Precision breeding for improved digestibility and herbicide resistance in bahiagrass; Fredy Altpeter and Aldo Merotto, Agronomy department University of Florida.

Final Progress Report

1. Introduction and analysis of a mutated acetolactate synthase (mALS) gene to confer herbicide resistance. This activity was completed before the funding period.

Acetolactate synthase gene of sorghum with one point mutation (to convert Tryptophan at position 545 into Leucine) in the coding region of gene (mALS) gene was recently found to confer herbicide tolerance to sugarcane (Dermawan et al. 2016). The construct used in the previous and this study contains all the genetic components from sorghum and no components of any plant pest or pathogen which is expected to facilitate regulatory approval when inserted into grasses. Bahiagrass seeds of cultivar "Argentine" were sterilized and used for initiation of callus tissue. mALS construct was coated onto gold particles and biolistically delivered into bahiagrass callus tissues. Transformed tissues were selected MS nutrient medium containing the ALS-inhibiting herbicide bispyribac sodium (BS). BS resistant tissues were regenerated into plantlets. Plantlets containing roots were transferred to soil and used for further molecular analysis. Typically, different gene transfer events differ in the location of the mALS gene integration into the plant genome. Therefore, expression of the mALS gene and the performance of the associated herbicide resistance trait may vary from event to event. Therefore, it is desirable to analyze a larger number (more than 10) of independent events to conclude about the success of the approach.

A. Molecular analysis of lines that carry a mutated ALS gene

RNA was isolated from the leaves of different bahiagrass lines regenerated on BS containing medium and used to confirm the expression of *mALS* gene by qRT-PCR using *mALS* specific primers. As shown in fig.1, *mALS* gene expression in bahiagrass lines varied from 0.25 in line BA20, to 5.8 times higher in line BA18 than the highly expressed internal control gene glyceraldehyde 3-phosphate dehydrogenase (GADPH). This level of expression is very high and compares favorably to the expression of the same gene in sugarcane (Dermawan et al. 2016). This high level of expression of the mALS gene will likely support a very high level of resistance to ALS-inhibiting herbicides.

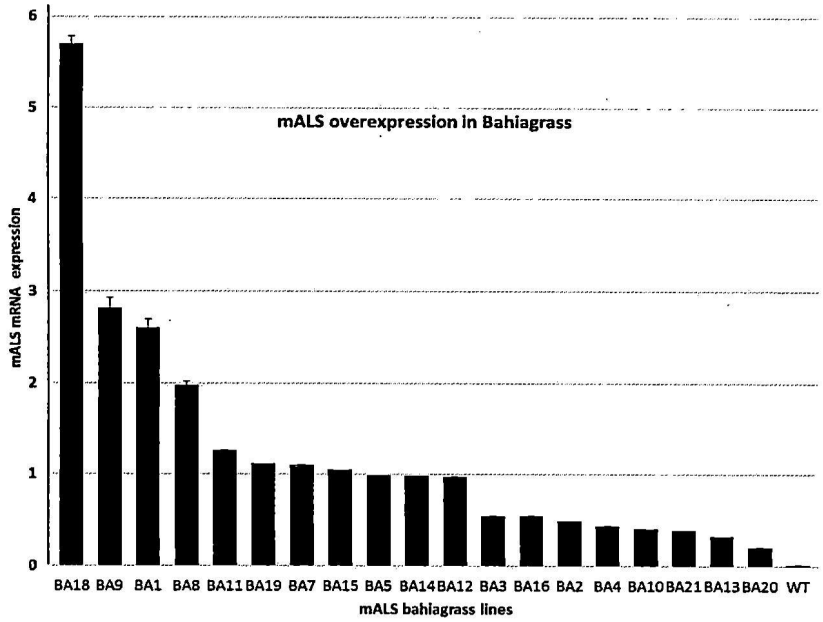

Fig. 1 *mALS* gene expression in mALS-bahiagrass lines. RNA was isolated from transformed and wild type bahiagrass lines and converted into cDNA. Real time PCR amplification was performed to quantify the expression of *mALS* gene. Vertical bars with different heights shows different levels of mALS expression in different bahiagrass lines. Relative *mALS* gene expression analysis was performed with respect to endogenous control gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

B. Tolerance of bahiagrass to different herbicides

The study was arranged in a completely randomized design with four replications. Treatments consisted of eleven bahiagrass genotypes and six herbicides. The bahiagrass genotypes used were one wild type and the mALS lines 1b, 3g, 5g, 7d, BA2, BA5, BA8, BA9, BA1 and BA18. Plants were initially growth in 25 cm pots and when thy reached approximately 40 cm height the soil was washed out from the roots and the rhizomes were cut in 5 cm length pieces having one individual tiller. Plants were established in 3.8 cm diameter and 21.0 cm length plastic SC10 cones (Stuewe & Sons) filled with Fafard 2b mix (Sungrow). The herbicides evaluated were metsulfuron-methyl (Cimaron Max, Part A), imazapic (Cadre 2 AS), imazaquim (Scepter 70WG), and metsulfuron methyl + chlorsulfuron (Telar XP) at 1, 2 and 5 X the recommended rate, and Imazapyr (Arsenal) and nicosulfuron (Accent) at 1, 2, 5, 10 and 20 X the recommended rate. The considered recommended rates were 31.8, 168.0, 430.5, 137.2, 25,2+7.9, and 60 g/ha for metsulfuron-methyl, imazapic, imazaquim, metsulfuron-methyl + chlorsulfuron, imazapyr and nicosulfuron, respectively. All herbicide treatments included 0.25% (v/v) nonionic surfactant. The herbicide treatments were applied using a cabinet sprayer calibrated for a spray volume of 189 $L \cdot ha^{-1}$ using a single even flow nozzle (Teejet 8001 EVS flat fan, Spraying Systems). Plants were maintained in greenhouse with a temperature of 25 ± 4 °C and were irrigated daily. The evaluations consisted of scoring the visual injury at 7, 14 and 21 after treatment (DAT) where zero corresponds to the absence of herbicide effects and 100 % to plant death. At 21 DAT

plants were cut at the soil level and dried at 60°C until constant weight for the evaluation of aboveground dry weight. Data were subjected to analysis of variance (ANOVA) and Tukey's test (P = 0.05) was used for mean separation. mALS bahiagrass tolerated upto 20X the label recommended rate of Imazapyr, Nicosulfuron, and upto 5 times the labeled rate of Metsulfuron, Imazapic, and Imazaquin. Response to 2X of the label rate of Imazapyr is shown in figure 3, all the mALS-bahiagrass treated lines were herbicide tolerant, while wild type could not survive even the 1x labeled rate.

