

SYNONYMOUS MUTATION GENE DESIGN TO OVEREXPRESS NATIVE  
ENZYMES IN PLANTS WHILE BYPASSING POSTTRANSCRIPTIONAL-GENE-  
SILENCING MECHANISMS

by

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ABSTRACT

Plants have gene-silencing mechanisms to prevent overexpression (OE) of native genes. OE of transgenes with a high level of identity to a native gene can trigger post-transcriptional-gene-silencing (PTGS) where mRNA is degraded into small interfering RNA (siRNA) approximately 21-nt long. In the present study, we tested transgene design using synonymous mutations to bypass PTGS. The *acetyl coenzyme A carboxylase* (*ACCase*) coding sequence was cloned from creeping bentgrass (*Agrostis stolonifera* L.) and used as a template for gene design. An additional non-synonymous mutation was included in the first position of the 1781 codon causing an isoleucine to leucine substitution known to provide resistance to *ACCase*-inhibition herbicides. Bentgrass calli were inoculated with the bentgrass or modified *ACCase* with or without the 1781 mutation. Six HR events were obtained from calli transformed with *ACCase* with synonymous mutations and the 1781 SNP. Whole plant dose response showed transgenic plants were resistant to the *ACCase*-inhibiting herbicide, sethoxydim. Transcription of the modified gene was confirmed, showing PTGS was avoided. Additionally, sethoxydim-resistant bentgrass was selected in vitro to compare to transgenic plants. Four sethoxydim-resistant events

were selected and the whole plant dose response confirmed high levels of sethoxydim resistance. Sequencing confirmed the 1781 leucine mutation.

Grass-derived promoters and terminators were tested for ability to control GUSPlus reporter gene expression. Maize (*Zea mays* L.) centromere-specific sequences were evaluated for promoter activity. The long terminal repeats (LTR) from retrotransposons CRM1 and CRM2, and the centromere repeat element CentC, were cloned from maize and evaluated for promoter activity. The CentC and the CRM LTRs were fused to GUSPlus gene. Constructs contained the luciferase reporter gene as an internal control to normalize GUS expression. Possible transcription terminator sequences were cloned from several grass species and tested in place of T-NOS, controlling GUSPlus transcription. All constructs were evaluated using a transient expression assay by microprojectile bombardment of rice callus tissue. Promoter activity of CentC was not significantly different from the *CaMV* 35S promoter. Among the terminator sequences evaluated, GUS expression was highest with the switchgrass pvubi2 3'UTR and was not significantly different from T-NOS.

INDEX WORDS: Gene silencing, ACCase, overexpression, PTGS, synonymous mutation, sethoxydim, fluazifop, anthocyanin, promoter, terminator, in vitro selection, herbicide, ACCase inhibitor, herbicide resistance

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## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW

The development of turfgrass lawns occurred during medieval times, and today has become an important part of the American landscape and economy. In 1304, Piero de' Crescenzi wrote the text *Liber Ruralium Commodorum* in which he described using turf for benches, methods for planting turf, and that turf should be mowed for a lawn to remain permanent (MacDougall, 1986). Centuries later, 16.4 million ha are in managed turf in the US (Milesi et al., 2005). The total economic impact of the turfgrass industry on the US economy in 2002 was approximately \$60 billion (Haydu et al., 2009). The golf course industry generated an estimated \$33.2 billion in gross profits in 2007, making it the largest constituent of the turfgrass industry (Haydu et al., 2008). Turfgrass used on golf courses and lawns is produced either by sod or seed production. Sod sales in the United States totaled \$1.4 billion in 2007 and involved nearly 1900 farms (USDA-ARS). Two-thirds of the grass seed produced in the US is grown in the Willamette Valley of Oregon (Young III, 2009). In 2010, grass seed production in Oregon resulted in \$220 million in sales produced on 198,000 ha (USDA-NASS, Oregon State University Extension Economic Information Office).

Among the turfgrasses, creeping bentgrass (*Agrostis stolonifera* L.) is an important turfgrass for the golf industry, as well as the Oregon grass-seed industry. Bentgrass can tolerate mowing heights as low as 3 mm, allowing it to be used on tees, greens, and fairways. Despite being adapted to cool climates, creeping bentgrass has become popular in warmer regions

because it provides a superior putting surface compared to the warm-season bermudagrass (*Cynodon* spp.) (Warnke, 2003).

One of the requirements for any golf green is complete monoculture, excluding broadleaf and grass weeds. Broadleaf weeds can be controlled through application of selective herbicides such as 2,4-D (2,4-dichlorophenoxyacetic acid). Weedy grass control is more difficult because of limited tolerance of creeping bentgrass to many pre-emergent and post-emergent herbicides. One grass weed that is particularly problematic for creeping bentgrass greens is perennial ryegrass (*Lolium perenne* L.). Methods for control of perennial ryegrass are limited. Chlorsulfuron (Corsair NuFarm Americas, Burr Ridge, IL) is labeled for use in established stands of creeping bentgrass when turf allowed to grow to over 0.5 in. Although labeled for use on creeping bentgrass, the label states that chlorsulfuron should not be used on golf course greens. The limited control options for perennial ryegrass in creeping bentgrass golf course greens, creates the need for alternative approaches for control.

### **Weed control in turf**

Weeds reduce the aesthetic value of lawns and golf course greens, and impact seed and sod production. Seed yield can be greatly reduced due to competition, and weed seed contamination can prevent seed certification. Grass seed certification limits impurities from other cultivars in the same species, noxious weeds, and weedy grass species (Rolston and Young III, 2009). Weed control is an effort to eradicate a weed population that currently exists. In contrast, weed management is an integrated pest management (IPM) system that includes weed prevention, cultural control methods, and chemical control (Buhler et al., 2000). Weed prevention is often described as the most important method of weed management; however, it has been de-emphasized in modern times due to the larger size of farms and herbicides available

to control weeds (Buhler, 2002). Currently, weed prevention takes the form of seed purity laws, noxious weed laws, and voluntary seed certification programs, as seen with Oregon's seed certification program. Cultural control aims to improve the competitiveness of turf against weeds. Cultural practices may include irrigation, selection of proper cultivars for the environment, mowing, and fertilization (Busey, 2003). For example, tall fescue (*Festuca arundinacea*) had significantly fewer crabgrass plants when mowed at 5.1 cm compared to 2.5 cm (Voigt et al., 2001).

In 1944, a major breakthrough in weed control came with the discovery of the herbicidal qualities of 2,4-D. Other herbicides preceded 2,4-D, but none were as cheap, selective, or practical (Crafts, 1960) for broadleaf control. Seventy years after development, 2,4-D is still used due to the same attributes it exhibited in 1944 (Hiradate et al., 2007). 2,4-D has been a useful tool for growers by selectively killing broadleaf weeds in grass crops. Conversely, grass weeds can be controlled in broadleaf crops. In the early 1970's, the observation that phenoxyphenoxypropionate acids reduce levels of cholesterol and triglycerides in animals, led to the idea that the effects of the compounds should be tested on plants (Boger and Sandmann, 1989). The phenoxyphenoxypropionate acids were found to inhibit lipid biosynthesis and growth of grass species, yet dicotyledonous species were unharmed. Since then, numerous herbicides have been developed from aryloxyphenoxypropionate (APP) compounds and a similar group of compounds, the cyclohexanediones (CHD).

Sherman et al. (1996) states that herbicide resistance can be due to three basic mechanisms including resistance at the site of action, metabolic detoxification, and prevention of the herbicide from reaching the site of action. Since then, other mechanisms have been found to confer herbicide resistance. Palmer amaranth (*Amaranthus palmeri* S. Wats.) was found to have

glyphosate resistance 6-8 fold higher than that of susceptible plants (Culpepper et al., 2006), and the resistance is due to a 5-160 fold increase in copy number of EPSPS (Gaines et al., 2010). Glyphosate-resistant horseweed (*Conyza canadensis* L.) transports glyphosate molecules into the vacuole by membrane transporters, where it is prevented from inhibiting EPSPS (Ge et al., 2014).

Resistance at the site of action can be exemplified by resistance to the grass-specific CHD and APP herbicides. Both compounds are now known to inhibit acetyl coenzyme A carboxylase (ACCase). Acetyl-coenzyme A is carboxylated by ACCase to form malonyl-coenzyme A in the first committed step of lipid biosynthesis. ACCase-inhibiting herbicides compete with acetyl-coenzyme A for its binding site in the in the ACCase carboxyl transferase (CT) domain (Delye, 2005). The herbicide molecule inserts into the CT domain and the enzyme can no longer function. The loss of lipid synthesis prevents newly forming cells in the meristem from producing cell membranes, resulting in cell death.

Plants possess two forms of the ACCase enzyme: eukaryotic and prokaryotic (Harwood, 1988). The eukaryotic ACCase is encoded by a single nuclear gene approximately 15,000 bp in length (Podkowinski et al., 1996). The resulting protein is a polypeptide with distinct functional domains (Harwood, 1988). The prokaryotic form is made up of four subunits coded for by four distinct genes. Three of these genes are located in the nuclear genome, and one gene is located in the chloroplast genome. The locations of these two types of ACCase differ in most plants. The chloroplast is known to be the primary site of lipid synthesis; however, there is ACCase present in the cytosol as well. Excluding the Poaceae, the prokaryotic ACCase is compartmentalized in the chloroplast, and the eukaryotic form is found in the cytosol (Sasaki et al., 1995). The Poaceae are unique in that they possess the eukaryotic form of ACCase in the cytosol as well as in the

chloroplast (Sasaki et al., 1995). There is a high degree of similarity between the two forms of ACCase enzymes in grasses, (Gornicki et al., 1994). Despite the high level of homology, the cytosolic form remains unaffected by ACCase-inhibiting herbicides, while the plastidic form is highly susceptible (Delye, 2005).

Specific point mutations in *ACCase* cause amino acid substitutions, preventing the binding of ACCase inhibitors while allowing normal enzymatic function (Delye et al., 2005). Currently, there are ten known single nucleotide polymorphisms (SNPs) conferring resistance to ACCase-inhibiting herbicides (Table 1.1). The most commonly reported mutation is a single isoleucine (ILE) to leucine (LEU) substitution at amino acid position 1781. Other known mutations in the ACCase CT domain are located at 1999, 2027, 2041, 2078, 2088, and 2096 amino acid positions. Each mutation confers resistance to one or both CHD and APP ACCase inhibitors. Resistance to the grass-specific herbicide sethoxydim (CHD) has been obtained in maize (Parker et al., 1990), and seashore paspalum (*Paspalum vaginatum* Sw.) (Heckart et al., 2010) by in vitro selection. In paspalum, the level of resistance due to a non-synonymous mutation at amino acid position 1781 caused by a single SNP, has been shown to increase sethoxydim resistance to over 15 times the field application rate (Heckart et al., 2010).

