RESEARCH

Obtaining Sethoxydim Resistance in Seashore Paspalum

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ABSTRACT

Herbicide resistance has been a sought-after trait for turfgrasses, however attempts to commercialize genetically modified (GM) turfgrasses have been unsuccessful. Sethoxydim (2-[1-(ethoxyimino]butyl)-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one) is a grass-specific herbicide, and resistance results from one of two possible single base-pair (bp) mutations. The most common mutation is an isoleucine (Ile) to leucine (Leu) substitution caused by an A to T mutation at position 1781 of acetyl coenzyme A carboxylase. Research was initiated to develop a novel source of resistance to sethoxydim in seashore paspalum (*Paspalum vaginatum* Swartz). The objectives of the present study were to develop in vitro selection and regeneration protocols to select for naturally occurring mutations conferring herbicide resistance. A dose response experiment was performed to determine the optimum sethoxydim concentration for selection. Callus was induced from immature inflorescences then plated on callus induction medium containing 10 μM sethoxydim for selection. Green plants were regenerated from resistant callus, the Ile to Leu mutation documented, and expression of herbicide resistance confirmed.

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Abbreviations: ACCase, acetyl coenzyme A carboxylase; APP, aryloxyphenoxypropionate; Asp, aspartate; BAP, benzylaminopurine; bp, base pairs; CHD, cyclohexandione; CT, carboxyl transferase; DAT, days after treatment; Gly, glycine; I_{50}, 50% injury; Ile, isoleucine; Leu, leucine; MK, Mauna Kea; MS, Murashige and Skoog; SR, sethoxydim resistant; PT, parental type; TCC, tissue culture control.

Seashore paspalum is a warm-season turfgrass that is adapted to coastal environments (Morton, 1973). This species is generally adapted to the same regions of the world as bermudagrass [*Cynodon dactylon* (L.) Pers.] and has numerous morphological characteristics that make it desirable as a turfgrass. Interest in the use of seashore paspalum as a turfgrass is largely related to its tolerance to salt and other abiotic stresses (Duncan and Carrow, 2000). Golf course architects recommend seashore paspalum for new courses in tropical or subtropical coastal areas where salt or water quality are issues. Many existing golf courses around the world have replaced bermudagrass with paspalum (Raymer et al., 2008).

The main difficulty in replacing bermudagrass with paspalum is bermudagrass re-establishment. Bermudagrass is highly competitive and difficult to eradicate once established (Lowe and Sweet, 2006). Invasion by bermudagrass and other weedy

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grasses can greatly reduce the aesthetic value and quality of paspalum turf. Currently there are no herbicides available that selectively control bermudagrass in seashore paspalum. Development of herbicide-resistant paspalum could provide an effective means of managing bermudagrass in paspalum and allow golf course and sporting venues to transition from bermudagrass to seashore paspalum.

Sethoxydim is a graminicide used to control perennial and annual grasses in numerous agricultural and ornamental crops. This herbicide is an acetyl coenzyme A carboxylase (ACCase) inhibitor and a member of the cyclohexanedione (CHD) family. Sethoxydim has been marketed in the United States under numerous trade names, including Poast, Poast Plus, Segment, and Vantage (BASF Corp., Research Triangle Park, NC). Sethoxydim and other ACCase inhibitors cause cell death by competing with acetyl-CoA for its binding site in ACCase (Délye, 2005). Therefore, the first step of fatty acid biosynthesis is halted and the product, malonyl coenzyme A, is not produced. Cell membranes are compromised without fatty acids and cell death ensues.

Spontaneous occurrence of sethoxydim resistance has been observed in numerous weedy species. Such resistance has arisen after the large-scale application of sethoxydim on an annual or semi-annual basis in various cropping systems (Delye and Michel, 2005). The most commonly reported mutation is a single Ile to Leu substitution at amino acid position 1781 in the carboxyl transferase (CT) domain (Delye and Michel, 2005) of the ACCase gene. An adenine to thymine transversion in the first position of the codon accounts for this substitution. Additionally, a mutation at the 2078 codon, causing a change from aspartate (Asp) to glycine (Gly), also confers resistance to sethoxydim. Both of these mutations also confer resistance to aryloxyphenoxypropionate (APP) herbicides, another group of ACCase inhibiting herbicides.

