds per tray with four replications, allowing the evaluation of 40 plants per line for each herbicide selector. The herbicide treatments were applied to 3-leaf rice, with recommended adjuvants, at a spray volume of 187 L/ha using the spray chamber.

Table 1. Herbicides tested on the EMS rice lines in the greenhouse, Shult Agricultural Research and Extension Center, Fayetteville, Arkansas 2022.

Seedlot ¹	Herbicide trade name	Common name	Formulation type ²	Application rate lb ai/gal	Formulation concentration lb ai/gal
Quiz	Provisia	quizalofop	L	0.1 (1X)	0.88
Assure	Provisia	quizalofop	L	0.1 (1X)	0.88
Fluri	Brake	fluridone	L	1.0 (1X)	1.2
Callisto	Callisto	mesotrione	SC	0.376 (4X)	4
Topra	Armezon	topramezone	SC	0.033 (2X)	2.8
Imaze	Newpath	imazethapyr	L	0.3 (4X)	2
Cleth	Dakota	clethodim	L	0.2 (1X)	2

¹Quiz = quizalofop; Fluri = fluridone; Topra = topramezone; Imaze -imazethapyr

²Abbreviations: L= liquid; SC = suspension concentrate

The plants were assessed for visible phytotoxicity 14 days after treatment to determine the level of tolerance to the rate of herbicide that was applied. Indicators such as color, necrosis, size, and overall appearance were considered in the damage assessment. Scores were assigned to each plant from 0-100 where 0 = no visible effect and 100 = dead. Scores in the 0-60 range indicate high tolerance to herbicide treatment. Scores in the 61-90 range indicate moderate tolerance. Scores in the 91-100 indicate sensitivity. Tissues were collected from the healthiest plants of each line, flash-frozen in liquid nitrogen, and stored at -80°C until processing. A total of 18 plants were sampled for HPPD sequence analysis and 6 plants for ALS sequence analysis (Appendix table 1).

Prior to DNA extraction, several forward and reverse primers were designed to amplify overlapping regions of the *ALS* and *HPPD* gene. Gene amplification was not successful for HPPD; therefore, only primers for ALS are presented here (Table 2).

Table 2. Primer pairs designed to amplify the rice *ALS* gene.

Primer Name	Sequence
OsALSF1	CACACTCTCCACCCCTCTCT
OsALSF2	TAAGAACCACCAGCGACACC
OsALSF3	GCCTTCCAGGAGACGCCATA
OsALSF5	ATCACCAACCACCTCTTCCG
OsALSF6	GCGCGTCCATGGAGATCC
OsALSR1	TGGGTCATTCAGGTCAAACATGTTTGACCTGAATGACCCA
OsALSR2	AAGAAGGCTTCCTGTATGACGCG
OsALSR3	CGTGGCCGCTTGTAGGTGTAATA
OsALSR5	ATCATGTCCTTGAATGCGCCGGCGCATTCAATTGG
OsALSR6	AATACACAGTCCTGCCATCACGTGATGGCAGG

A total of approximately 10-15 mg of leaf tissue were collected from three biological replicates in a 2.0 mL tube containing five 2.4 mm glass beads and stored at -80°C until processing. Leaf samples were ground using a mixer mill (MM400, Retsch GmbH, Haan, Germany) for 45 sec at 30 Hz and genomic DNA was extracted using a modified CTAB protocol as follows; 600 µL of CTAB buffer was added along with 1 µL of RNase to avoid RNA contamination. The sample was then transferred to a 60°C water bath. Next, 600 µL of chloroform was added, the tubes were vortexed for a few seconds, and centrifuged for 10 min at 13000 rpm. The supernatant was transferred to a new 1.5 mL tube, where 350 µL of cold isopropyl alcohol was added, and each tube was manually inverted multiple times. The samples were then incubated at -20°C for 1 to 2 h and centrifuged for 10 min at 13000 rpm to precipitate the DNA. Once the DNA pellets formed, the supernatant was discarded, and the pellet was washed with 500 µL of 70% alcohol. After discarding the alcohol, the pellet was dried using a

SpeedVac. The DNA pellet was dissolved in 50 μL of H_2O . All DNA samples were kept in a 60°C water bath for 5 min. The DNA was quantified using a NanoDrop (ND-1000, Thermo Scientific, Wilmington, DE).