The graminicide sethoxydim, (2-1-(ethoxyiminobutyl)-5-2-(ethylthio)propyl-3-hydroxy-2-cyclohexen-1-one) is used to control perennial and annual grasses in agricultural and ornamental crops. Sethoxydim has formerly been marketed under numerous trade names that include: Vantage<sup>®</sup>, Poast<sup>®</sup>, Rezult<sup>®</sup>, Torpedo<sup>®</sup>, Ultima<sup>®</sup>, and Conclude<sup>®</sup> by manufacturers BASF, Monterey, TopPro, and Segment. Sethoxydim is currently marketed under the name Sethoxydim Pro<sup>®</sup> by the BASF Corporation. Sethoxydim is absorbed through leaf surfaces, then is translocated throughout the plant and accumulated primarily in the meristematic tissue

(Ahrens, 1994). The interruption of lipid biosynthesis leads to membrane destruction in these actively growing areas, causing growth to halt and plants to die within 7-21 days. Sethoxydim has a half-life in soil of 25 days due to breakdown by soil microbial activity (Roslycky, 1986). Sethoxydim does not bind to soil colloids, and despite the lack of adsorption to the soil particles, sethoxydim has shown little or no leaching in various soils tested (Koskinen et al., 1993).

An example of an APP ACCase-inhibiting herbicide is fluazifop-p-butyl, R-2-4-5-(trifluoromethyl)-2-pyridinyloxyphenoxypropanate. Trade names of products containing fluazifop include: Fusilade 2000<sup>®</sup>, Fusilade DX<sup>®</sup>, Fusilade Turf and Ornamental<sup>®</sup>, Fusilade Fiv<sup>®</sup>, Fusilade Super<sup>®</sup>, Fusion<sup>®</sup>, Horizon<sup>®</sup>, Ornamec<sup>®</sup>, and Tornado<sup>®</sup>, from manufactures: Zeneca Agricultural Products, AgrEvo, and PBI/Gordon. Like sethoxydim, fluazifop is absorbed through leaf surfaces and accumulates in the meristem (Erlingson, 1988). Fluazifop can be degraded by soil microbes or hydrolysis and soil half-life is 15 days (Ahrens, 1994).

The differential manner in which each SNP confers resistance to CHDs and/or APPs creates the possibility that an *ACCase* with the correct two mutations could confer resistance to CHDs and APPs. The ability of a plant to be resistant to both groups of herbicides is important for preventing the production of HR weeds. Combining mutations to obtain resistance to both CHD and APP herbicides should provide the ability to rotate herbicides. The herbicide resistance that each HR SNP endows has been characterized in several species; however, it is unknown how multiple HR SNPs in a single gene would affect herbicide resistance or gene function.

Theoretically, herbicide-resistant (HR) grasses could be produced by adding a copy of the *ACCase* gene, which has been modified with HR point mutations. At one time, it was thought that introduction of an additional gene copy for a given trait would only enhance this trait, due to addition of extra protein. Such thinking was based on several key observations on gene dosage

effects. The aneuploid series of jimsonweed (*Datura stramonium* L.) first allowed the evaluation of dosage effects of chromosomes on plant morphology (Blakeslee et al., 1920). Primary and secondary trisomics of maize were later used by Carlson (1972) to assign isozyme loci to specific chromosome arms. When the locus is present on the trisome, primary trisomics show about 150% of the diploid enzyme activity. The secondary trisomics show twice as much activity as the diploid. An increase in ploidy level has also shown to increase gene expression. Guo et al. (1996) evaluated transcript levels of 18 genes in maize with ploidy levels ranging from monoploid to tetraploid. For most of the genes studied, they found that as gene dosage increased with each ploidy level, transcript level also increased. For example, transcript levels for *csu31* were compared to those of the diploid level. Monoploid expression was 34% of diploid, whereas triploid and tetraploid levels were 377% and 428%, respectively.

### **Gene silencing**

Addition of extra copies of a native gene appears to be a reasonable approach to over-express (OE) a native protein based on trisomic and polyploidy expression data, as well as the observed copy number increase in Palmer amaranth EPSPS. Arguably, the most well-known example of an addition of a native gene for the purpose of OE was the introduction of an extra chalcone synthase (CHS) gene in petunia (Napoli et al., 1990). It was hypothesized that the excess enzyme produced by the transgene would supplement the native enzyme at the first committed step of anthocyanin biosynthesis. The expected result was increased anthocyanin production due to an increase of substrates through the pathway, leading to increased pigment levels. Instead, 42% of the resulting plants had both native and introduced genes silenced, resulting in a white, striped, or spotted corolla phenotype. Both the transgene mRNA and homologous native gene mRNA were being degraded (Metzlaff et al., 1997) and, as it was later

determined, processed into small interfering RNAs (siRNA) (De Paoli et al., 2009). This specific form of gene silencing was initially termed cosuppression due to both genes being suppressed (Napoli et al., 1990). The authors also found that the sense transcript induced silencing at a greater level than the anti-sense transcript. The fact that sense transcripts caused silencing was unexpected, as plant cells should not be able to differentiate between transgene transcripts and native gene transcripts, as they are 100% identical.

OE of genes in plants could have many possible uses; however, the plant cell has mechanisms in place to prevent OE of its own genes as well as for transcriptional blocking of viral replication (Covey et al., 1997). In the past two decades, this phenomenon, termed post-transcriptional gene silencing (PTGS) or RNA interference (RNAi), has been extensively studied, beginning with the observations of Napoli et al. (1990). The introduction of an extra copy of *CHS* and resulting silencing is an example of sense-PTGS (S-PTGS) (Jorgensen et al., 2006). Two long-standing theories exist to explain the trigger of S-PTGS. First, S-PTGS could be triggered by expression levels that reach a threshold and are therefore down-regulated. The threshold theory is supported by experiments in which plant cells were transformed with transgenes carrying the coding sequence of viral coat proteins (CP) in order to gain viral resistance. The earliest example is tobacco transformed with the CP coding sequence from tobacco mosaic virus (TMV), resulting in resistance specifically to TMV (Abel et al., 1986). Later, Lindbo et al. (1993) transformed tobacco with tobacco etch virus (TEV) CP. Plants challenged with TEV exhibited viral resistance and CP mRNA levels in inoculated plants were 12 to 22-fold lower than those in non-inoculated plants. The authors showed that transgenes in both inoculated and non-inoculated plants were transcribed at similar rates, and suggest that mRNA levels may reach a threshold. When the threshold is surpassed, silencing mechanisms are

initiated. The idea that S-PTGS is triggered by surpassing a threshold is further supported by studies comparing transgene promoter strength. Que et al. (1997) analyzed strong and weak promoters driving a petunia CHS engineered into petunia. The result was a positive correlation between promoter strength and level of gene S-PTGS.

In addition to the threshold theory, Luo et al. (2007) proposed that aberrant transcripts trigger S-PTGS. Truncated and unpolyadenylated transcripts were produced in *Arabidopsis* engineered with constructs containing three copies of a  $\beta$ -glucuronidase (GUS) open reading frame. Additional aberrant transcripts were created in *Arabidopsis* plants containing constructs with a single GUS gene lacking a transcription terminator, resulting in unpolyadenylated mRNA. In both cases, degradation of GUS mRNA was observed. The two theories may not be mutually exclusive, as a gene that has high levels of transcription could have a greater probability of producing aberrant transcripts (Voinnet, 2008). Currently, the mechanism for sensing and initiation of S-PTGS by sense transgenes is not known (Jauvion et al., 2012, Le Masson et al., 2012, Parent et al., 2012)

Gene silencing can also occur through transcriptional-gene-silencing (TGS). TGS occurs when methylation of promoter sequences prevents transcription. Both PTGS and TGS can occur at the same time (Mette et al., 2000, Mourrain et al., 2007). Once S-PTGS is initiated by one of the possible triggers, the mRNA is degraded by a multi-step pathway. Fire et al. (1998) hypothesized that double-stranded (dsRNA) could be an aberrant RNA created by transgene transcription or during production of RNA molecules by bacteriophage polymerases. *Caenorhabditis elegans* was injected with sense RNA, anti-sense RNA, and dsRNA of a non-essential myofilament protein (UNC-22). Muscle twitching is observed when *UNC-22* is down regulated. The injection of sense and anti-sense RNA separately had no effect. Injection of

dsRNA produced severe twitching suggesting that dsRNA is a required step for gene silencing. The effect of dsRNA was observed in plants later that year. Waterhouse et al. (1998) transformed tobacco with constructs containing the coding region for a protease (PRO) of potato virus Y (PVY). Transgenic lines were obtained for sense PRO constructs and anti-sense PRO constructs. Sense and anti-sense lines were crossed creating progeny that produced dsRNA for PVY PRO. The authors observed greater silencing with dsRNA-producing progeny than with either parent.

Formation of dsRNA has been shown to be essential for S-PTGS; however, the polymerase responsible undetermined. The first RNA directed RNA polymerase (RDR), named *LeRDRI*, to be isolated was from tomato plants undergoing viroid infection (Schiebel et al., 1993). The dsRNA nature of viroid replication requires plant RDR. Gene knockouts of *SDEI* in *Arabidopsis* showed that S-PTGS depended on *SDEI*, which had high level of homology with *LeRDRI* (Dalmay et al., 2000). Concurrent experiments showed that S-PTGS also depended on the gene, *SGS-2*, which also had a high level of homology with *LeRDRI* (Mourrain et al., 2000). *SDEI* and *SGS-2* are now known as RNA dependent RNA polymerase 6 (RDR6). The exact mechanism of how RDR6 recognizes the single stranded substrate and produces dsRNA remains unknown (Willmann et al., 2011). The idea that transcription is arrested due to untranslatable RNA/RNA duplexes was proposed by Metzloff et al., (1997). Hamilton and Baulcombe (1999) observed that molecules of this size could be easily detected and were not. The authors hypothesized that the RNA was being degraded, but that the fragment size was too small to be detected. To test this, four types of PTGS were evaluated for low molecular weight small RNAs (sRNA). The authors tested plants with transgene induced cosuppression, transgene silencing in plants without a homolog, systemic spread of transgene silencing, and virus-infected plants.

They found sRNAs ~25 nt in length in all tissues, and these sRNAs were specific to the gene being silenced.

To determine how long dsRNA is processed into sRNAs, Bernstein et al. (2001) performed enzyme fractionation experiments in *Drosophila melanogaster* cells to isolate the enzyme responsible. They isolated a ribonuclease III, termed Dicer, responsible for cleavage of long dsRNA into sRNAs approximately 22 nt in length. Mlotshwa et al. (2008) analyzed two dicer-like (DCL) enzymes in *Arabidopsis* suspected of cleaving dsRNA produced from the sense transgene trigger. The authors analyzed knockouts of *DCL2* and *DCL4*; only *DCL2* knockouts exhibited down-regulation of sense transgene silencing. The results show that *DCL2* is required for cleavage of long dsRNA to 22-nt small interfering RNAs; however, Voinnet (2009) states that this may be an isolated example and other examples show that cleavage is dependent on *DCL4*. These siRNAs are considered primary siRNAs in the S-PTGS pathway (Baulcombe, 2007). Chan et al. (2004) found that *DCL3* cleaves transcripts into 24-nt fragments that guide methylation for TGS. The roles of *DCL2*, *DCL3*, and *DCL4* are now known to have overlapping function in transgene silencing (Dunoyer et al., 2010). *DCL2*, *DCL3*, *DCL4*, represent the pathway where 21-nt micro RNAs (miRNA) are produced. *DCL1* recognizes dsRNA caused by imperfect hairpins created by transcription of non-coding miRNA (MIR) genes, cleaves the 5' and 3' ends, and forms small primal RNA (priRNA). Subsequently, the rest of the strand is degraded into 21-nt mature miRNA (Voinnet, 2009). Micro RNAs can be used in the trans-acting RNA (tasiRNA) pathway. The tasiRNAs guide cleavage by finding a specific target site within mRNA that possess the corresponding sequence (Williams et al., 2005).