Transgenic approaches for obtaining sethoxydim resistance are not practical. First, the ACCase gene is very large with an estimated size of ≈12,000 bp (Podkowinski et al., 1996), which makes it difficult to manipulate with current transformation procedures. The cDNA sequences available from GenBank are also large (≈7,000 bp) not including the vector backbone. Secondly, there is a high level of homology that exists between the inserted gene and the native genes. Since only 20 to 26 bp of homology are needed to cause gene silencing of both the inserted gene and the native gene, this approach would likely result in silencing (Brodersen and Voinnet, 2006) and be lethal.

The ACCase exists in two forms: eukaryotic and prokaryotic (Harwood, 1988). The eukaryotic form is a single polypeptide with distinct functional domains, and is encoded by a nuclear gene. Sasaki et al. (1995) found that ACCase is compartmentalized in most plants, such that the prokaryotic form is in the chloroplast and the eukaryotic form in the cytosol. The Poaceae are unique in that they have the eukaryotic form of ACCase in both the cytosol and chloroplast (Sasaki et al., 1995). The two eukaryotic forms of ACCase in grasses are very similar, as are the genes that code for them (Gornicki et al., 1994). Even though there is homology between the plastidic and cystolic ACCases, the cystolic form is not affected by ACCase-inhibiting herbicides (Délye, 2005). The prokaryotic form is made up of four subunits and is coded by four genes, one of which is located in the chloroplast genome. Although the prokaryotic ACCase is inherently herbicide resistant, engineering grasses with these genes is not a practical option, as it could require the coordinated transformation of up to four genes into the nuclear and chloroplastic genomes.

The spontaneous appearance of resistant alleles in response to herbicide applications indicates that resistance can be obtained without transformation. Sethoxydim resistance was obtained in maize (*Zea mays* L.*)* by in vitro selection, providing an example of a graminaceous crop in which weedy grasses can be controlled via herbicide resistance (Parker et al., 1990). The resistance is now understood to originate from the single Ile to Leu substitution at position 1781 mutation occurring in the plastidic ACCase in grasses (Delye et al., 2005).

The goal of this research was to identify naturally occurring sethoxydim-resistant mutants in seashore paspalum suitable for use in a breeding program for the development of herbicide-resistant cultivars. Specific objectives were: (i) to develop protocols necessary for in vitro selection and regeneration of naturally occurring sethoxydim resistant (SR) mutants, (ii) to use these protocols to obtain sethoxydim resistant plants, (iii) to confirm the presence of resistance conferring mutations in these plants using molecular techniques, and (iv) to confirm expression of resistance to sethoxydim and other ACCase herbicides at the whole plant level.

MATERIALS AND METHODS

Tissue Culture

Explants were obtained from 10 genotypes including: eight experimental lines from The University of Georgia seashore paspalum breeding program, one collected ecotype, Mauna Kea (MK) (PI 647892), and the commercial seeded cultivar, 'Seaspray' (Table 1). Immature inflorescences were removed from floral shoots prior to emergence. The two racemes were separated and surface sterilized with 10% commercial bleach with several drops of Tween 80 for 10 min then rinsed with sterile water.

The induction medium consisted of MS basal salts (Murashige and Skoog, 1962) supplemented with B5 vitamins (Gamborg et al., 1968) and 2 mg L^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid) (Cardona and Duncan, 1997). The medium was brought to a pH of 5.8 and 2 g of Gelzan (Caisson Laboratories

Table 1. Summary of cell lines completing selection for sethoxydim resistance.

Cell line	Genotype	Cell line initiation	Calli through selection†	SR Calli‡	SR calli regenerated§	Calli positive for Leu1781
		Date			no.	
1	Mauna Kea	28 Nov. 2007	225	0	0	0
2	Mauna Kea	5 Dec. 2007	1350	3	0	0
3	Mauna Kea	12 Dec. 2007	225	0	0	0
4	Mauna Kea	9 Jan. 2008	1125	0	0	0
5	Mauna Kea	12 Jan. 2008	2025	29	2	3
6	Mauna Kea	21 Jan. 2008	450	7	0	0
7	Mauna Kea	6 Mar. 2008	1350	2	0	0
8	Mauna Kea	20 Mar. 2008	675	0	0	0
9	Seaspray	12 Jan. 2008	225	0	0	0
10	03-527.8	8 Jan. 2008	1575	0	0	0
11	03-527.8	21 Jan. 2008	900	0	0	0
12	03-527.8	16 May 2008	225	0	0	0
13	03-539.13	6 Mar. 2008	3825	11	0	0
14	03-539.13	13 Mar. 2008	1800	7	0	0
15	05-025.164	20 Mar. 2008	675	0	0	0
16	05-025.164	9 Apr. 2008	450	2	0	0
17	05-025.181	4 Mar. 2008	450	1	0	1
18	03-107C.1	4 Mar. 2008	450	0	0	0
19	03-098E.3	4 Mar. 2008	900	2	1	0
20	03-134F.17	4 Mar. 2008	225	0	0	0
21	03-525.22	20 Apr. 2008	1125	1	0	0
Totals			20,250	65	3	4

†Number of calli pieces completing three 3-wk cycles of selection for sethoxydim resistance.