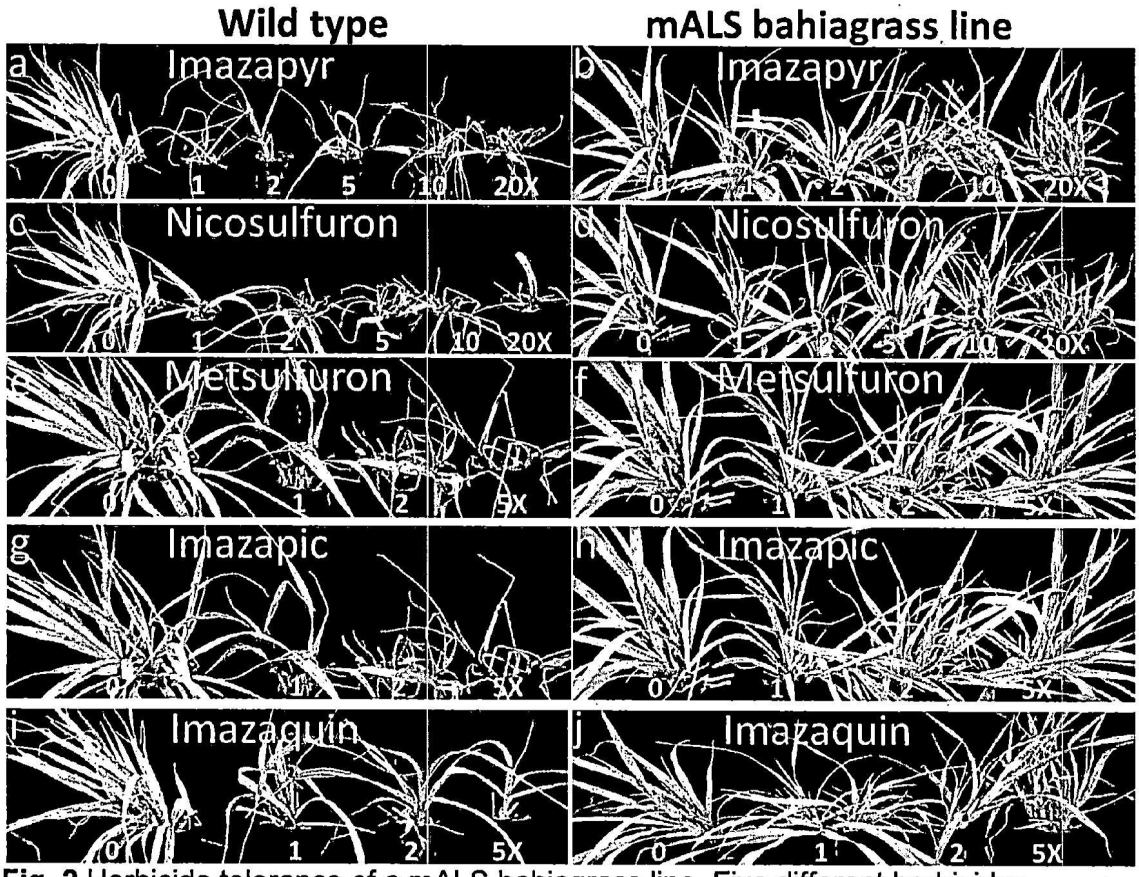

Fig. 2 Herbicide tolerance of a mALS bahiagrass line. Five different herbicides containing upto 5x or 20x concentration of recommended dose were sprayed on wild type and mALS bahiagrass line. Imazapyr and Nicosulfuron upto 20x, while Metsulfuron, Imazapic and Imazaquin upto 5x doses were used. Except Imazaquin (i), all four herbicides were able to suppress the growth of wild type bahiagrass (a, c, e and g), while mALS bahiagrass survived and grown very well upto 5x or 20x concentration of recommended herbicide doses for all the five herbicides (b, d, f, h, and j). Experiments were replicated three times and pictures were taken three weeks after herbicide treatments.