After DCL enzymes cleave dsRNA in their respective pathway, further processing of sRNAs is required to prevent further degradation. Park et al. (2002) hypothesized that HUA

ENHANCER 1 (HEN1) may have a role in gene silencing as HEN1 mutants in *Arabidopsis* have a similar phenotype to other mutants that interrupt gene silencing. The authors found that *hen1* plants failed to accumulate sRNA. It was later found the 3' end of siRNAs are methylated by HEN1 (Li et al., 2005). The siRNAs from *hen1* mutants failed to be methylated, and a uracil was added in place of the methyl group. The authors suggest that addition of a uracil group may cause the siRNA to be targeted for degradation. Hamilton and Baulcombe (1999) proposed that sRNAs were long enough to guide sequence specificity for further degradation of mRNA. Zamore et al. (2000) found that the target mRNA was cleaved into 21 to 23-nt fragments that were homologous to siRNAs. Hammond et al. (2000) transfected *Drosophila* cells with dsRNA followed by injection with the corresponding long mRNA. The mRNAs were degraded, and the authors performed enzyme fractionation to isolate the enzyme. The sRNAs corresponding to the degraded mRNA cofractionated with the enzyme, suggesting the enzyme, they term RNA-induced silencing complex (RISC), may be guided by a bound sRNA.

The primary siRNAs are bound to another dicer-like protein, Argonaut (AGO), which is part of the greater RISC (Hammond et al., 2001). AGO is characterized by two domains. The PAZ domain binds sRNAs to guide degradation of complementary mRNA (Ma et al., 2002). The AGO PIWI domain has characteristics indicating it has RNase activity. The PIWI domain possesses an aspartic acid-aspartic acid-histidine motif that is required for RNase activity (Rivas et al., 2005). Crystal structure analysis of PIWI shows a folding structure resembling RNase H (Song et al., 2004). The siRNAs in the RISC complex target complementary mRNAs transcribed from either the native gene or the transgene, as suggested by Hamilton and Baulcombe (1999). The mRNA is consequently cleaved by RISC, thus interrupting gene expression.

Initial silencing caused by primary siRNAs only travels to 10-15 surrounding cells (Himber et al., 2003). Yet, the silencing signal is known to travel throughout the plant, with the phenomenon termed transitive silencing. Transitivity refers to the process of primary siRNAs being amplified into secondary siRNAs (Brodersen and Voinnet, 2006). Transitive silencing was shown in tobacco undergoing S-PTGS for the *gusA* glucuronidase gene (Palauqui et al., 1997). Stalks of silenced plants were grafted to non-silenced scions, and silencing moved to all parts of the scion. Voinnet et al. (1998) evaluated transgenic tobacco plants expressing green fluorescent protein (GFP) as gene silencing spread systemically through the plant. The authors were able to observe the silencing signal as it spread through the plasmodesmata and the phloem. The movement of the silencing signal throughout the plant requires amplification of siRNA to surpass the initial 10-15 cells undergoing gene silencing.

Himber et al. (2003) also analyzed tobacco plants undergoing S-PTGS for GFP and found that the siRNAs 21-nt in length were responsible for transitive silencing. In contrast, Chen et al. (2010) utilized bioinformatics to analyze numerous data sets to determine the size of nucleotide that triggers secondary siRNA production in arabidopsis. They found that 22-nt siRNAs are secondary siRNA production triggers, and most 21-nt siRNAs are not. The authors suggest that their results are consistent with the findings of Mlotshwa et al. (2008), which show DCL2 as the dicer that processes mRNA into 22-nt siRNAs in S-PTGS.

Transitivity can be initiated via two pathways: primer dependent and primer independent (Brodersen and Voinnet, 2006). The primer independent pathway involves recognition of the cleaved mRNA being recognized by RDR6 as aberrant, and consequently converted to dsRNA (Luo and Chen, 2007). The degradation of these dsRNAs by dicer amplifies the amount of siRNAs in the cell. Secondary siRNAs produced to the 3' end of the primary siRNA implies

primer initiated secondary siRNA production (Moissiard et al., 2007). In this case, the primary siRNA binds to complementary regions of the target gene mRNA or transgene mRNA and primes dsRNA synthesis by RDR6 (Moissiard et al., 2007). RDR6 then synthesizes dsRNA from the primed template, making it available for dicer cleavage.

### **Overcoming gene silencing to overexpress native enzymes**

If it were possible to reliably OE native genes, numerous traits could be enhanced or added to a breeding program. Breeding programs commonly use varying forms of recurrent selection for improvement of a population. Fehr (1987) describes recurrent selection as a cyclic method of developing a population, evaluation of individuals, and selection of superior individuals to intercross to develop a new population. The goal of recurrent selection is to improve the population mean for single or multiple characteristics. OE could be used not to replace, but augment recurrent selection methodologies by increasing the mean for specific traits. In addition, the goal of recurrent selection is to improve the population mean without reducing genetic variation (Fehr, 1987). OE would only enhance a trait and would do nothing to decrease genetic diversity.

OE has been attempted with numerous genes by simply cloning the gene, ligating the gene to a promoter, and re-introducing it to the genome or into a genome of a closely related species. For example, petunia CHSA was transformed into a tobacco variety (W38) that possesses a pink corolla color (Wang et al., 2006). Transgenic plants exhibited S-PTGS. An alignment of cDNA sequences of the petunia CHSA and tobacco CHS showed 100 mismatches in the 1170 bp sequence. Between the two cDNA sequences, there were stretches of perfect identity up to 82 bp in length. As reviewed earlier, only 21-22 bp of identity between the introduced and native gene is required for both to be silenced. Petunia and tobacco belong to the

Solanaceae and their chalcone synthase genes are highly conserved orthologs. Fitch (1970) originally defines orthologs as a sub-class of homologous genes that are separated by a speciation event. A potential benefit of introducing orthologs is a level of assurance that the protein function will be the same, due to a high level of homology between the genes (Fang et al., 2010). But, due to the high level of identity between orthologs, it is likely to initiate S-PTGS. OE of native genes could be beneficial for a breeding program if S-PTGS could be reliably avoided. Traits such as pathogen resistance, abiotic stress resistance, and yield could be enhanced with OE of native genes. As genomes are being sequenced, thousands of potential genes are being identified and could be potentially used for OE.

There are several possibilities for overcoming S-PTGS. Genes with similar function, but different coding sequence can be taken from one organism and introduced into another. It is anticipated that the introduced heterologous gene will be below the threshold of homology required for PTGS. Fraser et al. (2002) engineered tomato with phytoene synthase (*CrtB*) taken from *Erwinia uredovora*, which has less than 35% similarity with the native tomato *CrtB*. Transgenic plants showed 2-4 fold elevation of fruit carotenoid levels. The *CrtB* transgene had low homology to the native gene and therefore avoided S-PTGS mechanisms (Römer and Fraser, 2005). Petunia chalcone isomerase (CHI) was expressed in tomato resulting in ~78 fold increase in flavonols, and S-PTGS was avoided despite both petunia and tomato belonging to the Solanaceae, which would suggest a high level of homology (Muir et al., 2001). It is important to note that silencing does not always occur, which, can lead to another extreme of off-target effects created by OE. OE of native phytoene synthase in tomato led to dwarf plants, due to the substrate being shunted to another pathway (Fray et al., 1995). In contrast to introducing the gene itself,

Bradley et al. (1998) up-regulated anthocyanin production in petunia by introducing a regulatory gene (*lc*) from maize.

As shown earlier with *CrtB* introduced into tomato and canola, orthologous genes from more distantly related species are used in attempts to overcome silencing. While introducing such heterologous genes may circumvent S-PTGS mechanisms there may be altered protein function (Fang et al., 2010). Altering protein function is an undesired result, as the original goal of OE is to increase specific protein levels with the same function in the cell. Probably the most widely known example of heterologous gene introduction is the introduction of the *Agrobacterium* enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (*EPSPS*) into plant species. In most plants, the native form of EPSPS is sensitive to the herbicide, glyphosate. Barry et al. (1992) screened EPSPS enzymes from numerous bacteria for insensitivity to glyphosate and kinetic activity. The EPSPS gene from the CP4 strain of *Agrobacterium* had the same function, as well as catalytic efficiency that were 10 fold higher than that of EPSPS from petunia. These qualities made it a suitable candidate for introduction into plants (Padgett et al., 1996). Another possible method of overcoming gene silencing is expression of viral repressors of PTGS. This was done in arabidopsis and tobacco transformed with a GUS reporter gene (Anandalakshmi et al., 1998, Endres et al., 2010, Mallory et al., 2002). GUS transgenes were silenced and introduction of the potyvirus protein HC-PRO, a known suppressor of PTGS, reversed the silencing of the GUS gene. Expression of HC-PRO not only down-regulated silencing for the *gusA* in tobacco, but also down-regulated viral silencing (Anandalakshmi et al., 1998). Expression of HC-PRO in tobacco increased pathogenicity of cucumovirus, potexvirus, and tobamovirus (Pruss et al., 1997).

Reducing similarity of introduced genes and native genes is another method for overcoming S-PTGS. Reduction of similarity was attempted using phytoene synthase in tomato by changing the coding sequence with synonymous mutations to reduce the identity to the native gene while keeping the original protein sequence (Drake et al., 1996). The result was elimination of gene silencing; however, there was no mention of success or failure of OE of the phytoene synthase protein. Kumar et al. (2006) also used a similar method to rescue silenced phenotypes. In this example the authors down-regulated their gene of interest, *salicylic acid-binding protein*, using a hairpin construct. To ensure the gene of interest was the actual target of silencing, the phenotype was rescued by expressing the same protein with a gene sequence containing synonymous mutations. These two examples show that PTGS mechanisms can be avoided by addition of synonymous mutations; however, neither show OE has been accomplished. Chapter 3 describes use of synonymous-mutation-gene design to OE *ACCase* with herbicide-resistance-point-mutations (Chapter 3).

A single SNP can provide a grass plant with high levels of ACCase-inhibitor resistance as seen with seashore paspalum. Creeping bentgrass plants were selected in vitro for sethoxydim resistance. In vitro selection was performed to determine the level of resistance conferred by a single SNP in a native gene to compare resistance conferred by a transgene with the same SNP. Sequencing of in vitro-selected lines revealed a 1781 ILE to LEU mutation. The same HR mutation was included in the gene modified with synonymous mutations.

