5-2023

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

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Spring 2023

Biology

Fulbright College of Arts and Sciences

The University of Arkansas

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 sulfonylurea[8].

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

Acknowledgements:

I would like to thank Dr. Nilda Roma Burgos, Dr. Gulab Rangani, and the rest of Dr. Burgos' research team for assisting me with this experiment and taking the time to educate me in crop science and agriculture. This opportunity has opened my eyes to the applications of biology outside of what I am accustomed to. I have learned much in my time in this research lab, and I am ever grateful for the experience.

I would also like to show appreciation to the remaining members of my thesis committee: Dr. Chapman, Dr. Pinto, and Dr. Zhuang. Thank you all for dedicating time out of your schedules to participate in my thesis defense.

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Introduction

Rice (*Oryza sativa*) is a food staple to much of the world, making up over 50% of the world's diet[28]. 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 study[25]. Due to an increasing global population, there is a need for increased food production without increasing the planted area[2]. 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 production[27]. 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 rice[24] 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 grain[26]. 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 entering[31]. 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 herbicides[20].

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 herbicides[32]. 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 metabolism[14]. 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 654[15].

In the instance of Ala122, multiple mutations have been reported, where Ala122 has been substituted for Tyr[14], Asn, Val, and Thr[22] and Ser[21]. 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 Tyr[34], Ile[7], Glu[17], Asn[18], Arg, Ala, Gln, Leu, Ser[33] and Thr[10]. All of these mutations induced ALS resistance. One substitution mutation in Ala205 has been documented, where Ala was substituted for Phe, conferring ALS-inhibitor resistance[3]. In a similar case, one mutation has been reported for Asn376, where substitution has occurred, replacing Asn with Glu, giving ALS-inhibitor resistance once again[16]. Arg377His is a reported mutation that, similar to previously mentioned mutations, also gives resistance to ALS-inhibitors[11]. Four mutations have been reported in Trp574, where Met, Leu[19], Gly[9] and Arg[12] also give ALS-inhibitor resistance to plants carrying the mutations. At Ser653 in the *ALS* gene, three mutations have been reported where Ile[30], Thr[1], and Asn[13], 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 resistance[5].

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 groups[20]. 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	Formulation concentration
				lb ai/gal	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

[1]Quiz = quizalofop; Fluri = fluridone; Topra = topramezone; Imaze -imazethapyr
[2]Abbreviations: 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	GCCTTCCAGGAGACGCCCATA
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 µL of RNAse-free water was added to the spin column membrane and centrifuged for 1 min at 10,000 rpm to elute the RNA[23].

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 µL of RNA, 4 µl of 5x reaction buffer, 1 µL of RiboLock, 2 µL of 10 µM oligonucleotide DNTP, and 1 µL 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 PCR[29]. The PCR mixture consisted of 5 µL of cDNA, 0.25 µL of Taq polymerase, 1 µL of forward primer, 1 µL of reverse primer and 3 µL 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.