Since attempts to amplify the *HPPD* gene failed, further research activities focused on amplifying the *ALS* gene for sequence analysis. Initially, amplification of the *ALS* gene was attempted using genomic DNA, but this produced multiple PCR fragments resulting from non-specific binding of primers. Succeeding attempts were done using cDNA. To do this, RNA was extracted using the Qiagen RNeasy Plant Mini Kit. First, 100 mg of plant material from three biological replicates was disrupted using a mixer-mill using liquid nitrogen. The powdered tissue was transferred into microcentrifuge tubes and 450 μL of buffer RLT was added to a maximum of 100 mg tissue powder and vortexed vigorously. The lysate was transferred to a QIAshredder spin column and placed in a 2 mL collection tube. The tubes were centrifuged for 2 min at full speed (13,000 rpm). The supernatant flowthrough product was then transferred to a new microcentrifuge tube taking care not to disturb the cell debris pellet. A 0.5 volume (approximately 200 μL) of 100% ethanol was added to the clear supernatant and subsequently mixed by pipetting. The sample was then transferred to a RNeasy Mini spin column in a 2 mL collection tube. The tubes were sealed and centrifuged for 15 seconds at 10,000 rpm. The flowthrough was discarded and 350 μL of buffer RW1 was added to the RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm to wash the spin column membrane. DNase I stock solution, 10 μL , was added to 70 μL of buffer RDD. This was mixed carefully by inverting the tube and centrifuged briefly to collect any residual liquid. Finally, 80 μL of the DNase I incubation mix was added to the RNeasy spin column membrane and incubated at room temperature for 15 min. After the previous steps, 500 μL of RPE was added to the RNeasy spin

column. This was then centrifuged for 15 seconds at 10,000 rpm and the flowthrough was discarded. This step was repeated, but the column was centrifuged for 2 min at 10,000 rpm. The RNeasy spin column was transferred to a new 1.5 mL collection tube, where 30 μ L of RNase-free water was added to the spin column membrane and centrifuged for 1 min at 10,000 rpm to elute the RNA²³.

Thermofisher's RevertAid RT Reverse Transcription Kit synthesis protocol was followed to convert RNA to cDNA for PCR amplification of the target gene. The PCR reaction consisted of 12 μ L of RNA, 4 μ L of 5x reaction buffer, 1 μ L of RiboLock, 2 μ L of 10 μ M oligonucleotide DNTP, and 1 μ L of RevertAid in that order. The reagents were mixed gently, centrifuged, and incubated for 60 min at 42°C to induce the cDNA synthesis. The reaction was terminated by heating the mixture at 70°C for 5 min. The product was stored at -20°C until it was used for PCR²⁹. The PCR mixture consisted of 5 μ L of cDNA, 0.25 μ L of Taq polymerase, 1 μ L of forward primer, 1 μ L of reverse primer and 3 μ L of water. The PCR protocol consisted of seven steps listed in Table 3.

Table 3. PCR Protocol for gene amplification

Cycle	Temperature	Time (Min)
1	95°C	1:00
2	95°C	1:00
3	56°C	1:00
4	72°C	2:00
5	GOTO step 2	39x
6	72°C	10:00
7	4°C	Indefinite Hold

Nearly the full-length *ALS* gene was amplified (using F5 and R2 primers listed in Table 2). The amplicon was purified and sent for sequencing using primers F5, F3, R3 and F2. The

expected product size was a band around 2 kb. The PCR products were visualized via gel electrophoresis. Multiple reactions were conducted until only the expected amplification product of the target gene was obtained. The PCR product was then purified and sent to Psomagen for sequencing. The resulting overlapping fragments from all individual R and S plants were assembled into one sequence manually to obtain the complete coding region of the *ALS* gene. The nucleotide sequences were translated into open reading frames using the online ExPASy translation tool.