‡Number of calli demonstrating exponential growth after three cycles of selection. SR, sethoxydim resistant.

§Number of SR calli producing viable plants after more than 6 wk on regeneration medium.

Inc., North Logan, UT) gelling agent was added. After autoclaving, the medium was poured into 100 × 15 mm Petri dishes. Four explants were placed on each plate, and the plates were sealed with Nescofilm (Karlan Research Products Co., Cottonwood, AZ). The explants were placed in the dark at 27°C. The callus generated was given a cell line designation based on the genotype and the date the explant was placed on induction medium.

The medium originally used for the induction of callus from bahiagrass (*Paspalum notatum* Flugge) (Altpeter and James, 2005) was modified by removing the auxin, thus allowing it to serve as the regeneration medium. The medium consists of MS/B5 basal medium supplemented with 1.24 mg L^{-1} CuSO4, and 1.125 mg L^{-1} BAP (6-benzylaminopurine). Calli of each sethoxydim-resistant (SR) line were placed on regeneration medium in a 4 × 4 grid, using five plates per line. Each callus was approximately 4 mm in size. These were placed in a growth chamber at 25°C with a 1-h dark, 23-h light photoperiod, and a light intensity of 66 to 95 μmol photons m^{-2} s^{-1} provided by cool-white fluorescent tubes. All plates were evaluated for regeneration at the end of a 30-d period. If shoots appeared, the cell lines were subcultured for an additional month on regeneration medium. To induce root growth, the shoots were transferred to MS/B5 basal medium without growth regulators. When root growth was adequate (≈30 days) the plants were removed from the medium and placed in pots containing a 1:1 mix of Fafard 3B (Conrad Fafard, Inc., Agawam, MA) mix and sand. The potted plants were maintained in a growth chamber for 1 wk as described, after which the plants were transferred to a greenhouse with a 10-h light, 14-h dark photoperiod at 24 to 32°C.

Callus Dose Response

The response of paspalum tissue to sethoxydim rate was determined using callus tissue generated from Seaspray. Laboratory-grade sethoxydim was obtained from Chemservice Inc. (West Chester, PA) and dissolved in methanol to create a stock solution with a concentration of 1 mg sethoxydim per 1 mL methanol. The sethoxydim concentrations evaluated were: 0, 2.5, 5, 7.5, 10, 25, 50, and 100 μM. The selection medium was the same as used for callus induction, with sethoxydim added after the autoclaved medium had cooled to approximately 55°C, thus preventing loss of activity from heat degradation (Campbell and Penner, 1985).

To measure callus growth, 0.5 g of callus tissue was weighed, separated into nine equal pieces, and placed in a 3 × 3 pattern on the medium of each plate. Six replicate plates for each of the eight sethoxydim concentrations were distributed on a rack in a growth room in a completely randomized design. At the 3-wk subculture period, the tissue from each plate was weighed and recorded. Only 0.5 g was subcultured from each plate and taken to the next growth period. This process was continued for 9 wk, providing three growth measurements for each plate. The weight from each plate at each of the measurement points (3, 6, and 9 wk) was divided by the initial weight to obtain the percent change for a 3-wk period (Parrot and Bouton, 1990). Callus growth in response to sethoxydim concentration was fitted to a negative exponential decay function using nonlinear regression (SAS Institute, 2008).

Selection of Sethoxydim-Resistant Cells

Callus tissue, approximately 6-mo old, was subcultured onto MS/B5 induction medium supplemented with 10 μM

sethoxydim, as determined by the dose response study. Calli approximately 4 mm in diameter were placed in 245 × 245 mm square bioassay plates in a 15 × 15 grid, for a total of 225 calli per plate. After 3 wk, the calli were subcultured to fresh plates. The process was repeated three times for a total selection period of 9 wk. Resistant calli were subcultured into 100 × 15 mm Petri dishes containing callus induction medium supplemented with 10 μM sethoxydim for 1 mo to obtain sufficient callus. This provided a total selection time of at least 12 wk.