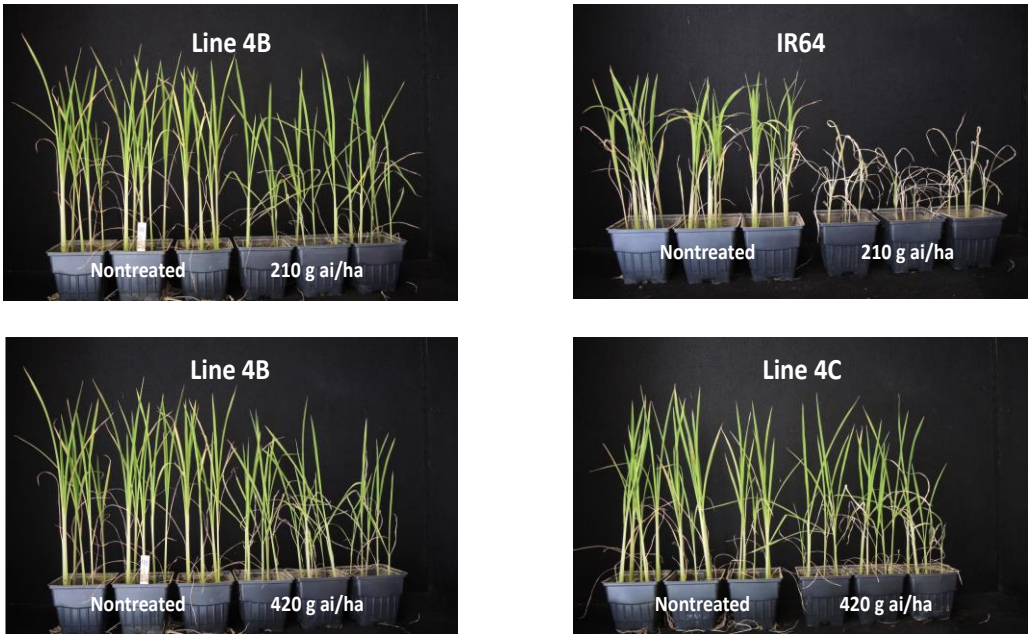


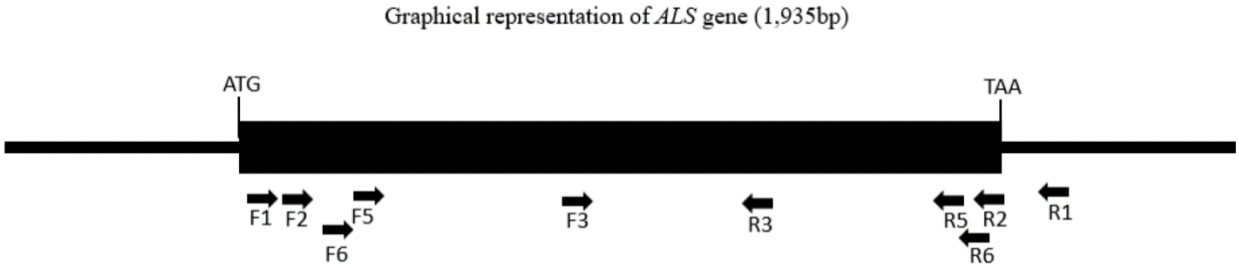
Table 4. Response of EMS rice lines to herbicides in the greenhouse

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

[1]Abbreviations: Quiz. = quizalofop; Cleth. = clethodim; Fluri. = fluridone; Topra. = topramezone; Meso. = mesotrione; Imaze. = imazethapyr;
[2]Rep. = replications
[3]X 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 SHhybrid ALS sequences. Highlighted in yellow are the amino acids known to 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 SHhybrid 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.