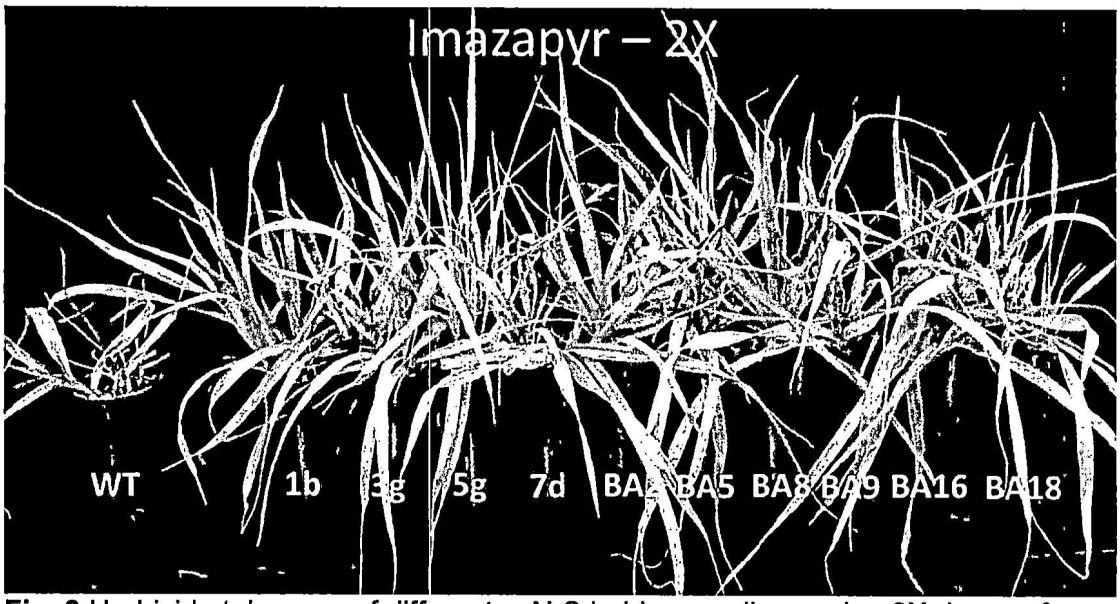

Fig. 3 Herbicide tolerance of different mALS bahiagrass lines using 2X doses of Imazapyr. Different bahiagrass lines were sprayed with two times higher than the recommended dose of Imazapyr. mALS bahiagrass lines showed tolerance against 2X dose of Imazapyr, while wild type (WT) plant was killed. Experiments were replicated three times and pictures were taken three weeks after herbicide treatments.

The injury caused by the herbicides was evaluated at 7, 14 and 21 days after treatment (DAT) using a visual evaluation scoring from zero (total absence of herbicide symptom) to 100 % (complete necrosis). This evaluation was carried out considering the untreated plants of each line as controls. The effects of the ALS-inhibiting herbicides starts to occur at 7 to 10 days causing reddish color in the new growing leaves. At 7 DAT, the injury caused by the herbicides on the wild type ranged from 20 to 47.5% (Table 1). However, absence of injury occurred in all mALS bahiagrass lines. The larger injury in the wild type line was caused by the herbicides imazapyr and imazapic and the lower herbicide effect was obtained with imazaquim (Table 1). In the evaluation at 14 DAT the level of injury increased in relation to the evaluation at 7 DAT (Table 2). In this evaluation even the 20 X of the recommended rate of imazapyr and nicosulfuron resulted in absence of effect in all mALS lines. At 21 DAT the herbicides resulted in injury ranging from 30 to 100% in the wild type and none symptoms were observed in all mALS lines (Table 3). In this evaluation the injury caused by the herbicides imazapyr and nicosulfuron at 2 X the recommended doses in the wild type was 96.3 and 85.0%, respectively. All mALS lines had no symptoms of the herbicides applied and consequently can be considered as resistant to the ALS-inhibiting herbicides.

Dry weight evaluated at 21 DAT was significantly reduced in the wild type for all herbicide and doses treatments in comparison with the untreated control (Table 4). This matches the observation that all herbicides used were lethal for wild type bahiagrass while mALS bahiagrass continued to grow like the untreated control. Therefore, all mALS lines had similar dry weight than the untreated control, which indicates that these lines are resistant to the herbicides up to the highest rates evaluated for all herbicides (20 X for imazapyr and nicosulfuron and 5 X for metsulfuron, imazapic, imazaquim and metsulfuron + chlorsulfuron (Table 4)). The rates up to 5X and 20X evaluated in this

study demonstrate the high level of resistance to the mALS lines. However, the resistance to the 2X doses is most relevant for practical field applications of herbicides, where boom overlapping and variations in herbicide preparations may cause higher than the intended 1x application rate. None of the mALS bahiagrass lines displayed injury symptoms at the 2X application rate. This confirms the success of the chosen approach and suggests that these compounds could be used without injury for weed control in bahiagrass mALS lines. The herbicide mixture metsulfuron + chlorsulfuron is used for Argentinian bahiagrass control in pastures in specific situations were the elimination of bahiagrass is desired. The mALS lines obtained in this study were not affected by this herbicide mixture up to 5X the recommended rate (Table 4), indicating that these bahiagrass lines are resistant to these compounds and herbicides with an alternative target enzyme like Roundup (epsp synthase) would be required to eliminate mALS bahiagrass. The herbicide imazapyr has a broad spectrum of weed control including cogon grass and a large residual activity. Several herbicide tolerant crops had been developed in the past based on the resistance to imazapyr. The dry weight of none of the mALS bahiagrass lines was affected by imazapyr even at application rates of 20X indicating very high level of resistance to this potent herbicide (Table 4).

Table 1 – Herbicide injury (%) at 7 days after spraying of bahiagrass lines treated with different ALS-inhibiting herbicides.

Herb/line	1b	3g	5g	7d	BA1	BA2	BA5	BA8	BA9	BA18	WT	Mean
Untreated	0	0	0	0	0	0	0	0	0	0	0	f[1] 0.0
Metsulfur. 1X	0	0	0	0	0	0	0	0	0	0	28.8	abcde 2.6
Metsulfur. 2X	0	0	0	0	0	0	0	0	0	0	30.0	abcde 2.7
Metsulfur. 5x	0	0	0	0	0	0	0	0	0	0	42.5	abc 3.9
Imazapic 1X	0	0	0	0	0	0	0	0	0	0	33.8	abcde 3.1
Imazapic 2X	0	0	0	0	0	0	0	0	0	0	41.3	abc 3.8
Imazapic 5X	0	0	0	0	0	0	0	0	0	0	46.3	ab 4.2
Imazapyr 1X	0	0	0	0	0	0	0	0	0	0	36.3	abcd 3.3
Imazapyr 2X	0	0	0	0	0	0	0	0	0	0	40.0	abc 3.6
Imazapyr 5X	0	0	0	0	0	0	0	0	0	0	41.3	abc 3.8
Imazapyr 10X	0	0	0	0	0	0	0	0	0	0	42.5	abc 3.9
Imazapyr 20X	0	0	0	0	0	0	0	0	0	0	47.5	a 4.3
Imazaquim 1X	0	0	0	0	0	0	0	0	0	0	20.0	cde 1.8
Imazaquim 2X	0	0	0	0	0	0	0	0	0	0	21.3	cde 1.9
Imazaquim 5X	0	0	0	0	0	0	0	0	0	0	20.0	cde 1.8
Mets+Chlo 1X	0	0	0	0	0	0	0	0	0	0	21.0	bcde 1.9
Mets+Chlo 2X	0	0	0	0	0	0	0	0	0	0	26.3	bcde 2.4
Mets+Chlo 5X	0	0	0	0	0	0	0	0	0	0	35.0	abcd 3.2
Nicosulf. 1X	0	0	0	0	0	0	0	0	0	0	20.0	cde 1.8
Nicosulf. 2X	0	0	0	0	0	0	0	0	0	0	25.0	cde 2.3
Nicosulf. 5X	0	0	0	0	0	0	0	0	0	0	32.5	abcde 3.0
Nicosulf. 10X	0	0	0	0	0	0	0	0	0	0	33.8	abcde 3.1
Nicosulf. 20X	0	0	0	0	0	0	0	0	0	0	37.5	abcd 3.4
Mean	B 0	B 0	B 0	B 0	B 0	B 0	B 0	B 0	B 0	B 0	A 31.4	