Resistant Calli Frequency

Individual cells were measured to determine cell size and thus estimate the frequency of resistant cells relative to the total number of cells placed on selection medium. To obtain individual cells, 2 g of callus tissue were added to 25 mL of liquid induction medium and placed on a shaker at 112 rpm. After growing in the dark at 25°C for 5 d, clear medium, absent of callus clusters, was extracted. Fifteen microliters of medium were placed on a slide and mixed with 2 μL of modified carbol fuchsin stain (Kao, 1975). Cells were observed in a microscope at 40X and measured using Image Pro Plus (Media Cybernetics Inc., Bethesda, MD). The diameter from a total of 100 cells was measured twice to calculate an average cell diameter. This value was used to estimate the volume of a single cell based on the assumption that cells were spherical. To find the approximate volume of a 4 mm callus piece, 100 representative calli were placed in a graduated cylinder to find the displacement in water and obtain the callus volume, which was divided by the volume of a single cell, thus providing an estimate of the number of cells in a 4 mm callus piece.

Data describing the number of resistant calli observed per plate completing the selection process were modeled as the probability of a resistant callus event per trial (plate of 225 calli) and tested for differences among genotypes using Proc GLIMMIX SAS (SAS Institute, 2008). In this analysis each plate of 225 calli was considered a replicate. Replicate numbers varied with genotype due to differences in the amount of tissue available for selection.

Molecular Characterization

Once SR paspalum lines were selected, the mutation causing the resistance was characterized. DNA was extracted from callus or leaves of regenerated plants via the CTAB method as described by Lassner et al. (1989).

Primers used for PCR amplification of the region surrounding the 1781 position were designed based on the amino acid sequence described by Delye et al. (2005). Two regions flanking the 1781 position were selected as targets for the forward and reverse primers due to their conservation among species. The nucleotide sequence from *Setaria viridis* ACCase (GenBank AF294805) (Delye et al., 2002) was translated using Generunner V.3.05 (Hastings Software, 1994) to find the nucleotide sequence corresponding to the conserved amino acid region. Twenty bps were selected in each homologous region and the BLAST function was used on GenBank to check homology between species. Individual bases were changed to match the highest number of grass species possible. The resulting primers amplify a 384-bp fragment of the ACCase gene that spans the A to T transversion which causes the Ile to Leu substitution at the 1781 position. The primers were designated SV384F (5' CGGGGTTTCAGTACATTTAT 3') and SV384R (5' GATCTTAGGACCACCCAACTG 3'). Annealing temperature was 53°C with an extension time of 30 s and 35 cycles. The same process was used to sequence the 2078 region. The primers developed for sequencing the 2078 region were SVAC2F (5'AATTCCTGTTGGTGTCATAGCTGTGGAG 3') and SVAC1R (5' TTCAGATTTATCAACTCTGGGTCAAGCC 3'). These primers amplify a fragment 520 bp in length that spans the coding region of the 2078 position.

Whole Plant Response to Acetyl Coenzyme A Carboxylase Inhibiting Herbicides

The SR plants regenerated from a sethoxydim-resistant cell line, SR11, were tested for resistance at the whole plant level in a dose-response experiment conducted in a greenhouse. In this experiment, SR11 was compared to two herbicide-susceptible controls; the original parental line, MK (PT); and a MK line regenerated from tissue culture (TCC). Plants were transplanted to Conetainers measuring 4 × 14 cm and tapering to 1 cm (Stuewe and Sons, Inc., Corvallis, OR) containing a 1:1 mix of Fafard 3B mix and sand and placed on benches under sodium lights in a greenhouse with a 16-h photoperiod maintained at 27/32°C day/night for 2 wk prior to treatment applications.

Herbicides were applied at a spray volume of 187 L ha^{-1} in an experimental spray chamber, and after drying, returned to the greenhouse bench and maintained under the conditions described above. Visual estimates of crop injury were recorded at 21 d after treatment (DAT) using a scale of 0 to 100%, where 0% equals no injury and 100% equals complete death. Estimates of herbicide rate at which 50% injury occurred (I_{50}) were calculated by interpolation.