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

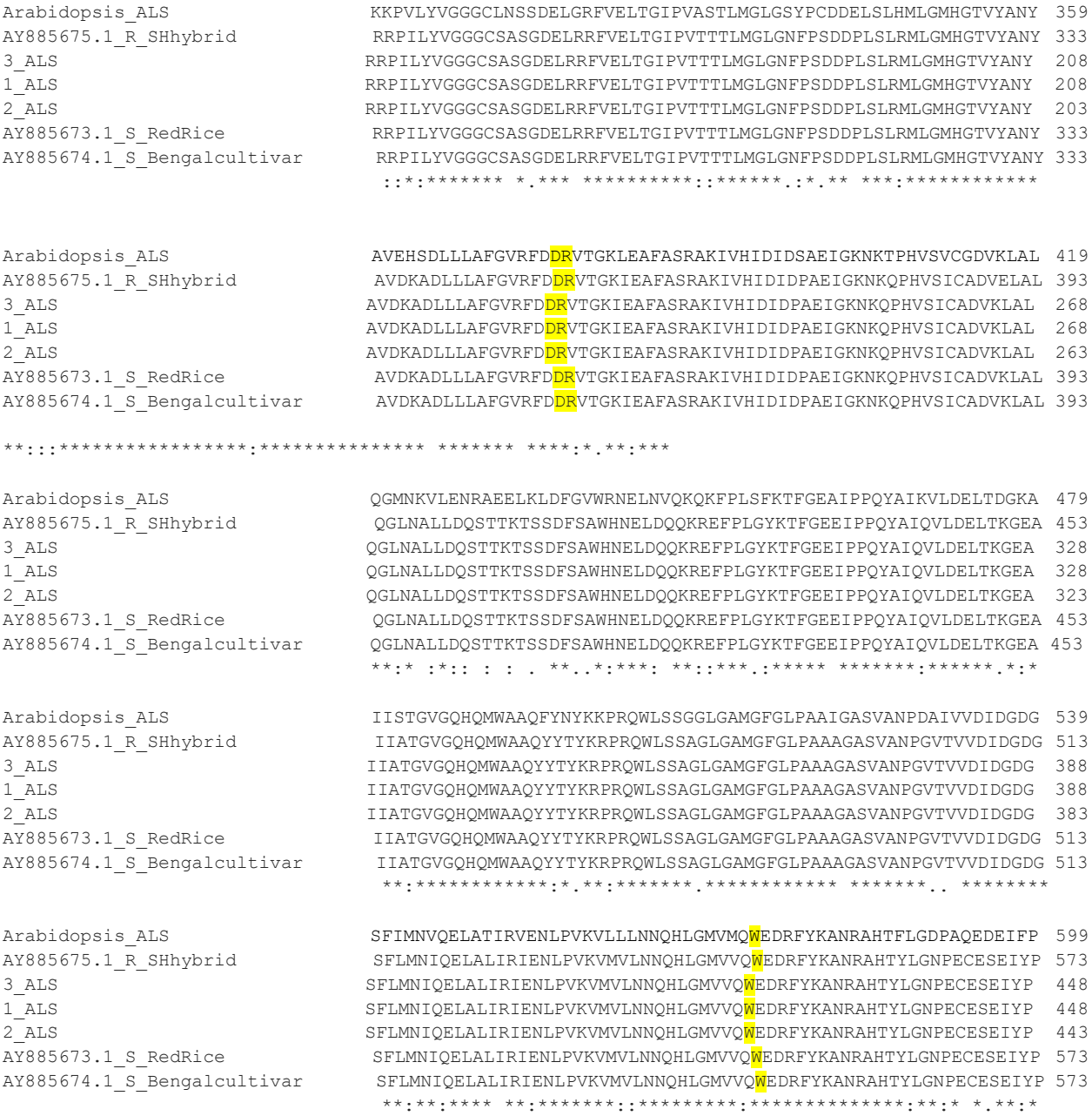


Figure 3c. Alignment of ALS sequences from various rice lines used in this study with *Arabidopsis thaliana*. Region 3.

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Arabidopsis_ALS                 NMLLFAAACGIPAARVTKKADLREAIQTMLDTPGPYLLDVICPHQEHVLPMIPSGGTFND  659
AY885675.1_R_SHhybrid           DFVTIAKGFNIPAVRVTKKSEVRAAIKKMLETPGPYLLDIIVPHQEHVLPMIPNGGAFKD  633
3_ALS                          DFVTIAKGFNIPAVRVTKKSEVRAAIKKMLETPGPYLLDIIVPHQEHVLPMIPNGGAFKD  508
1_ALS                          DFVTIAKGFNIPAVRVTKKSEVRAAIKKMLETPGPYLLDIIVPHQEHVLPMIPNGGAFKD  508
2_ALS                          DFVTIAKGFNIPAVRVTKKSEVRAAIKKMLETPGPYLLDIIVPHQEHVLPMIPNGGAFKD  503
AY885673.1_S_RedRice            DFVTIAKGFNIPAVRVTKKSEVRAAIKKMLDTPGPYLLDIIVPHQEHVLPMIPSGGAFKD  633
AY885674.1_S_Bengalcultivar     DFVTIAKGFNIPAVRVTKKSEVRAAIKKMLETPGPYLLDIIVPHQEHVLPMIPSGGAFKD  633
                                 ::: :* . .***.*****:::* **:.**:********:* ***********.**:*:*

Arabidopsis_ALS                                                  VITEGDGRIKY          670
AY885675.1_R_SHhybrid                                            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
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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-dimentionsal 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 acids[6]. 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, sulfonylurea, and triazolopyrimidine[20]. 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 herbicides[26].