### **Novel transcription regulatory sequences**

A possible source of transcription regulators is the centromeric region. The centromeric region has previously been thought to be transcriptionally silent, however, in the last decade has been shown to be actively transcribed (Topp et al., 2004). The centromere is predominantly

made up of non-coding sequences required for cell division. During cell division, spindle fibers attach to the centromere through a complex known as the kinetochore. The kinetochore is made up of four major components including: DNA, RNA, centromere-specific histone proteins, and other centromeric-specific proteins. Centromeres in plants can range between 3000 to 9000 kb and primarily consist of ~150-bp repeat elements (Kaszas and Birchler 1996). CentC is a tandem repeat element with a sequence length of 156 bp that can form long arrays interspersed with retrotransposons, specifically CRM retrotransposons (Ananiev et al., 1998, Zhong et al., 2002). Interestingly, centromeric proteins not only interact with genomic sequence of CentC and CRMs, but with the RNA sequences from these elements as well (Topp et al., 2004).

Topp et al. (2004) suggest that retrotransposons may have their own promoter elements and may cause read-through transcription of CentC. If CRMs have promoter activity, theoretically, these promoters could be cloned and used as promoters in expression vectors. Retrotransposons are classified as long terminal repeat (LTR) or non-LTR retrotransposons. LTR retro transposons have terminal repeats that can range from a few hundred base pairs to 5 kb (Kumar and Bennetzen, 1999). Non-centromeric LTR retrotransposons are known to contain promoters and terminators responsible for transcription of proteins needed for transposition (Kumar and Bennetzen, 1999). Non-centromeric LTR retrotransposons have been shown to have promoter activity in several species including human, mouse, rat, and rice (van de Lagemaat et al., 2003) (Romanish et al., 2007) (Hayashi and Yoshida, 2009). The first goal of Chapter 4 was to elucidate promoter activity of the LTR region from maize retrotransposons *CRM1* and *CRM2*. Additionally, the CentC sequence was also analyzed for promoter activity as transcripts have been found with CRM transcripts.

An often overlooked constituent of the expression vector is the transcription terminator. Terminator sequences have been shown to have a significant impact on levels of gene expression (Ingelbrecht et al., 1989, Mitsuhashi et al., 1996, Nagaya et al., 2010). The 3' untranslated region (3'UTR) possesses cis elements that are required for stabilization of the mRNA molecule, processing into mature mRNA, efficient translation, and transport from the nucleus to the cytoplasm. Most of what is known about the 3'UTR has been elucidated from organisms other than plants. Plant 3'UTRs are far less predictable in structure (Loke et al., 2005), therefore, in silico prediction of functional terminators, is difficult. Functional analysis has been done routinely using reporter genes. For example, Xing et al. (2010) fused possible soybean terminators to the yellow fluorescence protein (YFP) reporter gene. The authors found that expression levels of constructs with the putative terminators were at least as good as the control terminators from *PINII*. Similarly, Nagaya et al. (2010) fused six possible terminators, cloned from *Arabidopsis*, to  $\beta$ -glucuronidase (*GUS*) and/or *Renilla luciferase* reporter genes. One terminator from the heat shock protein was of interest as it exhibited 2.5 fold higher expression levels when compared to the T-NOS. The increase was seen with multiple promoters and reporter genes indicating that terminator function was independent of other sequences.

The second goal of Chapter 5 was to identify novel terminators derived from grass species to control expression of transgenes in grass species and therefore not currently regulated. Putative terminators were cloned from genes from maize (*Z. mays*), foxtail millet (*Setaria italica*), switchgrass (*P. virgatum*), wheat (*T. aestivum*) and rice (*O. sativa*). All sequences in Chapter 5 were evaluated in a similar fashion as Mann et al. (2010). Promoter sequences and LTR sequences were fused to the GUSPlus™ reporter gene and a luciferase gene was included in

the vector as an internal control. GUS activity was visualized by histochemical staining and quantified with a MUG assay.

## **Summary**

OE of native enzymes has the potential to add valuable traits to a breeding program. Gene-silencing mechanisms prevent molecular breeders from reliably overexpressing native genes. Twenty-one base pairs of identity are required between introduced and native genes to induce S-PTGS mechanisms. The amount of identity between the native and introduced genes can be reduced with the use of synonymous mutations. The use of synonymous mutations to OE native genes is not a new concept, however, has not been shown to be successful. This project aimed to overcome problems with OE by careful design and optimization of synthetic genes. Synthetic gene design was tested in petunia with two synthetic CHS genes and in creeping bentgrass with a synthetic ACCase gene possessing a SNP conferring resistance to ACCase inhibitors. Previously, we obtained seashore paspalum with resistance to ACCase-inhibiting herbicides through in vitro selection. The resistant paspalum plants exhibited resistance over 15 times the field rate. To evaluate the efficacy of an ACCase gene with synonymous mutations, creeping bentgrass was compared to HR bentgrass selected in vitro. In vitro-selected HR bentgrass was sequenced for HR SNPs and the same HR-conferring mutation was incorporated in the synthetic *ACCcase*.

Overexpression of a native gene with synonymous mutations may not be regulated by government agencies, but the gene-of-interest is not the only sequence to consider. TRS from species listed in APHIS's list of plant pathogens immediately deems a recipient plant a regulated event. With the current amount of genomic sequencing available, using sequences from the list of regulated species is a poor choice. In Chapter 5, LTR regions from LTR retrotransposons were

evaluated for promoter activity and 3' UTRs from genes from several grass species were evaluated for terminator activity. The goal of this research was to identify non-regulated TRSs that could ultimately be used to overexpress synthetic ACCase genes as well as providing sequences to be used routinely in expression vectors for transformation of other grass species.

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Table 1.1. Mutations in ACCase conferring resistance to ACCase inhibitors.

Position	Mutation	Species	Herbicide	Reference
1781	Ile to Leu	<i>Alopecurus myosuroides</i>	CHD & APP	(Brown et al., 2002) Delye et al., 2002a
	Ile to Leu	<i>Lolium rigidum</i>	CHD & APP	Zagnitko et al., 2001
	Ile to Leu	<i>Setaria viridis</i>	CHD & APP	Delye et al., 2002b
	Ile to Leu	<i>Avena fatua</i>	CHD & APP	Christoffers et al., 2002
	Ile to Leu	<i>Lolium multiflorum</i>	CHD & APP	White et al., 2005
	Ile to Thr	<i>Digitaria sanguinalis</i>	CHD & APP	Heckart, unpub.2014
	Ile to Ala	<i>D. sanguinalis</i>	CHD & APP	Heckart, unpub.2014
1999	Trp to Cys	<i>A. fatua</i>	APP	Liu et al., 2007
2027	Trp to Cys	<i>A.myosuroides</i>	APP	Delye et al., 2005
2041	Ile to Asn	<i>A.myosuroides</i>	APP	Delye et al., 2003
	Ile to Asn	<i>A.fatua</i>	APP	Liu et al., 2011
	Ile to Val	<i>L. rigidum</i>	APP	Powles and Yu, 2010
2078	Asp to Gly	<i>A.myosuroides</i>	CHD & APP	Delye et al., 2005
2088	Cys to Arg	<i>L. rigidum</i>	CHD & APP	Yu et al., 2007
2096	Gly to Ala	<i>A. myosuroides</i>	APP	Delye et al., 2005

## CHAPTER 2

### IN VITRO SELECTION OF SETHOXYDIM-RESISTANT CREEPING BENTGRASS<sup>2</sup>

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<sup>2</sup>Heckart, D.L., T.M. Tate, P.L. Raymer, and W.A. Parrott. To be submitted to *Crop Sci.*

## **Abstract**

In the United States, creeping bentgrass (*Agrostis stolonifera* L.) is an important turfgrass for the golf industry. Grass weeds are a recurrent problem for seed producers and turf managers. Herbicide-resistant creeping bentgrass would provide the seed producer and turf manager with an additional method for control of grass weeds. In the present study, in vitro selection was used to obtain creeping bentgrass resistant to the acetyl coenzyme A carboxylase (ACCase) inhibitor, sethoxydim. Selection occurred on medium containing 10  $\mu\text{M}$  sethoxydim and 13,725 calli were screened. Four individual sethoxydim-resistant events were selected and sequencing confirmed an A to C in the first position of the 1781 amino acid codon causing an isoleucine to leucine substitution. The whole plant dose response experiment showed the sethoxydim-resistant event, SR1, was resistant to sethoxydim  $> 3200 \text{ g ai ha}^{-1}$ . SR1 showed cross resistance the ACCase-inhibitor fenoxypop  $> 800 \text{ g ai ha}^{-1}$ .

## **Introduction**

Creeping bentgrass (*Agrostis stolonifera*) is a cool season turfgrass used widely around the United States for golf course turf. Weed infestation is a recurrent problem for golf course managers. Broadleaf weeds can be controlled through application of selective herbicides such as 2,4-D (2,4-dichlorophenoxyacetic acid). Control of weedy grasses is limited to mechanical control and application of non-specific herbicides. The lack of selective herbicides to control weedy grasses creates the need to find alternative approaches to enable chemical control. Development of herbicide-resistant creeping bentgrass would allow the end user to maintain aesthetic quality of turf and growers to maintain seed purity.

Acetyl-coenzyme A carboxylase (ACCase) inhibitors are grass-specific herbicides used to control weedy grasses in broadleaf crops. Overreliance on ACCase-inhibitors in cropping

systems has resulted in numerous weedy species developing resistance due to mutations in the ACCase gene (Delye and Michel, 2005). Resistance to ACCase-inhibiting herbicides has also purposely been obtained in maize (*Zea mays* L.) (Parker et al., 1990) and in seashore paspalum (*Paspalum vaginatum* Sw.) (Heckart et al., 2010) by in vitro selection. Currently, there are ten known mutations conferring resistance to these ACCase-inhibiting herbicides (Powles and Yu, 2010). The most commonly reported mutation is a single isoleucine (ILE) to leucine (LEU) substitution at amino acid (aa) position 1781 in the CT domain of the ACCase protein (Delye and Michel, 2005). Other known mutations in the ACCase CT domain are located at 1999, 2027, 2041, 2078, 2088, and 2096 aa positions. Whole-plant screening for herbicide-resistant bermudagrass (*Cynodon dactylon* L.) revealed a genotype resistant to ACCase inhibitors and did not possess any of the previously mentioned mutations (Grimshaw et al., 2014). The authors suggest a metabolic response or reduced herbicide uptake may be responsible for herbicide resistance.