Each of the three genotypes (SR11, PT, and TCC) was compared in four separate herbicide dose-response experiments. The herbicides tested are all ACCase inhibitors and registered for use on turfgrass in the U.S. Herbicides tested included CHD herbicides, sethoxydim (Segment, BASF Corp., Research Triangle Park, NC), and clethodim (Envoy Plus, Valent U.S.A. Corporation, Walnut Creek, CA) and the APP herbicides fluazifop-p-butyl (Fusilade II, Syngenta Crop Protection, Inc., Greensboro, NC) and fenoxaprop-p-ethyl (Acclaim Extra, Bayer Environmental Science, Montvale, NJ). The sethoxydim dose response experiment was a three by eight factorial with three genotypes and eight herbicide rates of 0, 50, 100, 200, 400, 800, 1600, and 3200 g ai ha^{-1}. In the clethodim, fluazifop, and fenoxaprop experiments, four replicates of each of the three genotypes were treated with seven rates of the appropriate herbicide including: 0, 50, 100, 200, 400, 800, and 1600 g a.i. ha^{-1}. Treatments of all experiments were arranged in randomized complete block designs.

Data for all whole plant response experiments were first analyzed using a two-way analysis of variance (SAS Institute, 2008) and subsequently analyzed within herbicide rate. Differences among genotype means at each herbicide rate were determined using Fisher's Least Significant Difference (LSD).

RESULTS

Callus Dose Response

Results of the callus dose response experiment are shown in Fig. 1. Even the lowest concentration of 2.5 μM sethoxydim

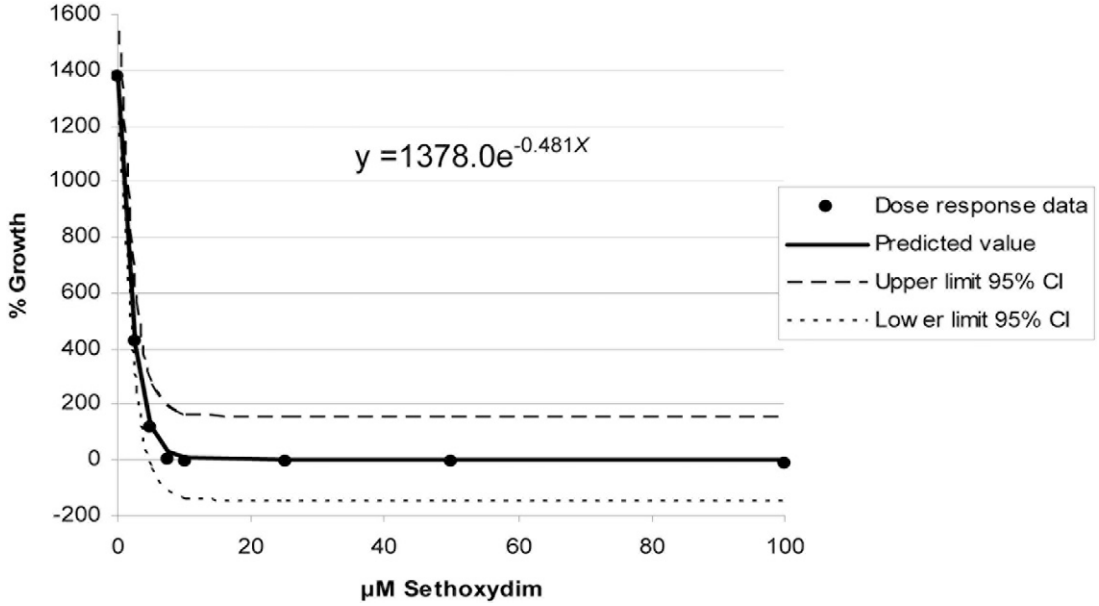

Figure 1. Growth response of seashore paspalum callus tissue after 9 wk exposure to varying concentrations of sethoxydim in the medium.

in the medium reduced callus growth dramatically and 7.5 µM sethoxydim prevented all growth of callus tissue. When these data were fitted to a negative exponential decay function using nonlinear regression, the resulting regression equation was: $(B_0e^{-B_1X}) = y = (1378.0e^{-0.481X})$. A concentration of 10 µM sethoxydim was chosen to ensure the efficacy of selection.

Selection of Sethoxydim Resistant Cells

Table 1 presents a summary of the selection process. Ten genotypes were used to generate 21 different cell lines that produced a total of 20,250 calli subjected to selection for sethoxydim-resistant cells. The selection process resulted in 65 SR calli, representing a mutation rate of one resistance event per 312 calli. Four of the 10 genotypes produced no SR calli. The six genotypes that produced SR calli were: MK, GA 05-025.164, UGA03-539.13, UGA05-025.181, UGA03-525.22, and UGA03-9E.3. The number of SR lines recovered varied, and ranged from zero to as high as nine per plate of 225 calli. The frequency of SR calli produced by genotypes ranged from 0 to 0.0051. Statistical analysis for differences in the probability of obtaining a resistant calli event indicated no significant differences ($p = 0.35$) among 10 genotypes.