An interesting discovery by Goulart, et al. (2012) suggests that mutations at Ser653 may affect germination rates, causing seeds to germinate at faster rates[13]. 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

References Cited

1. Beckie, Hugh J., Suzanne I. Warwick, and Connie A. Sauder. "Basis for Herbicide Resistance in Canadian Populations of Wild Oat (Avena Fatua)." *Weed Science* 60, no. 1 (2012): 10–18. https://doi.org/10.1614/WS-D-11-00110.1.
2. Benedetti, Lariza, et al. "Recurrent Selection by Herbicide Sublethal Dose and Drought Stress Results in Rapid Reduction of Herbicide Sensitivity in Junglerice." Agronomy, vol. 10, no. 11, 2020, p. 1619., https://doi.org/10.3390/agronomy10111619.
3. Brosnan, James T., Jose J. Vargas, Gregory K. Breeden, Logan Grier, Raphael A. Aponte, Stefan Tresch, and Martin Laforest. "A New Amino Acid Substitution (Ala-205-Phe) in Acetolactate Synthase (ALS) Confers Broad Spectrum Resistance to ALS-Inhibiting Herbicides." *Planta* 243, no. 1 (January 1, 2016): 149–59. https://doi.org/10.1007/s00425-015-2399-9.
4. Burgos, Nilda R., Jason K. Norsworthy, Robert C. Scott, and Kenneth L. Smith. "Red Rice (Oryza Sativa) Status after 5 Years of Imidazolinone-Resistant Rice Technology in Arkansas." *Weed Technology* 22, no. 1 (2008): 200–208.
5. Cao, Yi, Xinxin Zhou, and Zhaofeng Huang. "Amino Acid Substitution (Gly-654-Tyr) in Acetolactate Synthase (ALS) Confers Broad Spectrum Resistance to ALS-Inhibiting Herbicides." *Pest Management Science* 78, no. 2 (2022): 541–49. https://doi.org/10.1002/ps.6658.
6. Carol A. Mallory-Smith, and Jr. E. James Retzinger. "Revised Classification of Herbicides by Site of Action for Weed Resistance Management Strategies." *Weed Technology* 17, no. 3 (2003): 605–19.

7. Corbett, Cheryl-Ann L, and François J Tardif. “Detection of Resistance to Acetolactate Synthase Inhibitors in Weeds with Emphasis on DNA-Based Techniques: A Review.” *Pest Management Science* 62, no. 7 (2006): 584–97. https://doi.org/10.1002/ps.1219.
8. Crespo, Roberto J., Ana B. Wingeyer, Greg R. Kruger, Chance W. Riggins, Patrick J. Tranel, and Mark L. Bernards. “Multiple-Herbicide Resistance in a 2,4-D–Resistant Waterhemp (Amaranthus Tuberculatus) Population from Nebraska.” Weed Science 65, no. 6 (2017): 743–54. doi:10.1017/wsc.2017.39.
9. Deng, Wei, Yingjie Di, Jingxuan Cai, Yueyang Chen, and Shuzhong Yuan. “Target-Site Resistance Mechanisms to Tribenuron-Methyl and Cross-Resistance Patterns to ALS-Inhibiting Herbicides of Catchweed Bedstraw (Galium Aparine) with Different ALS Mutations.” *Weed Science* 67, no. 2 (2019): 183–88. https://doi.org/10.1017/wsc.2018.70.
10. Deng, Wei, Qian Yang, Yongzhi Zhang, Hongtao Jiao, Yu Mei, Xuefeng Li, and Mingqi Zheng. “Cross-Resistance Patterns to Acetolactate Synthase (ALS)-Inhibiting Herbicides of Flixweed (Descurainia Sophia L.) Conferred by Different Combinations of ALS Isozymes with a Pro-197-Thr Mutation or a Novel Trp-574-Leu Mutation.” *Pesticide Biochemistry and Physiology* 136 (2017): 41–45. https://doi.org/10.1016/j.pestbp.2016.08.006.
11. Fang, Jiapeng, Dongchen Yang, Zerui Zhao, Jinyi Chen, and Liyao Dong. “A Novel Phe-206-Leu Mutation in Acetolactate Synthase Confers Resistance to Penoxsulam in Barnyardgrass (Echinochloa Crus-Galli (L.) P. Beauv).” *Pest Management Science* 78, no. 6 (2022): 2560–70. https://doi.org/10.1002/ps.6887.
12. Fang, Jiapeng, Yuhua Zhang, Tingting Liu, Bojun Yan, Jun Li, and Liyao Dong. “Target-Site and Metabolic Resistance Mechanisms to Penoxsulam in Barnyardgrass (Echinochloa Crus-Galli (L.)