Plants possess two forms of the ACCase enzyme: eukaryotic and prokaryotic (Harwood, 1988). The eukaryotic ACCase is encoded by a single nuclear gene and the prokaryotic form is made up of four subunits coded for by four distinct genes. The locations of these two types of ACCase differ in most plants. Excluding the Poaceae, the prokaryotic ACCase is compartmentalized in the chloroplast and the eukaryotic form is found in the cytosol (Sasaki et al., 1995). The Poaceae are unique in that they possess the eukaryotic form of ACCase in the cytosol as well as in the chloroplast (Sasaki et al., 1995). Despite the high level of homology between the two forms of ACCase enzymes in grasses, (Gornicki et al., 1994), the cytosolic form remains unaffected by ACCase-inhibiting herbicides, while the plastidic form is highly susceptible (Delye, 2005). Acetyl-coenzyme A is carboxylated by ACCase to form malonyl-

coenzyme A in the first committed step of lipid biosynthesis. ACCase-inhibiting herbicides compete with acetyl-coenzyme A for its binding site in the ACCase carboxyl transferase (CT) domain (Delye, 2005).

The current study focusses on two groups of ACCase inhibitors including the cyclohexanediones (CHD) and the aryloxyphenoxypropionates (APP). The CHD sethoxydim, (2-[1-(ethoxyimino)butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one) is used to control perennial and annual grasses in agricultural and ornamental crops. Sethoxydim is currently marketed under the name Sethoxydim E-PRO® by BASF (Research Triangle Park, NC). Sethoxydim is absorbed through leaf surfaces then translocated throughout the plant and accumulated primarily in the meristematic tissue (Ahrens, 1994). The goal of this research was to identify naturally occurring sethoxydim-resistant creeping bentgrass mutants in vitro and regenerate resistant plants suitable for use in breeding herbicide-resistant cultivars.

## **Methods and Materials**

### ***Plant material***

Explant tissue used for callus induction was sterilized seeds of ‘Penn A-4’, ‘Penn A-1’, ‘Penncross’, ‘Crenshaw’, ‘Pick 96-2’, and ‘L93’ creeping bentgrass. Seeds were surface sterilized in 10% v/v Clorox with several drops of tween-20, rinsed three times with sterile water, and placed on callus induction medium, as described by Wang et al., (2003). All media were solidified with 2.5 g L<sup>-1</sup> Gelzan™ (Caisson Laboratories, North Logan, UT, USA), brought to pH 5.8, and autoclaved. Induction medium was poured into 100 x 15 mm Petri dishes. Approximately 100 seeds were placed on each plate and sealed with 3M Micropore™ tape (St. Paul, MN, USA). Seeds were placed in the dark at 27°C and callus tissue was transferred every three weeks.

Calli of each sethoxydim-resistant (SR) line were divided into 4 mm pieces and placed on shoot initiation medium (Wang et al., 2003). The calli were transferred to a growth chamber at 25 °C with a 1h dark, 23 h light photoperiod, and a light intensity of 66-95  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Shoots were sub-cultured on MS basal medium without growth regulators to induce root formation. Rooted plants were removed from the medium and placed in pots containing a 1:1 mix of Fafard<sup>®</sup> 3B (Agawam, MS) mix and sand. Potted plants were maintained in a growth chamber for one week, as described, then transferred to a greenhouse with a 10-hr light, 14-hr dark photoperiod at 24 °C to 32 °C.

### ***Dose response***

The response of bentgrass callus tissue to sethoxydim concentration was determined using callus tissue generated from the variety 'Penn A-4'. Laboratory-grade sethoxydim (Chemservice Inc. West Chester, PA) was diluted with methanol to 1 mg mL<sup>-1</sup>. The sethoxydim concentrations evaluated were: 0, 2.5, 5, 7.5, 10  $\mu\text{M}$ . Sethoxydim selection medium consisted of callus induction medium and addition of sethoxydim following autoclavation. Each dose level had seven replicate plates with 0.25 g of callus tissue per plate. Callus tissue was divided into nine equal pieces and plated in a 3 x 3 pattern. At three week intervals, calli were weighed and 0.25 g was re-plated. Weight measurements were divided by the initial weight to obtain the percent growth (Parrott and Bouton, 1990). Percent growth measurements were modeled using a negative exponential decay function using non-linear regression (SAS Institute, Inc. 2008. SAS OnlineDoc<sup>®</sup> 9.2. Cary, NC).

### ***Selection of sethoxydim-resistant cells***

Four month old callus tissue was sub-cultured onto selection medium containing 10  $\mu\text{M}$  sethoxydim. Two-hundred and twenty-five calli, approximately 4 mm in diameter, were placed

in 245 mm x 245 mm square bioassay dishes (Corning, Corning, NY) in a 15 x 15 grid. After three weeks, the calli were sub-cultured to fresh plates and the process was repeated three times. Resistant calli were amplified by sub-culturing for an additional three weeks on 10  $\mu$ M sethoxydim and regenerated as previously described.

### ***Whole plant evaluation***

DNA was extracted from leaves of regenerated plants via the CTAB method as described by Lassner et al., (1989). Previously published primers were used to amplify regions around the known locations of single-nucleotide-polymorphisms (SNPs) that confer resistance to ACCase-inhibiting herbicides (Heckart et al., 2010).

SR plants regenerated from a sethoxydim-resistant cell line, SR1 were tested for resistance at the whole plant level in a dose-response experiment conducted using four ACCase-inhibiting herbicides in a greenhouse. SR1 plants were compared to plants from the susceptible original parental line, 'Penn A-4'. Rooted plants were transferred to 4 x 14 cm Cone-Tainers™ (Stuewe and Sons Inc., Corvallis, Oregon) with a 1:1 mix of Fafard® 3B mix and sand. Cone-Tainers were placed under sodium lights in a greenhouse with a 16h photoperiod maintained at 27/32 °C day/night. Herbicides used included fenoxaprop, fluazifop, cycloxydim, and sethoxydim. Herbicides were applied at a spray volume of 187 l ha<sup>-1</sup> in a spray chamber. Each dose response experiment was a two by eight factorial with two genotypes and eight herbicide rates including: 0, 50, 100, 200, 400, 800, 1600, and 3200 g ai ha<sup>-1</sup>. Treatments were arranged in a randomized complete block design with four replications. Plants were allowed to dry and returned to the greenhouse. Visual estimates of crop injury were recorded at 21 and 28 d after treatment (DAT) and dose-response experiments were repeated twice. Injury was rated using a scale of 0 to 100, where 0 equals no injury and 100 equals complete death. Data for all whole

plant response experiments were analyzed using a two-way analysis of variance (SAS Institute, Inc. 2008. SAS OnlineDoc® 9.2. Cary, NC). The dose response data for ‘Penn A-4’ was fitted to a Mistic model, and SR1 injury was fitted to a linear model.

## **Results**

### ***Callus dose response***

Figure 2.1 shows callus response to varying concentrations of sethoxydim. A sethoxydim concentration of 2.5 to 5 µM greatly reduced callus growth. Callus growth was zero at 7.5 µM and would have been adequate for selection; however, previous experience with in vitro selection seashore paspalum has shown that increasing the concentration to 10 µM will prevent non-resistant escapes. Data from the dose-response experiment was fitted to a negative exponential decay function. The regression equation was  $y = (\beta_0 e^{-\beta_1 X}) = (12.5e^{-1.25X})$ .

### ***Selection of sethoxydim resistant cells***

Individuals from six cultivars were tested for callus formation for use in selection experiments (Table 2.1). Three out of six cultivars formed calli on callus induction medium as described by Wang et al. (2003). Callus growth for ‘Penn A-4’ was faster than of ‘Penn A-1’ or ‘Penncross’. Differences in growth rate resulted in selection on 57 plates each containing 225 four millimeter diameter calli (12,825 total) for ‘Penn A-4’; while only 675 and 225 calli completed the selection process for ‘Penn A-1’ and ‘Penncross’, respectively. During the third cycle of selection, four independent resistant events were selected from ‘Penn A-4’ calli. Resistant calli were subcultured on callus induction medium supplemented with 10 µM sethoxydim for an additional 3 weeks to confirm resistance and to increase callus tissue for regeneration. Sequencing confirmed the presence of the A to C transversion mutation at position 1781 in all four lines causing an ILE to LEU aa substitution (Figure 2.2).

### ***Whole plant response to acetyl coenzyme A-inhibiting herbicides***

Comparison among visual injury estimates taken at 21d and 28 d during experiment 1 and 2 (repetitions) showed no significant differences between ratings taken at 21 d and those taken at 28 d or between repetitions of the dose response experiments ( $p \leq 0.05$ ). Injury data was not significantly different between 21 dat, 28 dat or experiment replications; therefore, data was combined for each genotype at each rate. All four SR lines possessed the same nucleotide mutation and corresponding aa substitution; therefore, only SR line 1 (SR1) was compared to the parental type 'Penn A-4'. Figure 2.3 summarizes the results of four dose-response experiments using sethoxydim as well as three other ACCase inhibiting herbicides. SR1 demonstrated a high level of sethoxydim resistance with only minimal injury (8.4%) at the highest rate of sethoxydim (Figure 2.3, 2.4). The  $LD_{50}$  for SR1 was  $> 3200$  g a.i.  $ha^{-1}$ , compared to the parental line, 'Penn A-4' with an  $LD_{50}$  between 400 and 800 g a.i.  $ha^{-1}$ . Cycloxydim injury to SR1 plants was less than the susceptible 'Penn A-4' at all rates from 50 to 3200 g a.i.  $ha^{-1}$ ; however, injury at 3200 g a.i.  $ha^{-1}$  was substantial at 51.25%. SR1 injury was lower when compared to 'Penn A-4' at all fluazifop concentrations, but injury level was above 50% at 1600 g a.i. 3200 g a.i.  $ha^{-1}$ . Fenoxypop experiments showed that SR1 expresses high levels of resistance and did not exceed 20% at 3200 g a.i.  $ha^{-1}$ . These data indicate that SR1 bentgrass has approximately 4X tolerance to cycloxydim and fluazifop, 8X tolerance to fenoxypop, and even higher tolerance to sethoxydim. These data provide strong evidence of the presence of cross resistance to cycloxydim, fluazifop, and fenoxaprop in SR1 creeping bentgrass. The level of cross resistance present is adequate to provide effective control of susceptible weedy grasses without serious concerns over herbicide injury to SR1 creeping bentgrass.

## Discussion

In all, 13,725 calli were screened, resulting in four sethoxydim-resistant cell lines that regenerated into whole plants. Sequencing of the region around the 1781 codon revealed a previously unreported nucleotide mutation of A to C at the first position. The nucleotide mutation was unique; however, the aa substitution of ILE to LEU has been seen in seashore paspalum and several weedy species. Centipedegrass (*Ermochloa ophiuroides*), is naturally resistant to sethoxydim. The labeled rate for application of sethoxydim on centipedegrass is 320 g a.i. ha<sup>-1</sup>, and SR1 exhibits resistance at ten times this rate. SR1 shows varying levels of cross resistance to the other ACCase-inhibiting herbicides tested. In general, fluazifop, fenoxypop, and cycloxydim can also be applied to SR1 at field rate to control weedy grasses without concern of serious herbicide injury.