Results

Of the six rice lines planted and treated with herbicides, only four lines survived. The plants from parents treated with the ACCase inhibitors, quizalofop or clethodim, did not survive treatment with 1x the label rate of each herbicide (Table 4). The rice lines with survivors were imazethapyr, topramezone, fluridone, and mesotrione. Tolerance to HPPD inhibitors was heritable based on the response of the offspring to 2x and 4x rates of mesotrione (Figure 1). Sequencing of the *HPPD* gene was initially attempted; however, these attempts were unsuccessful and was discontinued. Thereafter, efforts were focused on sequencing the *ALS* gene.

Figure 1. Mesotrione-treated plant cultivars.

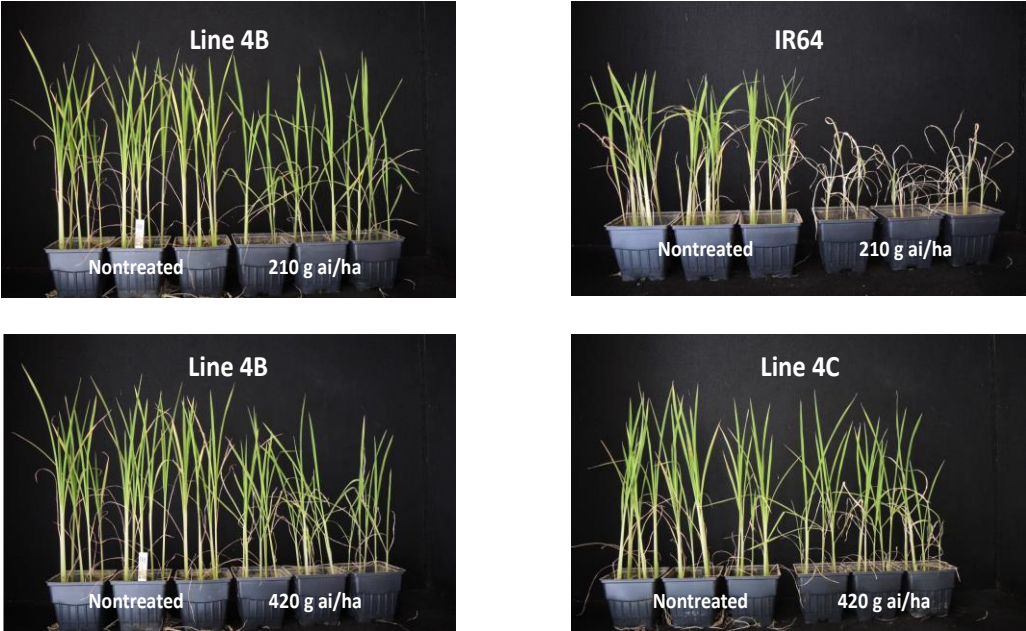


Table 4. Response of EMS rice lines to herbicides in the greenhouse

Seedlot ¹	Rep. ²	Rice injury per plant, 2 wk after treatment									
		1	2	3	4	5	6	7	8	9	10
		-----%-----									
Quiz.	1	100	100	100	100	100	100	100	100	100	100
Quiz.	2	100	100	100	100	100	100	100	100	100	100
Quiz.	3	100	100	100	100	100	100	100	100	100	100
Quiz.	4	100	100	100	100	100	100	100	100	100	100
Assure	1	95	95	95	100	100	95	100	100	X ³	100
Assure	2	95	95	95	95	95	100	95	X	95	95
Assure	3	95	95	95	90	95	X	90	90	90	90
Assure	4	X	90	90	90	90	90	95	95	90	X
Cleth.	1	100	100	100	100	100	100	100	100	100	100
Cleth.	2	100	100	100	100	100	100	100	100	100	100
Cleth.	3	100	100	100	100	100	100	100	100	100	100
Cleth.	4	100	100	100	100	100	100	100	100	100	100
Fluri.	1	85	65	55	95	80	90	45	40	60	X
Fluri.	2	45	50	50	45	45	45	40	40	35	35
Fluri.	3	55	85	45	50	65	65	60	35	60	70
Fluri.	4	90	35	35	X	65	50	35	35	40	85
Topra.	1	90	85	80	35	60	55	50	45	45	45
Topra.	2	0	0	0	0	X	0	0	X	X	X
Topra.	3	70	75	60	80	80	X	X	50	50	65
Topra.	4	60	60	55	55	70	80	65	65	85	80
Meso.	1	85	X	85	80	X	75	X	80	X	80
Meso.	2	80	X	80	80	85	75	X	85	85	85
Meso.	3	X	90	80	X	80	80	100	80	80	80
Meso.	4	80	80	80	80	X	X	80	X	80	85
Imaze.	1	55	50	X	45	45	50	45	50	50	55
Imaze.	2	60	50	55	X	X	X	65	45	40	X
Imaze.	3	55	60	60	60	70	60	50	X	55	50
Imaze.	4	45	40	45	45	40	45	X	X	35	X