[1] Means followed by different upper case letters within the column and by lower case letters within the line are significantly different (Tukey, 0.05%).

Table 2 – Herbicide injury (%) at 14 days after spraying of bahiagrass lines treated with different ALS-inhibiting herbicides.

Herb/line	1b	3g	5g	7d	BA1	BA2	BA5	BA8	BA9	BA18	WT	Mean
Untreated	0	0	0	0	0	0	0	0	0	0	0	h[1] 0.0
Metsulfur. 1X	0	0	0	0	0	0	0	0	0	0	28.8	bcdefg 3.4
Metsulfur. 2X	0	0	0	0	0	0	0	0	0	0	30.0	bcdefg 3.4
Metsulfur. 5x	0	0	0	0	0	0	0	0	0	0	42.5	abcdef 4.3
Imazapic 1X	0	0	0	0	0	0	0	0	0	0	33.8	abcdef 3.6
Imazapic 2X	0	0	0	0	0	0	0	0	0	0	41.3	abcdef4.1
Imazapic 5X	0	0	0	0	0	0	0	0	0	0	46.3	abcdef 4.3
Imazapyr 1X	0	0	0	0	0	0	0	0	0	0	36.3	abcdef 4.0
Imazapyr 2X	0	0	0	0	0	0	0	0	0	0	40.0	abcd 5.0
Imazapyr 5X	0	0	0	0	0	0	0	0	0	0	41.3	abc 5.6
Imazapyr 10X	0	0	0	0	0	0	0	0	0	0	42.5	a 6.5
Imazapyr 20X	0	0	0	0	0	0	0	0	0	0	47.5	ab 6.5
Imazaquim 1X	0	0	0	0	0	0	0	0	0	0	20.0	fg 2.0
Imazaquim 2X	0	0	0	0	0	0	0	0	0	0	21.3	efg 2.4
Imazaquim 5X	0	0	0	0	0	0	0	0	0	0	20.0	cdefg 2.3
Mets+Chlo 1X	0	0	0	0	0	0	0	0	0	0	21.0	defg 2.6
Mets+Chlo 2X	0	0	0	0	0	0	0	0	0	0	26.3	defg 2.8
Mets+Chlo 5X	0	0	0	0	0	0	0	0	0	0	35.0	abcde4.7
Nicosulf. 1X	0	0	0	0	0	0	0	0	0	0	20.0	efg 2.4
Nicosulf. 2X	0	0	0	0	0	0	0	0	0	0	25.0	efg 2.4
Nicosulf. 5X	0	0	0	0	0	0	0	0	0	0	32.5	bcdefg 3.4
Nicosulf. 10X	0	0	0	0	0	0	0	0	0	0	33.8	abcde4.9
Nicosulf. 20X	0	0	0	0	0	0	0	0	0	0	37.5	abcde5.0
Mean	B 0	B 0	B 0	B 0	B 0	B 0	B 0	B 0	B 0	B 0	A 40.9	

[1] Means followed by different upper case letters within the column and by lower case letters within the line are significantly different (Tukey, 0.05%).

Table 3 – Herbicide injury (%) at 21 days after spraying of bahiagrass lines treated with different ALS-inhibiting herbicides.

Herb/line	1b	3g	5g	7d	BA1	BA2	BA5	BA8	BA9	BA18	WT	Mean
Untreated	0	0	0	0	0	0	0	0	0	0	0	e[1] 0.0
Metsulfur. 1X	0	0	0	0	0	0	0	0	0	0	52.5	abcd 4.8
Metsulfur. 2X	0	0	0	0	0	0	0	0	0	0	62.5	abc 5.7
Metsulfur. 5x	0	0	0	0	0	0	0	0	0	0	93.8	ab 8.5
Imazapic 1X	0	0	0	0	0	0	0	0	0	0	72.5	ab 6.6
Imazapic 2X	0	0	0	0	0	0	0	0	0	0	82.5	ab 7.5
Imazapic 5X	0	0	0	0	0	0	0	0	0	0	97.5	ab 8.9
Imazapyr 1X	0	0	0	0	0	0	0	0	0	0	70.0	abc 6.4
Imazapyr 2X	0	0	0	0	0	0	0	0	0	0	96.3	ab 8.8
Imazapyr 5X	0	0	0	0	0	0	0	0	0	0	97.5	ab 8.9
Imazapyr 10X	0	0	0	0	0	0	0	0	0	0	100.0	a 9.1
Imazapyr 20X	0	0	0	0	0	0	0	0	0	0	100.0	a 9.1
Imazaquim 1X	0	0	0	0	0	0	0	0	0	0	30.0	bcd 2.7
Imazaquim 2X	0	0	0	0	0	0	0	0	0	0	30.0	bcd 2.7
Imazaquim 5X	0	0	0	0	0	0	0	0	0	0	50.0	d 4.5
Mets+Chlo 1X	0	0	0	0	0	0	0	0	0	0	67.5	a 6.1
Mets+Chlo 2X	0	0	0	0	0	0	0	0	0	0	72.5	ab 6.6
Mets+Chlo 5X	0	0	0	0	0	0	0	0	0	0	95.0	ab 8.6
Nicosulf. 1X	0	0	0	0	0	0	0	0	0	0	82.5	ab 7.5
Nicosulf. 2X	0	0	0	0	0	0	0	0	0	0	85.0	ab 7.7
Nicosulf. 5X	0	0	0	0	0	0	0	0	0	0	95.0	ab 7.7
Nicosulf. 10X	0	0	0	0	0	0	0	0	0	0	100.0	a 9.1
Nicosulf. 20X	0	0	0	0	0	0	0	0	0	0	100.0	a 9.1
Mean	B 0	B 0	B 0	B 0	B 0	B 0	B 0	B 0	B 0	B 0	A 74.9	

[1] Means followed by different upper case letters within the column and by lower case letters within the line are significantly different (Tukey, 0.05%).