Although this novel mutation could allow control of many grasses in creeping bentgrass, two of the major grass weeds in creeping bentgrass are annual bluegrass (*Poa annua*) and red fescue (*Festuca rubra* L.), which also possess the 1781 LEU mutation (Delye and Michel, 2005) and would therefore not be controlled by a herbicide resistance system based on this mutation. The herbicide ethofumesate (Prograss<sup>®</sup>) has been labeled for control of annual bluegrass in creeping bentgrass. Testing of the experimental herbicide, methiozoline (PoaCure<sup>®</sup>), on golf course on golf course greens in Georgia has shown methizolin to be highly effective for control of annual bluegrass with minimal turf injury (McCullough et al., 2013). Conversely, creeping bentgrass can also be considered a weed if found in a seed-production field or putting green of another species. Treatment with herbicides with other mechanisms of action, including glyphosate, will still control sethoxydim-resistant bentgrass, because the mutation in ACCase only confers resistance to some ACCase inhibiting herbicides.

In summary, creeping bentgrass with high levels of sethoxydim resistance has been selected in vitro. HR creeping bentgrass also showed cross resistance to other ACCase-inhibiting herbicides. Herbicide resistance is a result of a natural single base mutation in the ACCase gene and resulting plants are not transgenic; therefore, are not subject to current government regulations. This HR turfgrass may be a valuable product for seed producers and end users.

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Table 2.1. Summary of creeping bentgrass cell lines completing in vitro selection for sethoxydim resistance.

	Genotype	Calli Through Selection <sup>†</sup>	SR Calli <sup>‡</sup>	SR Calli Regenerated <sup>§</sup>	Calli positive for Leucine 1781
1	'Penn A-4'	12,825	4	4	4
2	'Penn A-1'	675	0	0	0
3	'Penncross'	225	0	0	0
4	'Pick 96-2'	0	0	0	0
5	L93	0	0	0	0
6	'Crenshaw'	0	0	0	0

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

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

<sup>§</sup>Number of SR calli producing viable plants after more than 4 wk on regeneration medium.

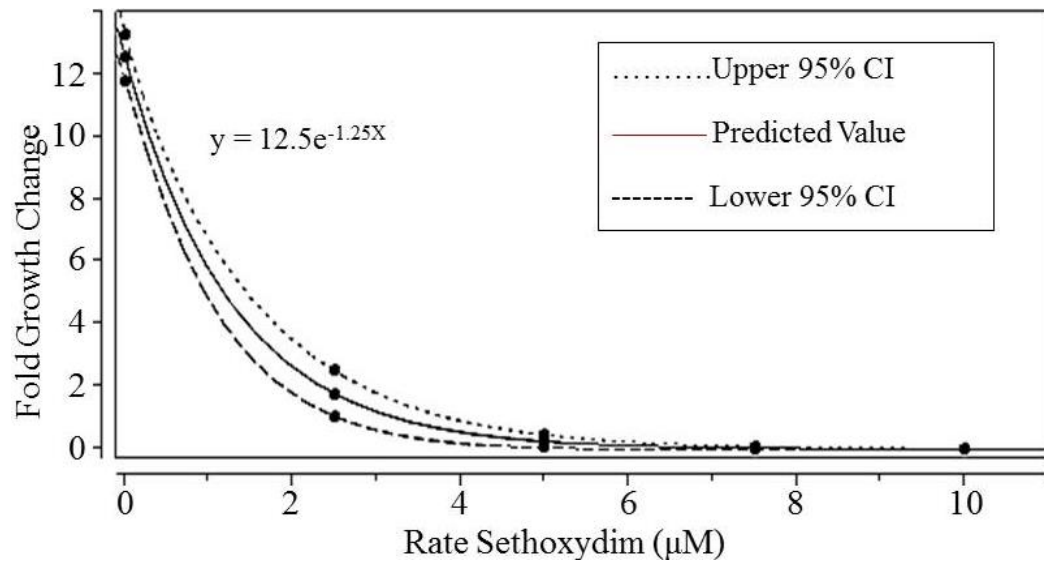


Figure 2.1. Callus tissue response of creeping bentgrass after nine weeks on varying concentrations of sethoxydim selection. Callus tissue was derived from seeds ‘Penn A-4’ and 0.25 g was placed on each plate. Calli were transferred after three weeks and underwent three cycles of selection. Data from five replicate plates for each concentration were modeled using an exponential decay function.

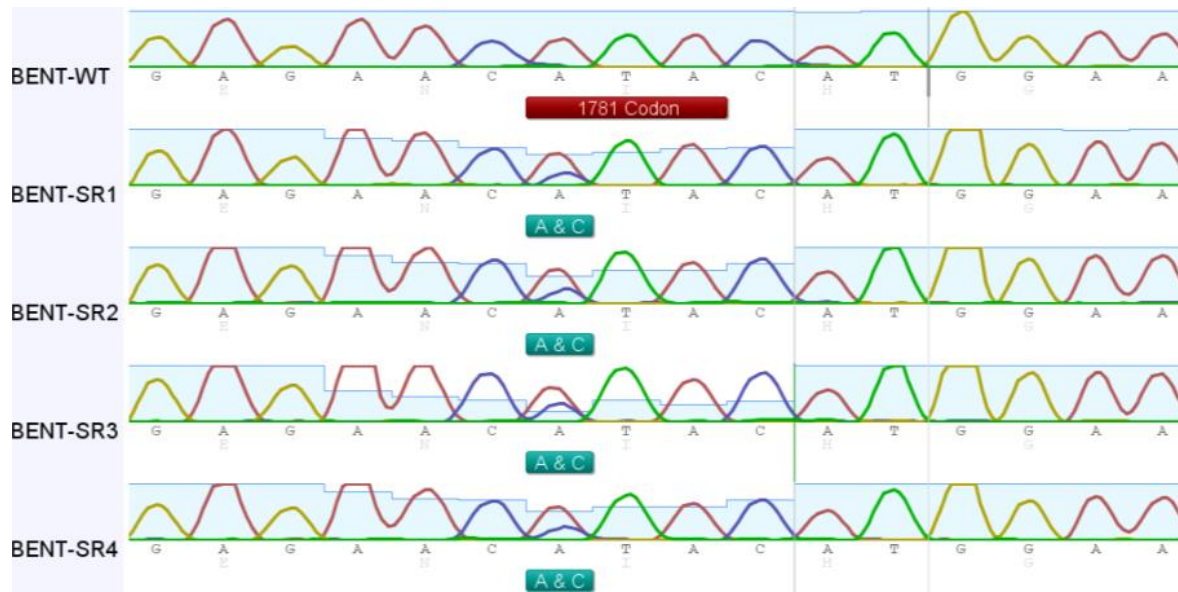


Figure 2.2. Alignment of PCR-amplified sequences from wild-type (WT) ‘Penn A-4’ and sethoxydim-resistant lines SR1-4. Creeping bentgrass has multiple alleles; therefore, a mutation is indicated by a double peak at the first position of the 1781 codon. The A to C mutation causes an isoleucine to leucine, known to confer resistance to ACCase-inhibiting herbicides.

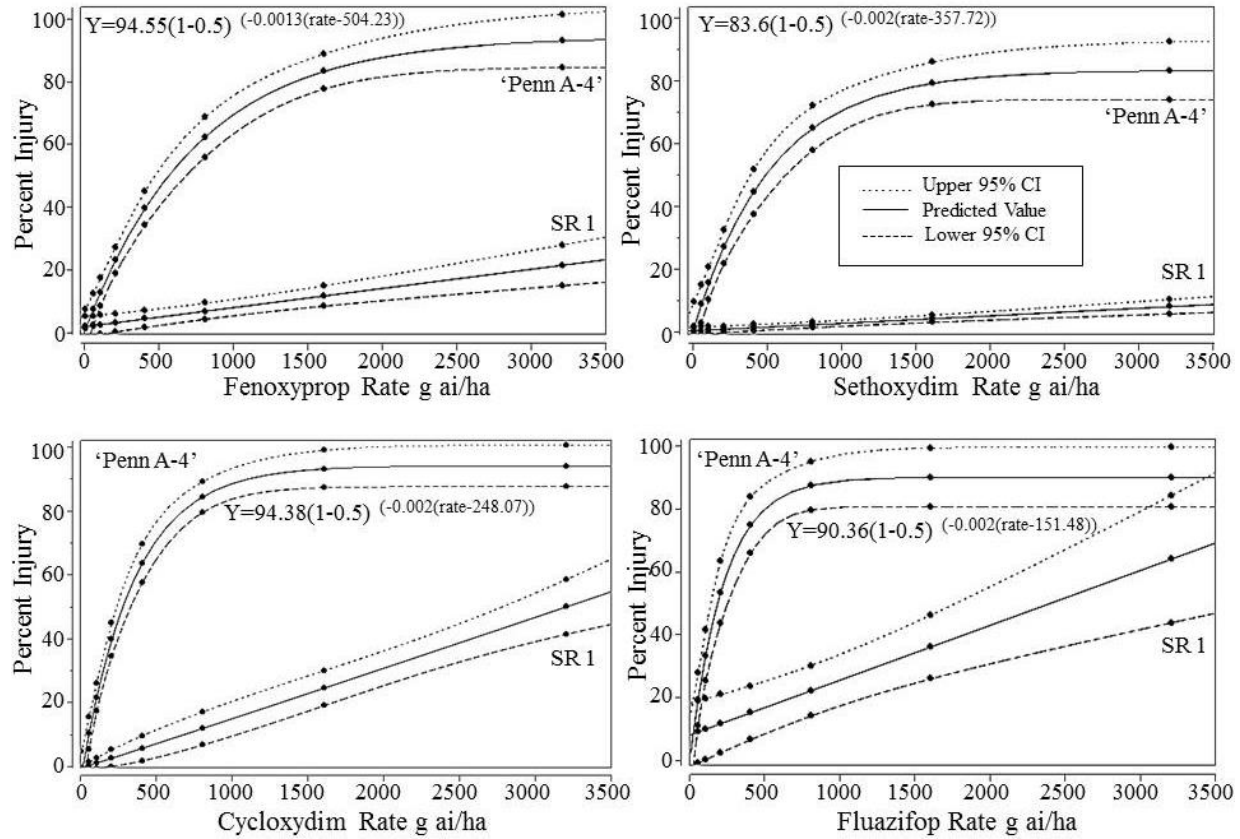


Figure 2.3. Whole-plant response of SR1 and parent type 'Penn A-4' to four ACCase-inhibiting herbicides. Measurements were taken at 21 and 28 days after treatment and herbicide dose experiments were repeated twice, excluding cycloxydim, where one experiment was performed. Dose response for 'Penn A-4' was modeled using a Misticlich model and response of SR1 was fitted to a linear model.