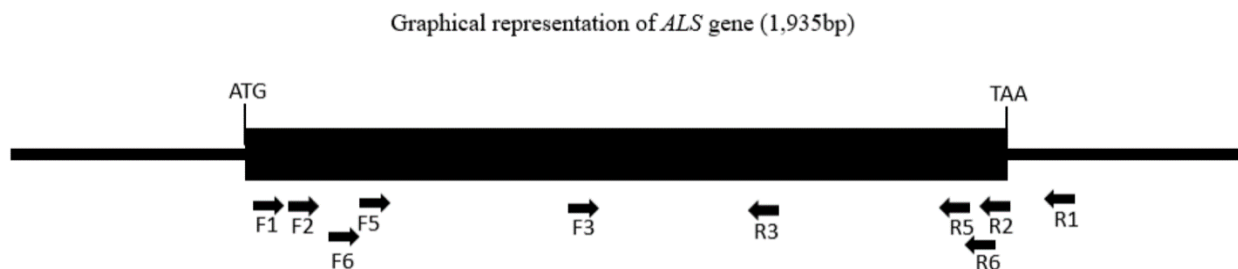
¹Abbreviations: Quiz. = quizalofop; Cleth. = clethodim; Fluri. = fluridone; Topra. = topramezone; Meso. = mesotrione; Imaze. = imazethapyr;

²Rep. = replications

³X means seed did not germinate

Using cDNA from the imazethapyr rice line, the F5R1 primer pair produced a ~1800 bp PCR fragment. The sequence of this ALS region revealed a missense mutation from serine to asparagine at serine 653 when aligned with the ALS sequence of Bengal rice cultivar. Figure 2 depicts where F5 and R1 are located on the *ALS* gene.

Figure 2. Graphical representation of *ALS* gene with location of primer pairs.



The sequencing results from Psomagen for the *ALS* gene are presented in Figure 3. Six of the seven known spots of mutation are accounted for, displaying the correct amino acid at each of their respective sites. However, in the spot of serine 653, an asparagine can be seen having been substituted for serine.

Multiple sequence alignment was done using UniProt Align Tool using *Arabidopsis*, red rice, Bengal cultivar and SHybrid *ALS* sequences. Highlighted in yellow are the amino acids known to be involved in ALS-herbicide resistance. Highlighted in green is the alteration at Ser653 positions in three rice lines, 1, 2, and 3. The same mutation (S653N) was also found in ALS-resistant SHybrid plants derived from a cross between red rice and Clearfield® rice and is used as one of the references in this comparison.

Figure 3a. Alignment of ALS sequences from various rice lines used in this study with *Arabidopsis thaliana*. Region 1.