Two of the 65 SR cell lines were lost prior to regeneration and of the 63 SR lines remaining, only two regenerated, SR11 and SR31. These two lines originated from the same cell line derived from MK initiated on 12 Jan. 2008. The callus tissue of the two lines that regenerated was dense and yellow compared to that of a majority of the lines, which was white and soft. Thus, the two primary qualifiers for a successful genotype subjected to in vitro selection are its ability to mutate for resistance and its capability for regeneration.

Molecular Characterization

ACCase amplicons were obtained from 63 of the 65 SR lines, and only four contained the A to T transversion at position 1781 (Table 1). The possibility cannot be excluded that mutations conferring herbicide resistance at positions other than those examined could have occurred in these SR cell lines.

Resistant lines are heterozygous for the mutation as shown by the presence of a double peak at the point of the mutation in the electropherogram presented in Fig. 2. One peak represents the wild-type allele, and the other the mutated allele. Therefore, plants regenerated from SR11 and SR31 callus are known to possess the expected Ile to Leu mutation. It should be noted however, that a number of the plants regenerated from SR11 and SR31 callus did not contain the 1781 mutation but rather had the wild-type sequence at this position. Since sethoxydim resistance can also be conferred by an Asp to Gly mutation at the 2078 position; DNA from SR11 and SR31 was analyzed for the presence of this mutation. Sequence data indicated the wild-type sequence at position 2078 for SR11 and SR31. Therefore, the resistance expression of SR11 and SR31 can be attributed to the presence of the Ile to Leu mutation at 1781.

Resistant Calli Frequency

The average volume of a single callus cell was measured to be 1.3582×10^{-5} µL. This provides an approximation of 258,000 cells per 4-mm callus piece. Thus, the 20,250 calli put through selection contained approximately 5.2 billion cells. Assuming that only a single mutant cell was responsible for each SR cell line, the frequency of resistant cells in this experiment was one per 8×10^7 cells. Four of the 65 resistant calli were confirmed to contain the A to T mutation at the 1781 position for a frequency of one in 1.3×10^9cells (Table 1).

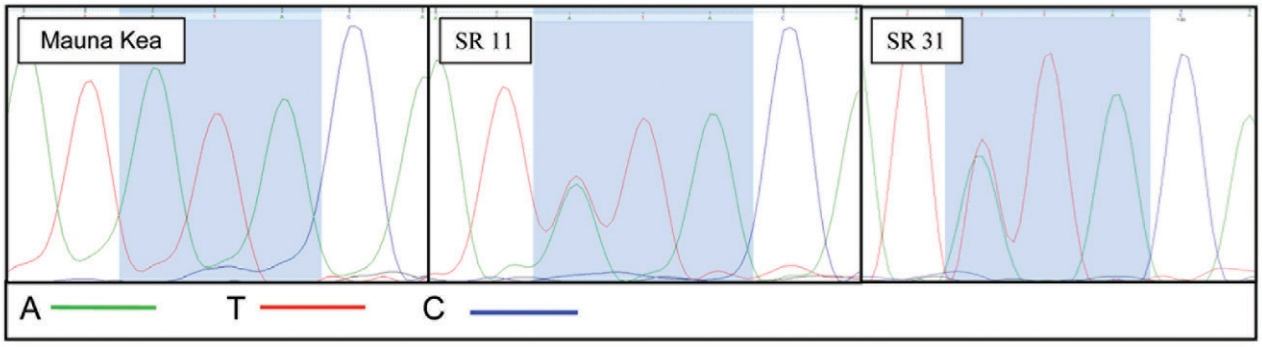

Figure 2. Electropherograms of wild type Mauna Kea parent compared with the sethoxydim resistant (SR) mutant lines, SR11 and SR31. The codon for acetyl coenzyme A carboxylase (ACCase) 1781 aa is highlighted in the blue background. Since seashore paspalum is a diploid and the mutation occurred in one of the two alleles, the chromatograph shows an equal mix of mutated to nonmutated ACCase genes.