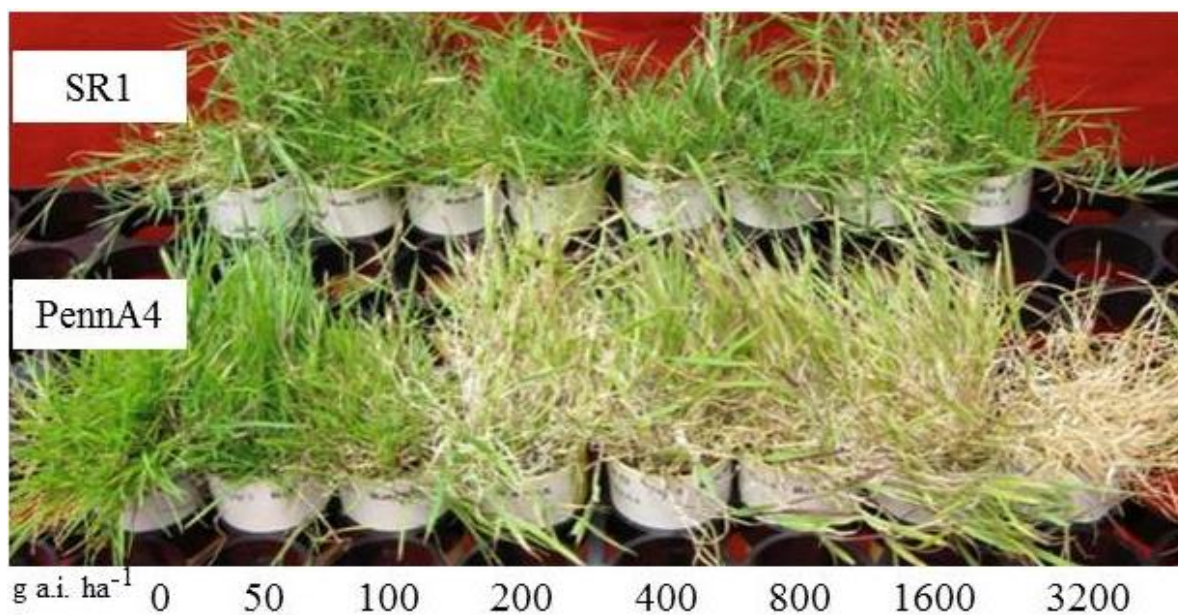


Figure 2.4. Whole-plant response of SR1 compared to the parental type 'Penn A-4' to different rates of sethoxydim ranging from 0 to 3200 g a.i. ha<sup>-1</sup>. Plants were evaluated 28 days after treatment following foliar application of sethoxydim.

## CHAPTER 3

### SYNONYMOUS MUTATION GENE DESIGN TO OVEREXPRESS ACCASE IN CREEPING BENTGRASS TO OBTAIN RESISTANCE TO ACCASE-INHIBITING HERBICIDES <sup>3</sup>

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<sup>3</sup>Heckart, D.L., P.L. Raymer, B.M. Schwartz, and W.A. Parrott. To be submitted to *Transgenic Research*.

## **Abstract**

Engineering plants with an extra copy of their own gene to obtain overexpression has many possible applications for crop improvement. Overexpression of a native gene can cause expression of both introduced and native genes to be silenced by posttranscriptional gene silencing (PTGS) mechanisms. PTGS mechanisms rely on sequence identity between the transgene and native genes; therefore, designing genes with synonymous mutations may avoid PTGS. For proof of concept, the sequence of acetyl-coA carboxylase (*ACCase*) from creeping bentgrass (*Agrostis stolonifera* L.) was altered with synonymous mutations. A native bentgrass *ACCase* was cloned and used as a template for the modified gene. Wild-type and modified genes were further modified with a non-synonymous mutation, coding for an isoleucine to leucine substitution at position 1781, known to confer resistance to *ACCase*-inhibiting herbicides. Five-hundred calli of creeping bentgrass ‘Penn A-4’ were inoculated with *Agrobacterium* containing either the WT or modified genes, with or without the herbicide-resistance mutation. Six herbicide-resistant-transgenic events containing the modified gene with the 1781 mutation were obtained. Transcription of the modified *ACCase* was confirmed in transgenic plants, showing that gene-silencing mechanisms were avoided. Transgenic plants were confirmed to be resistant to the *ACCase*-inhibiting herbicide, sethoxydim, providing evidence that the modified gene was functional. The result is a novel herbicide-resistance trait and shows that overexpression of a native enzyme with a gene designed with synonymous mutations is a viable strategy.

## **Introduction**

Grass weeds can be difficult to control in golf-course greens and grass-seed-production fields. Economic losses and loss of aesthetics can be minimized if the cultivars grown are herbicide-resistant (HR). HR turf would be unique from other examples of HR crops, as the trait

could be used by both the producer and the end-user. A common approach to obtaining HR crops is through insertion of the *Agrobacterium CP4 EPSPS* gene. Alternatively, Scotts Miracle Gro<sup>®</sup> transformed Kentucky bluegrass (*Poa pratensis* L.) and St. Augustine grass (*Stenotaphrum secundatum* Walt.) with a modified *EPSPS* from *Arabidopsis*, which is not covered by APHIS regulation (APHIS, 2011, Gregoire, 2012) Details on how the *Arabidopsis EPSPS* was modified was not made public (Wang et al., 2014).

In vitro approaches are an alternative to the use of transgenics. In vitro selection has been used to select maize (*Zea mays* L.), seashore paspalum (*Paspalum vaginatum* Sw.), and creeping bentgrass (*Agrostis stolonifera* L.) for resistance to acetyl coenzyme A carboxylase (ACCase)-inhibiting herbicides (Heckart et al., 2010, Heckart et al., 2014, Parker et al., 1990). In paspalum and bentgrass, resistance is due to a non-synonymous mutation at amino acid position 1781 of ACCase, which increased sethoxydim resistance to over 15 and 8 times the field application rate, respectively (Heckart et al., 2010, Heckart et al., 2014).

ACCase catalyzes the first committed step of fatty acid biosynthesis, and ACCase-inhibiting herbicides compete with acetyl coenzyme A for its binding site (Delye, 2005). Inactivation of ACCase ceases lipid production, resulting in loss of cell membrane integrity and cell death. *ACCase* coding sequences in Poaceae are ~7 kb. Specific point mutations in *ACCase* cause amino acid substitutions that prevent the binding of ACCase-inhibitors while allowing normal enzyme function (Delye et al., 2005). Currently, there are eight known single nucleotide polymorphisms (SNPs) conferring resistance to these herbicides (Powles and Yu, 2010). The most commonly reported mutation is a single isoleucine (ILE) to leucine (LEU) substitution at amino acid (aa) position 1781. Other known HR substitutions in ACCase are located at amino acid positions 1999, 2027, 2041, 2078, 2088, and 2096. Each mutation confers resistance to one

or both groups of ACCase inhibitors, including the cyclohexanediones (CHD) and aryloxyphenoxypropionates (APP).

A third alternative to the use of heterologous genes or in vitro selection is the re-introduction of a native gene containing a SNP for resistance. Theoretically, HR Poaceae cultivars could be produced by overexpression (OE) of *ACCase* modified with a SNP that confers resistance to ACCase-inhibiting herbicides. Arguably, the most well-known example of addition of a native gene for the purpose of OE was the introduction of an extra chalcone synthase gene in petunia (Napoli et al., 1990). Both the transgene mRNA and homologous native gene mRNA were degraded in a phenomenon that was termed cosuppression (Metzlaff et al., 1997, Napoli et al., 1990). Cosuppression is now understood to be a form of post-transcriptional gene silencing (PTGS). Silencing results from degradation of mRNA into small interfering RNA (siRNA) approximately 21-25 nt in length (Hamilton and Baulcombe, 1999, Zamore et al., 2000) by enzymatic cleavage by the ribonuclease, Dicer (Bernstein et al., 2001). Cleavage of primary transcripts is guided by siRNAs bound to the dicer-like protein Argonaut (Hammond et al., 2001). These observations indicate that only ~21 bp of identity are required between native and introduced genes for silencing mechanisms to degrade transcripts from both. Thus, to obtain reliable OE of an introduced gene, the sequence identity of the introduced gene to any native gene must be low.

Due to the perfect identity but for one SNP, an alternative approach may be required to OE *ACCase* and overcome gene silencing. Drake et al. (1996) were able to bypass gene silencing by introduction of synonymous mutations. Synonymous mutations reduce the identity between the native gene and transgene, while conserving the amino acid sequence. Kumar et al. (2006) also utilized a gene design with synonymous mutations to express a native protein. The native

gene had previously been silenced, while the use of an optimized transgene with synonymous mutations restored the wild-type (WT). These two examples show that PTGS mechanisms can be avoided by addition of synonymous mutations.

In the present study, synonymous-mutation-gene design was tested by overexpressing a modified creeping bentgrass *ACCase* in creeping bentgrass to obtain plants resistant to ACCase inhibitors, while avoiding gene silencing. To accomplish this, a native ACCase gene was cloned and used as a template for gene design. Additionally, the WT and modified gene were further modified by addition of the 1781 SNP conferring resistance to ACCase-inhibiting herbicides.

## **Methods and Materials**

### ***Cloning bentgrass ACCase***

The sequence for bentgrass *ACCase* was unknown; therefore, it had to be cloned before it could be used as a template for the modified *ACCase*. Fifteen *ACCase* sequences from eight species (Table 3.1) were aligned using Geneious<sup>®</sup> (Biomatters Ltd. Auckland, NZ) and revealed highly conserved regions throughout the gene that were used for primer design. Primers were also designed from the untranslated regions (UTRs) to obtain the 5' and 3' sequences that include the start and stop codons. RNA was extracted from bentgrass cultivar 'Penn A-4' using TRI Reagent<sup>®</sup> (Ambion<sup>®</sup> Life Technologies, Grand Island, NY). First strand cDNA synthesis was performed with RNA to cDNA EcoDry<sup>™</sup> Premix Oligo dT kit (Clontech Laboratories Inc. Mountain View, CA) according to the manufacturer's protocol. The 5' and 3' ends were amplified with primer sets 5'UTR1/BG750R and BG10F/3'UTR1 using Phusion<sup>®</sup> proofreading polymerase (New England Biolabs, Ipswich, MA) (Table 3.2). All fragments were sequenced by Genewiz Inc. (South Plainfield, NJ). Bentgrass-specific primers including the start codon (BGSTARTF) and stop codon (BGSTOPR) were designed from the sequenced fragments. Full

length ACCase cDNA sequences were amplified using GoTaq<sup>®</sup> Long PCR Master Mix (Promega, Madison, WI). The PCR conditions were 94 °C/2 min, followed by 30 cycles of 92 °C/ 30 s, 60 °C/30 s, 68 °C/7 min, followed by a 10 min extension at 72 °C. PCR The resulting cDNA sequence was 6.88 kb in length.