P. Beauv).” *Journal of Agricultural and Food Chemistry* 67, no. 29 (July 24, 2019): 8085–95. https://doi.org/10.1021/acs.jafc.9b01641.

13. GOULART, I C G R, F O MATZENBACHER, and A MEROTTO JR. “Differential Germination Pattern of Rice Cultivars Resistant to Imidazolinone Herbicides Carrying Different Acetolactate Synthase Gene Mutations.” *Weed Research* 52, no. 3 (2012): 224–32. https://doi.org/10.1111/j.1365-3180.2012.00909.x.
14. Han, H., Yu, Q., Purba, E., Li, M., Walsh, M., Friesen, S., & Powles, S. B. (2012). “A novel amino acid substitution Ala-122-Tyr in ALS confers high-level and broad resistance across ALS-inhibiting herbicides.” *Pest management science*, *68*(8), 1164–1170. https://doi.org/10.1002/ps.3278
15. Heap, I. The International Survey of Herbicide Resistant Weeds. Online. Internet. Wednesday, April 12, 2023 . Available www.weedscience.com
16. Li, Mei, Qin Yu, Heping Han, Martin Vila-Aiub, and Stephen B Powles. “ALS Herbicide Resistance Mutations in Raphanus Raphanistrum: Evaluation of Pleiotropic Effects on Vegetative Growth and ALS Activity.” *Pest Management Science* 69, no. 6 (2013): 689–95. https://doi.org/10.1002/ps.3419.
17. Liu, Weitang, Guohui Yuan, Long Du, Wenlei Guo, Lingxu Li, Yaling Bi, and Jinxin Wang. “A Novel Pro197Glu Substitution in Acetolactate Synthase (ALS) Confers Broad-Spectrum Resistance across ALS Inhibitors.” *Pesticide Biochemistry and Physiology* 117 (2015): 31–38. https://doi.org/10.1016/j.pestbp.2014.10.005.
18. MASSA, D, B KRENZ, and R GERHARDS. “Target-Site Resistance to ALS-Inhibiting Herbicides in Apera Spica-Venti Populations Is Conferred by Documented and Previously

Unknown Mutations." *Weed Research* 51, no. 3 (2011): 294–303. https://doi.org/10.1111/j.1365-3180.2011.00843.x.