Table 4 – Aboveground dry weight (g) at 21 days after spraying of bahiagrass lines treated with different ALS-inhibiting herbicides.

Herb/Line	1b	3g	5g	7d	BA1	BA2	BA5	BA8	BA9	BA18	WT
Untreated	A[1] 0,842 a	AB 0,556 a	A 0,480 a	A 0,8648 a	A 0,767 a	ABC 0,852 a	A 0,766 a	AB 0,675 a	AB 0,637 a	A 0,430 a	A 0,887 a
Metsulfur. 1X	A 0,803 ab	B 0,467 abc	A 0,434 ab	A 0,7775 ab	A 0,729 ab	AB 0,991 a	A 0,761 ab	AB 0,813 ab	AB 0,605 abc	A 0,596 abc	B 0,135 c
Metsulfur. 2X	A 0,518 ab	AB 0,623 ab	A 0,432 ab	A 0,748 a	A 0,563 ab	ABC 0,592 ab	A 0,628 ab	AB 0,581 ab	AB 0,494 ab	A 0,402 ab	B 0,154 b
Metsulfur. 5x	A 0,416 ab	AB 0,539 ab	A 0,840 a	A 0,5365 ab	A 0,598 ab	C 0,585 ab	A 0,618 ab	B 0,421 ab	AB 0,423 ab	A 0,686 ab	B 0,135 b
Imazapic 1X	A 0,694 ab	AB 0,674 ab	A 0,603 ab	A 0,6793 ab	A 0,817 a	AB 0,915 a	A 0,734 a	AB 0,724 ab	AB 0,474 ab	A 0,550 ab	B 0,146 b
Imazapic 2X	A 0,852 ab	AB 0,923 ab	A 0,504 abc	A 0,681 abc	A 1,008 a	ABC 0,754 ab	A 0,759 ab	AB 0,666 abc	AB 0,761 ab	A 0,381 bc	B 0,134 c
Imazapic 5X	A 0,636 ab	AB 0,808 a	A 0,428 ab	A 0,9093 a	A 0,822 a	AB 0,922 a	A 0,760 a	AB 0,636 ab	AB 0,485 ab	A 0,451 ab	B 0,091 b
Imazapyr 1X	A 0,634 ab	AB 0,808 a	A 0,462 ab	A 0,732 a	A 0,776 a	AB 1,001 a	A 0,980 a	AB 0,726 a	AB 0,770 a	A 0,510 ab	B 0,134 b
Imazapyr 2X	A 0,645 abc	AB 0,814 ab	A0,5235 abc	A 0,531 abc	A 1,041 a	ABC 0,782 ab	A 0,728 ab	AB 0,623 abc	AB 0,580 abc	A 0,514 ab	B 0,133 c
Imazapyr 5X	A 0,638 ab	AB 0,538 ab	A 0,4315 ab	A 0,625 ab	A 0,830 a	ABC 0,602 ab	A 0,871 a	AB 0,851 a	AB 0,532 ab	A 0,679 ab	B 0,123 b
Imazapyr 10X	A 0,687 a	AB 0,901 a	A 0,4598 ab	A 0,744 a	A 0,947 a	ABC 0,678 a	A 0,554 ab	AB 0,698 a	AB 0,627 ab	A 0,414 ab	B 0,059 b
Imazapyr 20X	A 0,853 ab	AB 0,877 ab	A 0,4653 ab	A 0,599 abc	A 1,003 a	ABC0,528abc	A 0,580 abc	AB 0,596 abc	B 0,460 ab	A 0,536 ab	B 0,081 c
Imazaquim 1X	A0,852 abcd	A 1,158 a	A0,5223 abc	A 0,989 abc	A 0,873 a	AB 1,105 ab	A 0,953 abc	AB0,828abcd	AB 0,487 ab	A0,673abcd	B 0,314 d
Imazaquim 2X	A0,786 abcd	AB1,039 abc	A 0,6273 ab	A 0,915 abc	A 1,141 a	A 1,187 a	A0,773 abcd	AB 1,078 ab	AB0,790abcd	A 0,523 ab	B 0,254 d
Imazaquim 5X	A 0,663 abc	AB 0,808 ab	A 0,4203 ab	A 1,006 abc	A 0,970 ab	ABC 0,886 ab	A 1,022 a	A 1,151 a	AB 0,663 abc	A 0,824 ab	B 0,198 c
Mets+Chlo 1X	A 0,720 ab	AB 0,891 a	A 0,4003 ab	A 0,986 a	A 0,982 a	ABC 0,594 ab	A 0,926 a	AB 0,789 a	AB 0,691 ab	A 0,522 ab	B 0,160 b
Mets+Chlo 2X	A 0,836 a	AB 1,036 a	A 0,706 ab	A 1,153 a	A 1,005 a	ABC 0,812 a	A 0,701 ab	AB 0,919 a	A 0,789 a	A 0,818 a	B 0,216 b
Mets+Chlo 5X	A 0,746 a	AB 1,109 a	A 0,632 ab	A 0,993 a	A 1,091 a	ABC 0,703 ab	A 1,013 a	AB 0,921 a	AB 0,745 a	A 0,601 ab	B 0,154 b
Nicosulf. 1X	A 0,555 ab	AB 0,783 a	A 0,4635 ab	A 0,603 abc	A 0,858 a	AB 0,965 a	A 0,616 ab	AB 0,625 ab	AB 0,543 ab	A 0,517 ab	B 0,161 b
Nicosulf. 2X	A 0,700 ab	AB 0,601 ab	A 0,4826 ab	A 0,812 a	A 0,822 a	AB 0,964 a	A 0,525 ab	AB 0,816 a	AB 0,726 ab	A 0,587 ab	B 0,202 b
Nicosulf. 5X	A 0,614 ab	AB 0,901 a	A 0,4716 ab	A 0,959 a	A 0,972 a	ABC 0,892 a	A 0,582 ab	AB 0,564 ab	AB 0,580 ab	A 0,523 ab	B 0,115 b
Nicosulf. 10X	A 0,611 ab	AB 0,719 a	A 0,4314 ab	A 0,998 a	A 1,013 a	BC 0,481 ab	A 0,600 ab	B 0,491 ab	AB 0,508 ab	A 0,577 ab	B 0,104 b
Nicosulf. 20X	A 0,637 a	B 0,477 ab	A 0,2913 ab	A 0,640 a	A 0,836 a	ABC 0,533 ab	A 0,647 a	AB 0,738 a	AB 0,453 ab	A 0,515 ab	B 0,048 b