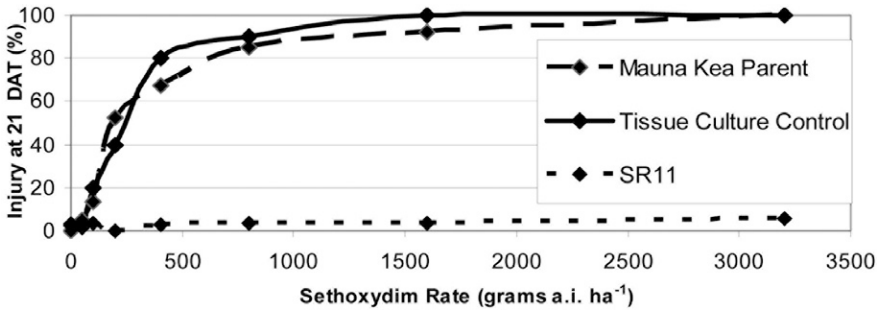

Figure 3. Whole-plant response of three seashore paspalum genotypes to sethoxydim rate measured as injury at 21 days after treatment (DAT).

Whole Plant Response to Acetyl Coenzyme A Carboxylase Inhibiting Herbicides

The effects of sethoxydim rate on injury ratings at 21 DAT of each of the three tested genotypes are presented in Fig. 3. For this experiment only SR11 was evaluated due to availability of plant material. The two-way analysis of variance indicated significant genotype by herbicide rate effects for injury ratings at 21 DAT. Line SR11 showed excellent herbicide resistance, even at the highest rate of 3200 g a.i. ha^{-1} (Fig. 4). In contrast, both PT and TCC

Figure 4. Whole-plant response of three seashore paspalum genotypes to different rates of sethoxydim 21 days after treatment (DAT). PT is the parental type (Mauna Kea), TCC is the tissue-culture-derived control developed from Mauna Kea, and SR11 is the sethoxydim resistant (SR) line.

Table 2. Response of parental type (PT), tissue culture control (TCC), and sethoxydim resistant (SR11) to other acetyl coenzyme A carboxylase (ACCase) inhibiting herbicides.

	Plant Injury 21 DAT‡								
	Clethodim (Envoy)			Fenxoxyprop (Acclaim)			Fluazifop (Fusilade)		
Herbicide rate†	PT	TCC	SR11	PT	TCC	SR11	PT	TCC	SR11
	%								
0	13.8a§	2.5a	6.2a	8.8a	16.2a	11.2a	12.5b	35.0a	6.2b
50	85.0a	70.0a	22.5a	47.5a	60.0a	10.0b	63.8a	53.8a	10.0b
100	60.0ab	67.0a	42.0b	68.8a	70.0a	8.8b	76.2a	66.2a	12.5b
200	56.3a	52.5a	25.0b	82.5a	91.2a	10.0b	58.8a	70.0a	12.5b
400	85.0a	75.0a	80.0a	92.5a	96.2a	13.8b	83.8a	86.2a	20.0b
800	100.0a	95.8a	100.0a	93.8a	98.8a	12.5b	97.5a	95.0a	50.0b
1600	100.0a	100.0a	100.0a	98.8a	100.0a	8.8b	100.0a	100.0a	40.0b

†Grams a.i. ha^{-1}.

‡DAT = days after treatment.

§Means on the same row (herbicide rate) and within a herbicide group (i.e., Clethodim) followed by the same letter are not considered to be significantly different at 0.05 according to a protected LSD.

had injury scores of 30% or greater at rates of 200 g a.i. ha^{-1}, and injury scores of 80% or greater at rates ≥800 g a.i. ha^{-1}. When mean injury scores were compared for each of the three genotypes at each herbicide rate, SR11 had significantly less injury than PT or TCC at all rates above 100 g a.i. ha^{-1}. The maximum injury score observed on SR11 was 5.5 at 3200 g a.i. ha^{-1}, a rate 15 times greater than the labeled rate for centipedegrass [*Eremochloa ophiuroides* (Munro) Hack], a turfgrass species naturally tolerant to sethoxydim. Estimates of I_{50} for the three genotypes were 189, 276, and >3200 g a.i. ha^{-1} for PT, TCC, and SR11, respectively. Injury due to clethodim rate of each of the three tested genotypes showed SR11 to have sustained significantly less injury than PT and TCC at the 200 g a.i. ha^{-1} rate only (Table 2). There were no differences in plant injury among the three genotypes at all rates above 400 g a.i. ha^{-1}.