Arabidopsis_ALS	MAAATTTTSSSISFSTKPSPPSSSKSLPISRFSLPFLNPNKSSSSRRRG-IKSSSP	59
AY885675.1_R_SHybrid	MAT--TAAAAA--TLSAAAT-----AKTGRKNHQRRHVLPARGR	36
3_ALS	-----	0
1_ALS	-----	0
2_ALS	-----	0
AY885673.1_S_RedRice	MAT--TAAAAA--TLSAAAT-----AKTGRKNHQRRHVLPARGR	36
AY885674.1_S_Bengalcultivar	MAT--TAAAAA--TLSAAAT-----AKTGRKNHQRRHVLPARGR	36
Arabidopsis_ALS	SSISAVLNNTTNTTTPSPKPKPETFISRFAPDQPRKGADILVEALERQGVETVFAYP	119
AY885675.1_R_SHybrid	VGAAAVR---CSAVSPVTPSPAPPATPLRPWGPAEPRKGADILVEALERCGVSDVFAYP	93
3_ALS	-----	0
1_ALS	-----	0
2_ALS	-----	0
AY885673.1_S_RedRice	VGAAAVR---CSAVSPVTPSPAPPATPLRPWGPAEPRKGADILVEALERCGVSDVFAYP	93
AY885674.1_S_Bengalcultivar	VGAAAVR---CSAVSPVTPSPAPPATPLRPWGPAEPRKGADILVEALERCGVSDVFAYP	93
Arabidopsis_ALS	GGASMEIHQALTRSSSIRNVLPRHEQGGVFAAEGYARSSGKPGICATSFGPATNLVSL	179
AY885675.1_R_SHybrid	GGASMEIHQALTRSPVITNHLFRHEQGEAFAASGYARASGRVGCVATSGPATNLVSAL	153
3_ALS	-----SGYARASGRVGCVATSGPATNLVSAL	28
1_ALS	-----SGYARASGRVGCVATSGPATNLVSAL	28
2_ALS	-----ASGRVGCVATSGPATNLVSAL	23
AY885673.1_S_RedRice	GGASMEIHQALTRSPVITNHLFRHEQGEAFAASGYARASGRVGCVATSGPATNLVSAL	153
AY885674.1_S_Bengalcultivar	GGASMEIHQALTRSPVITNHLFRHEQGEAFAASGYARASGRVGCVATSGPATNLVSAL	153
	:*: :*:*****.*	
Arabidopsis_ALS	ADALLDSVPLVAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVMDVEDIPRIIEEAF	239
AY885675.1_R_SHybrid	ADALLDPVPMVAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVLDVEDIPRVIQEAF	213
3_ALS	ADALLDSVPMVAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVLDVEDIPRVIQEAF	88
1_ALS	ADALLDSVPMVAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVLDVEDIPRVIQEAF	88
2_ALS	ADALLDSVPMVAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVLDVEDIPRVIQEAF	83
AY885673.1_S_RedRice	ADALLDSVPMVAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVLDVEDIPRVIQEAF	213
AY885674.1_S_Bengalcultivar	ADALLDSVPMVAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVLDVEDIPRVIQEAF	213
	***** **:*****.*	
Arabidopsis_ALS	FLATSGRPGPVLVDVPKDIQQQLAIPNWEQAMRLPGYMSRMPKPPEDSHLEQIVRLISES	299
AY885675.1_R_SHybrid	FLASSGRPGPVLVDIPKDIQQQMAVPVWDTSMNLPGYIARLPKPPATELLEQVLRVLGSES	273
3_ALS	FLASSGRPGPVLVDIPKDIQQQMAVPVWDTSMNLPGYIARLPKPPATELLEQVLRVLGSES	148
1_ALS	FLASSGRPGPVLVDIPKDIQQQMAVPVWDTSMNLPGYIARLPKPPATELLEQVLRVLGSES	148
2_ALS	FLASSGRPGPVLVDIPKDIQQQMAVPVWDTSMNLPGYIARLPKPPATELLEQVLRVLGSES	143
AY885673.1_S_RedRice	FLASSGRPGPVLVDIPKDIQQQMAVPVWDTSMNLPGYIARLPKPPATELLEQVLRVLGSES	273
AY885674.1_S_Bengalcultivar	FLASSGRPGPVLVDIPKDIQQQMAVPVWDTSMNLPGYIARLPKPPATELLEQVLRVLGSES	273
	:**:*****:*:* :*:*****:***** .***:***:**	

Figure 3b. Alignment of ALS sequences from various rice lines used in this study with *Arabidopsis thaliana*. Region 2.