[1] Means followed by different upper case letter within the line and preceded by lower case letters within the column are significantly different (Tukey, 0.05%)

2. Development and analysis of the brown midrib gene (BMR) using genome editing

Mutation in BMR gene is reported to produce brown midrib phenotype in maize, sorghum and sugarcane (Jung and Altpeter 2016). We isolated partial fragment of BMR gene containing 2nd exon from bahiagrass and targeted using genome editing tool TALEN, to create mutation in the coding region (2nd exon) of the BMR gene. TALEN construct was developed and transformed into bahiagrass with biolistic gene transfer prior to the project start.

A. Molecular analysis of original lines and their progenies

Genomic DNA was isolated from leaf tissues of TALEN transformed plantlets and tested for the presence of TALEN cassette by PCR. Out of 35 tested lines, 19 lines were positive for the presence of TALEN (Fig. 4A). TALEN mediated mutagenesis of BMR gene is expected to delete the nucleotide sequences present between two TALEN binding arms. To test the TALEN mediated mutation of BMR gene in bahiagrass, genetic region present between two TALEN binding arms were amplified by PCR and digested by *Xho*I restriction enzyme. Non-mutated BMR gene will be digested by *Xho*I while mutated BMR gene will be resistant to *Xho*I digestion. As shown in figure 4B, *Xho*I resistant DNA bands of higher size (upper DNA band) were observed in four different original bahiagrass lines 15, 16, 23 and 39, suggests the TALEN mediated mutation of BMR gene in these lines. However, presence of smaller size bands (lower band) in addition to *Xho*I resistant band of higher size, indicates either the chimeric nature of transformed tissues or presence of different BMR allelic variants.

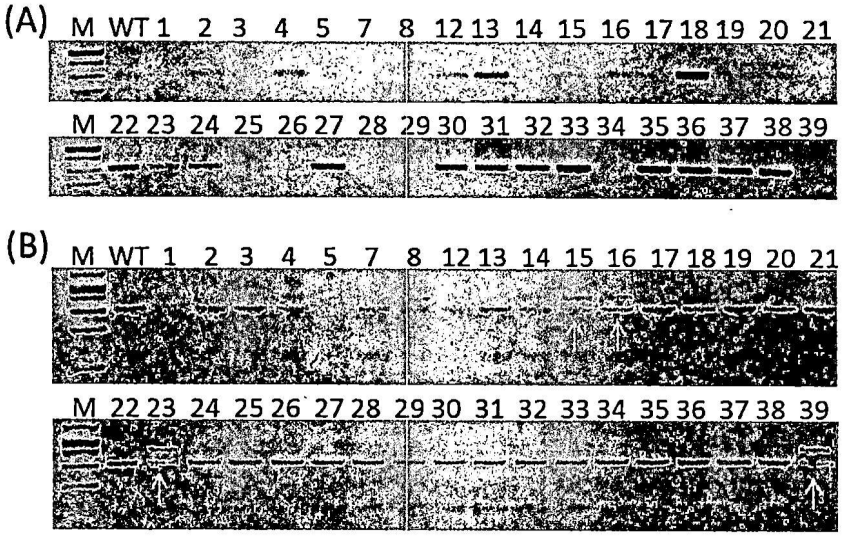

Fig. 4 Agarose gel electrophoresis to confirm the presence of TALEN and TALEN mediated BMR mutation in bahiagrass lines. (A)TALEN transformed bahiagrass lines were confirmed by PCR, and presence of band in each lane indicates the presence of TALEN construct in transformed bahiagrass lines. (B) DNA region of BMR gene flanked by TALEN binding arms were amplified by PCR and subjected to *Xho*I restriction enzyme digestion. Presence of upper band in arrow marked lines 15, 16, 23 and 39 indicates the mutation in BMR gene.

To further confirm the inheritance of TALEN mediated BMR mutation in bahiagrass, vegetative progenies derived from seeds were used for capillary

electrophoresis of target PCR amplicons. Capillary electrophoresis has the potential to resolve even single base deletion in DNA. Capillary electrophoresis derived electropherograms of four original mutant lines and their seed derived progenies are shown in figure 5. Presence of additional peaks in BMR lines compared to WT, suggests the presence of mutations in BMR PCR amplicons produced by TALEN mediated BMR mutation in bahiagrass.

mutant lines, and WT is wild type bahiagrass. The mutation in BMR gene. Presence of additional peaks in BMR lines compared to WT suggests the different size BMR gene products due to deletion in BMR gene.

3. Suppression of the brown midrib gene with RNA interference

2nd exon of BMR gene from bahiagrass was cloned into sense and antisense orientation to produce double stranded RNA into bahiagrass for suppression of corresponding BMR gene expression. BMR-RNAinterference construct was biolistically transformed into callus of bahiagrass and four different bahiagrass lines were regenerated.

A. Molecular analysis of original lines and their progenies

Quantitative expression analysis was performed to test the RNAi mediated suppression of BMR gene in bahiagrass lines. As shown in figure 5, lines 3G and 7D showed 10% suppression (90%) and 25% suppression (75% expression) of BMR gene respectively compared to 100% expression of BMR gene in wild type.