SR11 showed significantly less injury than PT and TCC at all fenoxaprop and fluazifop rates above 50 g a.i. ha^{-1} (Table 2). SR11 was injured <15% at all fenoxaprop rates up to 1600 g a.i. ha^{-1}. The estimates of I_{50} for PT, TCC, SR11, were 56, 22, and >1600g a.i. ha^{-1}, respectively. Estimates of I_{50} for fluazifop for PT, TCC, and SR11 were 36, 37, and 800 g a.i. ha^{-1}, respectively.

DISCUSSION

After screening more than 20,250 calli, SR cells were identified and ultimately regenerated seashore paspalum plants highly resistant to sethoxydim. The mutation conferring herbicide resistance was characterized as a single nucleotide change in the codon for position 1781 of ACCase. This mutation was estimated to occur once in each 1.74 billion cells but only 2 of the 5.22 billion cells screened resulted in regenerable plants with the mutation. Reports on frequency of spontaneous mutations have been highly variable. Ruiter et al. (2003) found the mutation frequency in the native *als* gene of *Brassica napus* L. to be 2×10^7. They also reported spontaneous mutations in the transgenic *bar* gene in *B. napus* L. and tobacco (*Nicotiana tabacum* L.) occur at the higher rate of 4×10^4. In contrast, the A to T mutation in paspalum was 65 and 32,500 fold lower than reported rates for *Brassica* and tobacco, respectively. Although the frequency at which the single specific base change occurs is low, the use of large plates made it possible to efficiently screen large numbers of callus pieces in 5 mo.

The whole plant dose response data indicate that SR11 has sethoxydim resistance 15 times or greater than the registered use rate on centipedegrass. This high level of resistance should be adequate to provide effective control of most susceptible weedy grasses without concerns of crop (SR11) injury. Bermudagrass however, will likely require repeated applications of sethoxydim to achieve control. Johnson (1987) reported 96% bermudagrass control after 2 yr of spring and fall applications with sethoxydim at rates of 340 g a.i. ha^{-1}.

The data also provides evidence of the presence of cross resistance to fluazifop and fenoxaprop in SR11. The level of cross resistance present is adequate to provide effective control of susceptible weedy grasses without serious concerns over herbicide injury. However, cross resistance to clethodim is minimal and cannot be used for control of weedy grasses in SR paspalum. Minimal resistance to clethodim was expected and has been reported in several grass species with the same Ile to Leu substitution at position 1781 (Delye et al., 2005). It should be noted that SR11 is likely to have resistance to additional ACCase herbicides not evaluated in these studies. However, the ability to control SR11 using herbicides with other modes of action such as glyphosate and glufosinate is not impacted by this mutation.

In vitro selection for herbicide resistance has been attempted in numerous crops, including in vitro selection for glyphosate resistance. Glyphosate resistance requires two single point mutations in the native EPSPS gene (Sidhu et al., 2000). Thus the possibility of obtaining glyphosate resistance through in vitro selection is

extremely low due to the fact that both mutations would have to occur spontaneously and simultaneously for any level of glyphosate resistance to occur. In contrast, in vitro selection has been successfully used to develop resistance to herbicides in numerous crops where single point mutations are all that is needed to confer resistance (Chaleff and Parsons, 1978; Miller and Hughes, 1980; Chaleff and Ray, 1984; Swanson et al., 1988; Swanson et al., 1989; Parker et al., 1990; Newhouse et al., 1991; Rajasekaran et al., 1996; Wright and Penner, 1998). However, in vitro selection proved unsuccessful for developing herbicide resistance to ACCase herbicides in Kentucky bluegrass (*Poa pratensis* L.*)* (Somers, 1996). It was stated the failure in the latter was due to improper tissue culture protocols, illustrating the importance of having established callus induction and plant regeneration protocols.

Herbicide resistance is a trait that has been actively pursued for many crops. Arguably the most notable of these are the Roundup Ready (RR) products (Monsanto Co., St. Louis, MO). Although, a glyphosate-resistant creeping bentgrass (*Agrostis stolonifera* L.) has been developed (Cerdeira and Duke, 2006), the commercial deployment of RR in turfgrasses has yet to occur. Development of turfgrass with transgenically derived herbicide resistance traits such as glyphosate or glufosinate face steep regulatory concerns before they can be released. In contrast, environmental releases of plants with herbicide resistance obtained by nontransgenic means are not subject to these government regulations, except in Canada. Thus, in vitro selection for herbicide resistance is an attractive alternative technology to transformation and greatly improves the potential for both domestic and international commercialization of herbicide-resistant turfgrass cultivars.

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