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Arabidopsis_ALS      KKPVLVYVGGGCLNSSDELGRFVELTGIPVASTLMGLGSPCDDELSLHMLGMHGTVYANY 359
AY885675.1_R_SHybrid RRPILYVGGGCSASGDELRRFVELTGIPVTTTLMGLGNFSPDDPLSLRMLGMHGTVYANY 333
3_ALS                RRPILYVGGGCSASGDELRRFVELTGIPVTTTLMGLGNFSPDDPLSLRMLGMHGTVYANY 208
1_ALS                RRPILYVGGGCSASGDELRRFVELTGIPVTTTLMGLGNFSPDDPLSLRMLGMHGTVYANY 208
2_ALS                RRPILYVGGGCSASGDELRRFVELTGIPVTTTLMGLGNFSPDDPLSLRMLGMHGTVYANY 203
AY885673.1_S_RedRice RRPILYVGGGCSASGDELRRFVELTGIPVTTTLMGLGNFSPDDPLSLRMLGMHGTVYANY 333
AY885674.1_S_Bengalcultivar RRPILYVGGGCSASGDELRRFVELTGIPVTTTLMGLGNFSPDDPLSLRMLGMHGTVYANY 333
      :*:***** *.*** *****:*****:*.** ***:*****

Arabidopsis_ALS      AVEHSDLLAFGVRFDDRVTGKIEAFASRAKIVHIDIDSAEIGKNKTPHVSVCQDVKLAL 419
AY885675.1_R_SHybrid AVDKADLLAFGVRFDDRVTGKIEAFASRAKIVHIDIDPAEIGKNKQPHVSI CADVELAL 393
3_ALS                AVDKADLLAFGVRFDDRVTGKIEAFASRAKIVHIDIDPAEIGKNKQPHVSI CADVKLAL 268
1_ALS                AVDKADLLAFGVRFDDRVTGKIEAFASRAKIVHIDIDPAEIGKNKQPHVSI CADVKLAL 268
2_ALS                AVDKADLLAFGVRFDDRVTGKIEAFASRAKIVHIDIDPAEIGKNKQPHVSI CADVKLAL 263
AY885673.1_S_RedRice AVDKADLLAFGVRFDDRVTGKIEAFASRAKIVHIDIDPAEIGKNKQPHVSI CADVKLAL 393
AY885674.1_S_Bengalcultivar AVDKADLLAFGVRFDDRVTGKIEAFASRAKIVHIDIDPAEIGKNKQPHVSI CADVKLAL 393

**::*****:***** ***** ***:*.**:*

Arabidopsis_ALS      QGMNKVLENRAEELKLDGFGVWRNELNVQKQKFPPLSFKTFGEAIPPQYAIQVDELDTGKA 479
AY885675.1_R_SHybrid QGLNALLDQSTTKTSSDFSAWHNELDQKREFPLGYKTFGEEIPPQYAIQVDELDTKGEA 453
3_ALS                QGLNALLDQSTTKTSSDFSAWHNELDQKREFPLGYKTFGEEIPPQYAIQVDELDTKGEA 328
1_ALS                QGLNALLDQSTTKTSSDFSAWHNELDQKREFPLGYKTFGEEIPPQYAIQVDELDTKGEA 328
2_ALS                QGLNALLDQSTTKTSSDFSAWHNELDQKREFPLGYKTFGEEIPPQYAIQVDELDTKGEA 323
AY885673.1_S_RedRice QGLNALLDQSTTKTSSDFSAWHNELDQKREFPLGYKTFGEEIPPQYAIQVDELDTKGEA 453
AY885674.1_S_Bengalcultivar QGLNALLDQSTTKTSSDFSAWHNELDQKREFPLGYKTFGEEIPPQYAIQVDELDTKGEA 453
      **:* :*: : . . **..*:*: **:*:*:*:***** *****:*****.*:*