B. Lignin content and in vitro digestibility

One of the RNAi suppressed BMR bahiagrass line (7D) had significantly higher level of potentially digestible neutral detergent fiber and significantly lower level of un-digestible fiber fraction as determined by incubation in rumen fluid for 120 hr and 240 hr (Table 6). This result was highly correlated with highly significant suppression (25%) of target gene in 7D as compared to wild type bahiagrass (Fig. 6). However, lignin and neutral detergent fiber content of TALEN mediated BMR mutant, BMR-RNAi suppressed bahiagrass lines were similar to wildtype.

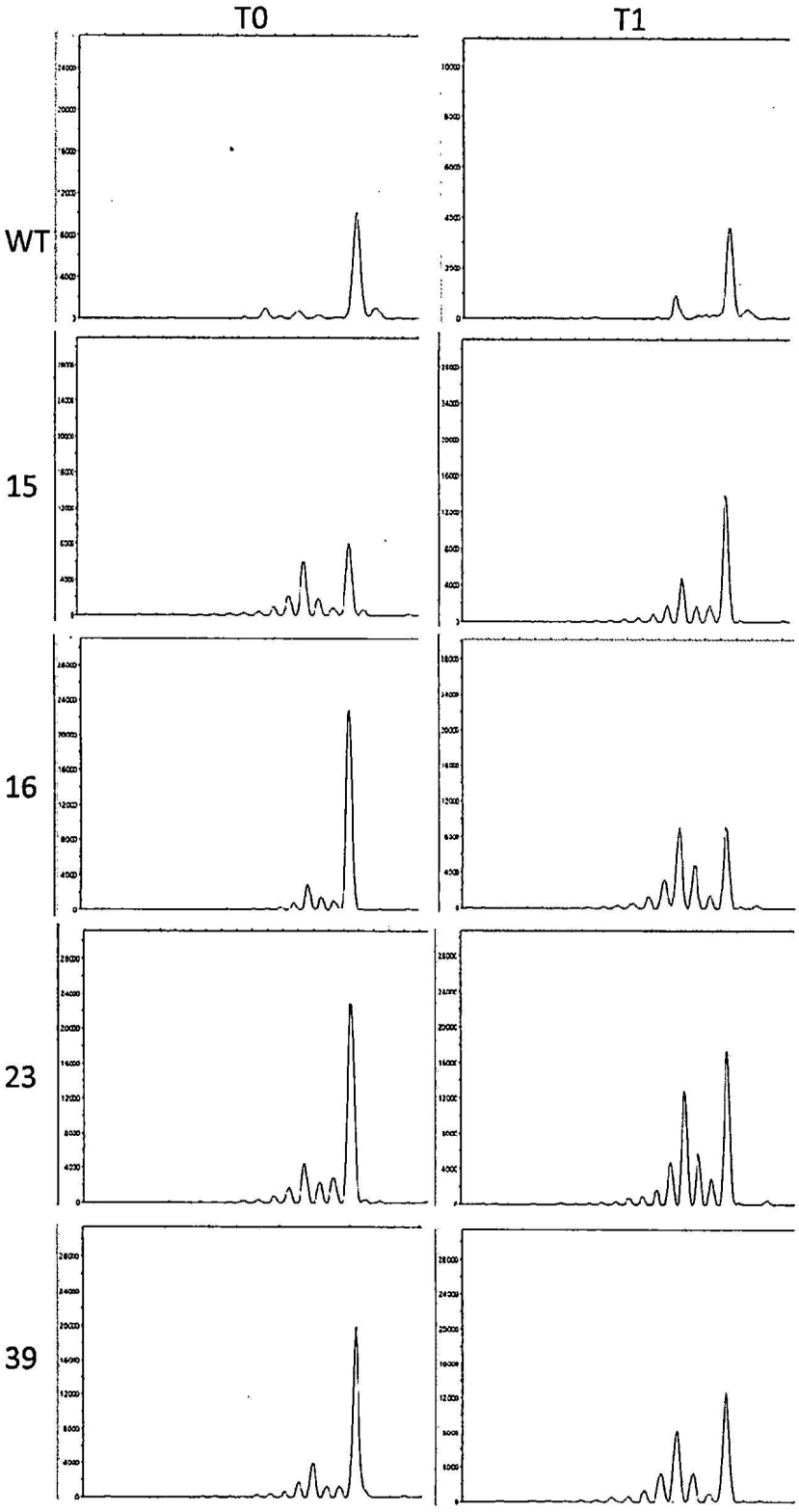

Fig. 5 Capillary electropherograms showing TALEN mediated mutation in BMR gene of bahiagrass. Fluorescently labeled PCR amplified DNA flanking the BMR mutation site was subjected to capillary electrophoresis. Capillary electrophoresis was performed in original lines (T0) and their progenies (T1). 15, 16, 23 and 39 indicate different BMR

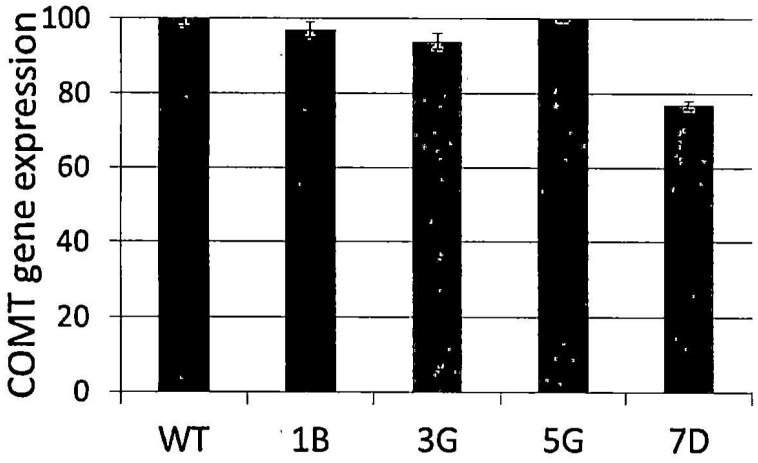

Fig. 6 Expression of BMR gene in BMR-RNAi bahiagrass lines. RNA isolated from four BMR-RNAi lines and wild type (WT) plants of bahiagrass was converted into cDNA and used for expression analysis using quantitative PCR. Expression of BMR gene was performed in triplicate.