19. Milani, Andrea, Laura Scarabel, and Maurizio Sattin. "A Family Affair: Resistance Mechanism and Alternative Control of Three Amaranthus Species Resistant to Acetolactate Synthase Inhibitors in Italy." *Pest Management Science* 76, no. 4 (2020): 1205–13. https://doi.org/10.1002/ps.5667.
20. MP44 2023 Arkansas recommended chemicals for weed and Brush Control. (n.d.). Retrieved April 14, 2023, from https://www.uaex.uada.edu/publications/pdf/mp44/publications-supplemental.pdf
21. Murphy, Brent P., and Patrick J. Tranel. "Target-Site Mutations Conferring Herbicide Resistance." *Plants* 8, no. 10 (2019). https://doi.org/10.3390/plants8100382.
22. Panozzo, Silvia, Laura Scarabel, Valentina Rosan, and Maurizio Sattin. "A New Ala-122-Asn Amino Acid Change Confers Decreased Fitness to ALS-Resistant Echinochloa Crus-Galli." *Frontiers in Plant Science* 8 (2017). https://www.frontiersin.org/articles/10.3389/fpls.2017.02042.
23. Qiagen RNeasy Plant Mini Kit. https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en
24. Qiu, Jie, Yongjun Zhou, Lingfeng Mao, Chuyu Ye, Weidi Wang, Jianping Zhang, Yongyi Yu, et al. "Genomic Variation Associated with Local Adaptation of Weedy Rice during De-Domestication." *Nature Communications* 8, no. 1 (May 24, 2017): 15323. https://doi.org/10.1038/ncomms15323.
25. Rao, A.N. "Rao, A. N., and Hiroshi Matsumoto. Weed Management in Rice in the Asian-Pacific Region. Asian-Pacific Weed Science Society, 2017.," n.d.

26. Rajguru, Satyendra N., et al. “Mutations in the Red Rice ALS Gene Associated with Resistance to Imazethapyr.” Weed Science, vol. 53, no. 5, 2005, pp. 567–577., https://doi.org/10.1614/ws-04-111r1.1.
27. Rice. (n.d.). Retrieved April 13, 2023, from https://www.arfb.com/pages/arkansas-agriculture/commodity-corner/rice/#:~:text=Arkansas%20farmers%20produce%20more%20than,and%20its%20top%20agricultural%20export.
28. Rice sector at a glance. (n.d.). Retrieved April 13, 2023, from https://www.ers.usda.gov/topics/crops/rice/rice-sector-at-a-glance/
29. Thermofisher RevertAid RT Reverse Transcription Kit. https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0012955_RevertAid_RT_UG.pdf
30. Torra, Joel, Aritz Royo-Esnal, Yolanda Romano, María Dolores Osuna, Ramón G. León, and Jordi Recasens. “Amaranthus Palmeri a New Invasive Weed in Spain with Herbicide Resistant Biotypes.” *Agronomy* 10, no. 7 (2020). https://doi.org/10.3390/agronomy10070993.
31. University of Michigan. https://extension.umn.edu/herbicide-mode-action-and-sugarbeet-injury-symptoms/amino-acid-synthesis-inhibitor-herbicides#:~:text=Acetolactate%20synthase%20(ALS%20)%20enzyme%20inhibitors,acetolactate%20synthase%20(ALS%20enzyme).
32. Wedger, Marshall J., Nilda Roma-Burgos, and Kenneth M. Olsen. “Genomic Revolution of US Weedy Rice in Response to 21st Century Agricultural Technologies.” *Communications Biology* 5, no. 1 (September 8, 2022): 885. https://doi.org/10.1038/s42003-022-03803-0.

33. Yu, Qin, Heping Han, and Stephen B Powles. “Mutations of the ALS Gene Endowing Resistance to ALS-Inhibiting Herbicides in Lolium Rigidum Populations.” *Pest Management Science* 64, no. 12 (2008): 1229–36. https://doi.org/10.1002/ps.1624.

34. Zhao, Ning, Yanyan Yan, Lu’an Ge, Baolin Zhu, Weitang Liu, and Jinxin Wang. “Target Site Mutations and Cytochrome P450s Confer Resistance to Fenoxaprop-P-Ethyl and Mesosulfuron-Methyl in Alopecurus Aequalis.” *Pest Management Science* 75, no. 1 (2019): 204–14. https://doi.org/10.1002/ps.5089.