Arabidopsis_ALS      IISTGVGQHQMWAQFYNYKKPRQWLSSGGLGAMGFGLPAAIGASVANPDAIVVDIDGDG 539
AY885675.1_R_SHybrid I IATGVGQHQMWAQYYTYKRPRQWLSSAGLGAMGFGLPAAAGASVANPGVTVVDIDGDG 513
3_ALS                I IATGVGQHQMWAQYYTYKRPRQWLSSAGLGAMGFGLPAAAGASVANPGVTVVDIDGDG 388
1_ALS                I IATGVGQHQMWAQYYTYKRPRQWLSSAGLGAMGFGLPAAAGASVANPGVTVVDIDGDG 388
2_ALS                I IATGVGQHQMWAQYYTYKRPRQWLSSAGLGAMGFGLPAAAGASVANPGVTVVDIDGDG 383
AY885673.1_S_RedRice I IATGVGQHQMWAQYYTYKRPRQWLSSAGLGAMGFGLPAAAGASVANPGVTVVDIDGDG 513
AY885674.1_S_Bengalcultivar I IATGVGQHQMWAQYYTYKRPRQWLSSAGLGAMGFGLPAAAGASVANPGVTVVDIDGDG 513
      **:******:*.**:******.***** ***** ***:*.**:*

Arabidopsis_ALS      SFIMNVQELATIRVENLPVKVLLLNQHLGMVQWEDRFYKANRAHTFLGDPAQEDEF 599
AY885675.1_R_SHybrid SFLMNIQELALIRIENLPVKVMVLNNQHLGMVVQWEDRFYKANRAHTYLGNPECESEIYP 573
3_ALS                SFLMNIQELALIRIENLPVKVMVLNNQHLGMVVQWEDRFYKANRAHTYLGNPECESEIYP 448
1_ALS                SFLMNIQELALIRIENLPVKVMVLNNQHLGMVVQWEDRFYKANRAHTYLGNPECESEIYP 448
2_ALS                SFLMNIQELALIRIENLPVKVMVLNNQHLGMVVQWEDRFYKANRAHTYLGNPECESEIYP 443
AY885673.1_S_RedRice SFLMNIQELALIRIENLPVKVMVLNNQHLGMVVQWEDRFYKANRAHTYLGNPECESEIYP 573
AY885674.1_S_Bengalcultivar SFLMNIQELALIRIENLPVKVMVLNNQHLGMVVQWEDRFYKANRAHTYLGNPECESEIYP 573
      **:****** **:******:*****:***** ***** ***:*.**:*

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Figure 3c. Alignment of ALS sequences from various rice lines used in this study with *Arabidopsis thaliana*. Region 3.

Arabidopsis_ALS	NMLLFAAACGIPAARVTKKADLREAIQTMLDTPGPYLLDVICPHQEHVLPMPISGGTFND	659
AY885675.1_R_SHybrid	DFVTIAKGFNIPAVRVTKKSEVRAAIAKKMLETPGPYLLDIIVPHQEHVLPMPISGGAFKD	633
3_ALS	DFVTIAKGFNIPAVRVTKKSEVRAAIAKKMLETPGPYLLDIIVPHQEHVLPMPISGGAFKD	508
1_ALS	DFVTIAKGFNIPAVRVTKKSEVRAAIAKKMLETPGPYLLDIIVPHQEHVLPMPISGGAFKD	508
2_ALS	DFVTIAKGFNIPAVRVTKKSEVRAAIAKKMLETPGPYLLDIIVPHQEHVLPMPISGGAFKD	503
AY885673.1_S_RedRice	DFVTIAKGFNIPAVRVTKKSEVRAAIAKKMLDTPGPYLLDIIVPHQEHVLPMPISGGAFKD	633
AY885674.1_S_Bengalcultivar	DFVTIAKGFNIPAVRVTKKSEVRAAIAKKMLETPGPYLLDIIVPHQEHVLPMPISGGAFKD	633
	::: :* . .***.*****:::* **:.**:*:*****:* *****.*:*:*	
Arabidopsis_ALS	VITEGDGRIKY	670
AY885675.1_R_SHybrid	MIPDGDGRTVY	644
3_ALS	M-----	509
1_ALS	MILDGDG----	515
2_ALS	MILDGDG----	510
AY885673.1_S_RedRice	MILDGDGRTVY	644
AY885674.1_S_Bengalcultivar	MILDGDGRTVY	644