Table 6. Lignin and in vitro digestible neutral detergent fiber content in TALEN mediated BMR mutant and BMR-RNAi bahiagrass lines.

Lines	aNDF	% uNDFom 30hr	% uNDFom 120hr	% uNDFom 240hr	% NDFDom 30hr	% NDFDom 120hr	% NDFDom 240hr	Avg. lignin
M36	57.93 ±0.1^{C}	18.57 ±0.4^{BCD}	13.30±0.4^{AB}	10.30±0.2^{A}	67.93±0.8^{BCD}	77.18±0.7^{E}	82.23±0.3^{CD}	3.30 ±0.8^{A}
WT	62.17 ±0.3^{AB}	12.77 ±0.8^{E}	9.97±0.6^{CD}	8.80±0.1^{AB}	79.47±1.4^{A}	84.00±0.9^{BC}	85.90±0.1^{BC}	2.37 ±0.8^{A}
1B	58.83 ±0.4^{BC}	14.40 ±0.5^{DE}	13.10±0.3^{AB}	10.70±0.7^{A}	75.53±0.6^{A}	77.77±0.4^{E}	81.83±1.2^{AB}	3.00 ±0.8^{A}
3G	61.73 ±1.2^{AB}	12.87 ±1.1^{E}	8.70±0.2^{DE}	6.37±0.6^{C}	79.23±1.4^{A}	85.93±0.2^{AB}	89.70±0.8^{A}	2.53 ±0.2^{A}
5G	61.57 ±0.4^{AB}	15.70±1.3^{DE}	9.27±0.1^{DE}	6.90±0.3^{BC}	74.47±2.2^{AB}	84.97±0.3^{AB}	88.73±0.6^{AB}	3.23 ±0.7^{A}
7D	65.07 ±0.7^{A}	17.10±0.6^{CDE}	7.03±0.1^{E}	5.90±0.2^{C}	73.73±0.9^{ABC}	89.17±0.2^{A}	90.97±0.4^{A}	2.63 ±0.7^{A}
15	61.70 ±0.2^{AB}	20.37±0.2^{ABC}	14.40±0.3^{A}	9.97±0.2^{A}	66.97±0.3^{CD}	76.70±0.5^{E}	83.90±0.3^{CD}	2.13 ±0.1^{A}
16	61.60 ±0.6^{AB}	22.60±0.6^{AB}	12.37±0.5^{ABC}	10.30±0.3^{A}	63.33±0.9^{D}	79.90±0.7^{CDE}	83.33±0.4^{CD}	3.73 ±0.3^{A}
23	63.20 ±0.2^{A}	23.13±0.2^{AB}	10.57±0.2^{BCD}	9.77±0.3^{A}	63.43±0.3^{D}	83.30±0.4^{BCD}	84.57±0.5^{CD}	3.43 ±0.3^{A}
39	64.57 ±0.27^{A}	24.10±0.2^{A}	13.50±0.8^{A}	11.20±0.2^{A}	62.60±0.3^{D}	79.10±1.2^{DE}	82.63±0.4^{CD}	3.40 ±0.1^{A}

Means ± standard error followed by different capital letters within the column are significantly different at $p<0.05$ (Scheffe's test)
WT, wildtype; M36, Random mutant line; 1B, 3G, 5G, 7D, BMR-RNAi lines; 15, 16, 23, 39, TALEN mediated BMR mutant lines;
aNDF-Neutral Detergent Fiber content (protein and starch free basis);
aNDFom- Neutral Detergent Fiber content (organic matter free basis);
uNDFom – Undigested Neutral Detergent Fiber content (organic matter free basis);
NDFDom- Neutral Detergent Fiber Digestibility (organic matter free basis);

Impact and conclusions for future work

Lignin content and in vitro digestibility:
A modest improvement in digestibility was observed in one bahiagrass line following RNAinterference suppression of the BMR gene COMT. Stronger suppression of COMT (more than 80% suppression) is typically required for significant reduction in lignin content which was not confirmed in any of the analyzed bahiagrass lines. However, moderate suppression of COMT has been reported to affect lignin monomer (S/G) ratios which also influence digestibility. Future improvement should include alternative RNAi constructs or TALEN pairs targeting different regions of the COMT gene. Most recently we successfully generated brown midrib sugarcane by targeting the 1st exon region of the COMT gene (Jung and Altpeter 2016). This region has now been cloned by us from bahiagrass and would allow translation of this successful approach to bahiagrass. Genome editing with TALEN or other tools has recently been treated as exempt from deregulation by USDA-Aphis on a case by case basis and as long as no transgenic footprint is left in the final improved cultivar.

Herbicide resistance:
The level of herbicide resistance achieved with a single point mutation of the ALS gene was impressive. Resistance also included a range of herbicides. The most relevant of these herbicides for weed control in bahiagrass including being imazapyr for difficult to control weeds like cogon grass and nicosulfuron for less problematic weeds. The regulatory costs associated with a commercial release of mALS bahiagrass generated with this gene transfer approach will likely be reduced compared to a transgenic approach since only sequences from a related grass (Sorghum) were used but may still be significant. However, an alternative approach to avoid any regulatory costs is the random chemical mutagenesis of the bahiagrass ALS gene. Chemical mutagenesis is considered traditional plant breeding, does not require any gene transfer and it is currently not subject to regulation by USDA-APHIS. We recently used chemical mutagenesis to generate bahiagrass with improved turf quality (Kannan et al. 2015). The success of the here reported project confirms the potential of such a chemical mutagenesis strategy to generate a mutated ALS gene for herbicide resistance. We will shortly submit a proposal to the FCA to carry out chemical mutagenesis to identify random mutants of the bahiagrass ALS gene. The required tissue culture and selection and analytical procedures following chemical mutagenesis have already been established with the here completed project and will drive the success.