Discussion and Implications for Future Research

Ser653Asn confers resistance to ALS-inhibitor herbicides while also providing specific resistance to a group of ALS herbicides called imidazolinones (IMI)¹³. In this mutation, adenine replaces guanine in serine. The Ser653Asn mutation endows resistance by changing the 3-dimensional shape of the enzyme's substrate binding pocket. In doing so, the binding affinity for the herbicide is weakened, allowing the enzyme to continue biosynthesis of branched chain amino acids⁶. The mutation was found in the EMS rice line that had been selected with imazethapyr, the offspring of which was sprayed with 4x the labeled rate of Newpath herbicide. The active ingredient in Newpath herbicide is imazethapyr, which is in the IMI family of herbicides. This confirms that the imazethapyr rice line carried a strong resistance-endowing ALS mutation. In weed species, this mutation has been reported to confer resistance not only to imidazolinone herbicides, but also to pyrimidinylthio-benzoate, sulfonylamino-carbonyltriazolinone, sulfonyleurea, and triazolopyrimidine²⁰. Through further research, it has also been determined that the mutation Ser653Asn is also the mutation that allows several

Clearfield® rice cultivars, a commonly planted type of herbicide-resistant rice, to exhibit immunity to IMI herbicides²⁶.

An interesting discovery by Goulart, et al. (2012) suggests that mutations at Ser653 may affect germination rates, causing seeds to germinate at faster rates¹³. One of the possible mutations at Ser653 that is mentioned by the authors is Ser653Asn. The authors, however, did not present any opinion on specific benefits an accelerated germination rate could confer. Further studies would help characterize the benefits of a fast-emerging crop cultivar. Future investigations can be based on the hypothesis that the mutation conferring accelerated germination allow plants to compete better with weeds at the seedling and juvenile growth stage. Rapid germination, hypothetically, allow faster establishment in the field, giving rice plants the ability to grow taller and larger faster than weed seedlings.

Other future research for *ALS* and *HPPD* genes still remain as well. In regard to *ALS*, a tolerance assay must be conducted to determine how high of a dose the Ser653Asn line of rice can tolerate without interfering with yield potential. If the line can resist a higher dosage than that of previously established herbicide resistance rice, it would be an advantageous and novel trait that could be used in the agricultural industry. Additionally, the Ser653Asn line of rice may have the increased ability to detoxify imazethapyr, which could be endowing the high resistance mentioned earlier. If this is the case, this line would become a novel line, as it would then have two mechanisms working together, as opposed to the one found in lines like Clearfield® cultivars. This would be a finding of great importance to the agricultural and requires further investigation.

With respect to *HPPD*, another tolerance assay would also be in order, as to determine to what extent the line can tolerate herbicide application without yield interference as well as to

determine if increased detoxification ability is present. However, new primer pairs must be designed to move forward with research in this area.

APPENDIX

Appendix table 1: Rice plants sampled for *HPPD* and *ALS* gene analysis.

Herbicide	Trade name	Rate	Herbicide ID	Rep	Plant/tube ID
imazethapyr	Newpath	4x	1	R1	1D
imazethapyr	Newpath	4x	1	R2	1A, 1F
imazethapyr	Newpath	4x	1	R3	1E
imazethapyr	Newpath	4x	1	R4	1C,1B
topramezone	Armezon	2x	2	R1	2E,2F
topramezone	Armezon	2x	2	R2	2A,2B
topramezone	Armezon	2x	2	R3	2C
topramezone	Armezon	2x	2	R4	2D
fluridone	Brake	1x	3	R1	3F
fluridone	Brake	1x	3	R2	3D,3C
fluridone	Brake	1x	3	R3	3B,3E
fluridone	Brake	1x	3	R4	3A
mesotrione	Callisto	4x	4	R1	4C
mesotrione	Callisto	4x	4	R2	4A,4B
mesotrione	Callisto	4x	4	R3	4E
mesotrione	Callisto	4x	4	R4	4F,4D

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