### ***ACCase gene design with synonymous mutations***

The cDNA sequence was translated into an amino acid sequence using Geneious<sup>®</sup> software. The amino acid sequence was then back-translated into a nucleotide sequence using Gene Designer 2.0 (DNA 2.0, Menlo Park, CA). Back translation of the amino acid sequence requires knowledge of codon bias to ensure rare codons were not used; however, bentgrass has too few known coding sequences to determine bias. Wheat (*Triticum aestivum* L.) was possibly the closest relative to creeping bentgrass (Edwards et al., 2011) with known codon usage at the time, and was therefore used for back-translation. An alignment was made between the cDNA sequence and the modified sequence to find regions of identity of 21 bp or higher. Codons were swapped to reduce any stretches of perfect identity over 21 bp, while ensuring rare codons were not introduced. The resulting modified gene included 1538 synonymous mutations, and the longest stretch of 100% identity, when compared to the native cDNA sequence, was 14 bp. The amino acid sequence of the modified protein was aligned with the native ACCase to ensure 100% identity. The modified gene was synthesized without any further modification by Genscript (Piscataway, NJ).

### ***Vector construction***

Four genes were used in this study. These included: 1) wild-type ACCase with no mutations, 2) wild-type ACCase with an A to T mutation in the first position of the 1781 aa codon, 3) modified ACCase with no mutations 4) modified ACCase with an A to T mutation in

the first position of the 1781 aa codon. The pCambia 1305.2 plasmid (Cambia, Canberra, AU) was digested with *MauBI* and *SbfI* to remove the 35S promoter, GUSPlus™ reporter gene, and a portion of the NOS terminator (T-NOS). Vectors were assembled with In-Fusion™ cloning (Clontech Laboratories Inc. Mountain View, CA). The ACCase genes were under control of the switchgrass (*Panicum virgatum* L.) PvUbi2 promoter (Mann et al., 2011) and T-NOS. PvUbi2, T-NOS, and the modified gene were amplified with In-fusion primers (Table 3.2). The modified gene was amplified in three fragments, 2667, 2650, and 1600 bp in length by primer sets SynACF1IF-F/ SynACF1IF-R, SynACF2IF-F/ SynACF2IF-R, and SynACF3IF-F/ SynACF1IF-R, respectively. An additional set of primers was designed to include the HR SNP in the 1781 codon. The HR SNP was included in the 15 bp overhang in the reverse primer for fragment two (SynACF2LEUIF-R) and in the forward primer for fragment three (SynACF3LEUIF-F). Fragments were amplified using Phusion® proofreading polymerase (New England Biolabs, Ipswich, MA).

The bentgrass gene without the HR SNP was amplified using GoTaq® Long PCR Master Mix and the primer set BGStartIF-F/BGStopIF-R to add InFusion ends. Two fragments were amplified to include the 1781 mutation. The mutation was included in the 3' 15 bp overhang of the 5 –kb fragment one and the 5' 15 bp overhang of the 1.88-kb fragment two. Primers used to include the 1781 HR mutation were BG1781LEUF and BG1781LEUR. The 5-kb fragment was amplified with GoTaq® Long PCR Master Mix and the 1.8-kb fragment was amplified with Phusion proofreading polymerase. The molar ratio of plasmid to insert for the cloning reaction was 2:1. Each construct was assembled in a single In-Fusion reaction that included all fragments for the construct, including promoter and terminator. Leading and lagging strands were

sequenced for each plasmid to ensure all portions were properly joined and that no unintentional SNPs were included.

### ***Plant material***

Callus induction and transformation procedures followed protocols as described by Luo et al., (2004) with minor adjustments. Media were solidified with 2.5 g L<sup>-1</sup> Gelzan™ (Caisson, North Logan, UT). Plates were sealed with Micropore™ tape (3M, St. Paul, MN).

*Agrobacterium* st. LBA4404 containing each construct was maintained in a freeze stocks and streaked on solid YM plates containing 50 mg L<sup>-1</sup> kanamycin. Cultures were grown for three days at 20 °C and re-suspended in liquid callus induction medium with 100 µM acetosyringone. Five-hundred calli were inoculated with each construct, including the control plasmid pCambia 1305.2. Selection of transgenic events occurred in two stages. First, selection occurred on callus induction medium supplemented with 200 mg L<sup>-1</sup> hygromycin and 150 mg L<sup>-1</sup> timentin to select for transgenic events. Hygromycin-resistant events were transferred to medium supplemented with 10 µM sethoxydim. Sethoxydim (Chemservice Inc. West Chester, PA) was diluted with methanol to a concentration of 1 mg mL<sup>-1</sup>. Sethoxydim-resistant events were transferred to shoot induction medium as described by Luo et al., (2004) and placed in a growth chamber at 25 °C with a 1-h dark, 23-h light photoperiod, with a light intensity of 66-95 µmol photons m<sup>-2</sup> s<sup>-1</sup>. Regenerating plants were placed on rooting medium consisting of MS basal medium without growth regulators (Murashige and Skoog, 1962).

### ***Molecular characterization of transgenic events***

PCR was performed on all regenerated plantlets to confirm the presence or absence of the transgene. DNA was extracted using the CTAB method as described by Lassner et al. (1989).

The primer set SynACF1IF-F/ SynACF3IF-R was used to amplify the complete modified ACCase transgene. PCR conditions and reagents were the same as conditions described earlier for amplification of the creeping bentgrass ACCase. Four PCR reactions were used to evaluate transcription in transgenic events. RNA was extracted using TRI Reagent<sup>®</sup> and cDNA was produced using SuperSript<sup>®</sup> III reverse transcriptase (Life Technologies). The primer set SYNACF/ SYNACR was used to check transcription of the transgene and primers VirGF and VirGR were used to test for contamination from residual *Agrobacterium*. The third primer set, BG2921F and BG3300R was used to amplify a fragment of the native ACCase that spans an intron, serving as a DNA quality check for gDNA samples and as a check for gDNA contamination in cDNA samples. The primer set, HYG370F/HYG720R, confirmed transcription of the *hpt* gene. In a previous experiment, sethoxydim-resistant creeping bentgrass obtained through in vitro selection possessed the 1781 LEU mutation (Heckart et al., 2014). To ensure the transgenic events were not resistant due to a mutation in the native gene, the region from the 1781 codon to the 2096 codon was amplified and sequenced. Primers used to amplify the sequence were BG4880F and BG6300R. Leading and lagging strands were sequenced for 10 plants from events one and two, and five plants from events three and four.

### ***Transgenic plant response to sethoxydim***

Response of transgenic plants to sethoxydim was tested using a single rate in vitro, and with a dose response in soil. For in-vitro analysis, a dose-response experiment was conducted to determine the minimum sethoxydim concentration needed to kill the ‘Penn A-4’ control. Rooting medium was supplemented with 0, 0.5, 1, 2.5, 5, 7.5, or 10  $\mu$ M sethoxydim. Rooted ‘Penn A-4’ plants were placed on medium in 100 x 20 mm petri dishes (Bioexpress, Kaysville, UT) in a 3x3 grid with five replications per concentration. Plant health was evaluated after three weeks.

Following the dose response, plants from each event were placed on medium containing 0.5  $\mu\text{M}$  sethoxydim. Each plate contained a single transgenic event and a control 'Penn A-4' plant, and was evaluated after 3 weeks. Ten plants from each of the first four events were evaluated.

For whole-plant dose response, rooted plants were transferred to Cone-Tainers<sup>TM</sup> measuring 4 x 14 cm (Stuewe and Sons Inc., Corvallis, Oregon) containing a 1:1 mix of Fafard<sup>®</sup> 3B mix and sand. Plants were transferred to the greenhouse ~ 30 °C placed under sodium lights in a with a 16-h photoperiod. Plants were maintained at a height of 4 cm. Dose- response experiments followed general guidelines as described by Heckart et al. (2010). Herbicide resistance was evaluated with Sethoxydim G-PRO<sup>TM</sup> (BASF Corp., Florham Park, NJ). Transgenic plants were treated with eight rates of sethoxydim, including: 0, 50, 100, 200, 400, 800, 1600 and 3200 g ai ha<sup>-1</sup>. Sethoxydim was applied at a spray volume of 187 L ha<sup>-1</sup> in a spray chamber and allowed to dry. Treatments were arranged in a randomized complete block design with five replications. Plant injury was evaluated visually at 14, 21, and 28 d after treatment. Evaluation of plant injury was performed using a scale of 0 to 100, where 0 equals no injury and 100 equals complete plant death. In addition to visual rating, digital image analysis was performed to obtain percent green cover (PCG) in each Cone-Tainer. Photos of each Cone-Tainer were taken with a GoPro<sup>®</sup> Hero3 Black edition fitted with a 10X magnification lens. The lens and camera were attached to a PVC pipe fitted with eight 5 mm super bright white LEDs (model NTE30045, Radioshack, Ft. Worth, TX). If necessary, plants were trimmed to ~2 cm prior to taking pictures. The camera apparatus was placed over each Cone-Tainer and distance from the lens to the top of the plant was ~1 cm. Image capture settings were: video resolution 720, wide field of view, 7 mp wide photo resolution, Protune setting was on, 6500 k white balance, GOPRO color, ISO limit 400, low sharpness, and exposure set to zero. Images were

analyzed using SigmaScan<sup>®</sup> Pro (Systat Software, San Jose, CA). Hue was adjusted to 50-150 and brightness was 5-99. SigmaScan<sup>®</sup> forced a PCG reading for pots where plants were completely dead; therefore, these pots were set to a PCG of zero. PCG was adjusted by removing the black background outside of the Cone-Tainer.

## Results

A total of 500 calli were inoculated for each of the five constructs, including pCambia 1305.2 control. Hygromycin selection produced 37 to 60 resistant events per construct (Table 3.3). Six sethoxydim-resistant events were selected from calli inoculated with the modified *ACCase* with the 1781 substitution and none were selected from other constructs. All events were PCR-positive for the complete synthetic gene (Figure 3.1a.). All events showed expression of the *ACCase* transgene and *hygromycin phosphotransferase* (Figure 3.1 b and c, respectively). The events were negative for *Agrobacterium* and genomic DNA contamination (Figure 3.1 d and c, respectively). Alignments of genomic sequences from transgenic events and the ‘Penn A-4’ control revealed that no unintended mutations were in the region spanning the 1781 to 2096 codons.

The in vitro dose response experiment showed that all doses were lethal to ‘Penn A-4’. Therefore; the lowest dose of 0.5  $\mu\text{M}$  sethoxydim was used to test transgenic events. Due to availability of tissue, the first four events were used for further analysis. All four transgenic events tested showed high levels of sethoxydim resistance compared to the ‘Penn A-4’ controls (Figure 3.2). ‘Penn A-4’ plants were completely killed and all transgenic events survived and showed vigorous growth at 0.5  $\mu\text{M}$  sethoxydim. Following the in vitro analysis, transgenic events were planted in soil for evaluation of response to foliar applied Sethoxydim G-Pro<sup>®</sup>. Data for visual injury at 28 dat for ‘Penn A-4’ were fitted to a Misticich model, using nonlinear

regression, and the resulting equation was:  $y = \beta_0(1-0.5e^{-\beta_1(\text{rate}-\chi^p)}) = 101(1-0.5e^{-0.0093(\text{rate}-1.3)})$  (Figure 3.3). PCG was modeled using nonlinear regression to an exponential decay model, and the resulting equation was  $\beta_0(e^{-\beta_1\chi}) = y = (63.15 \cdot (e^{-0.0095\chi}))$  (Figure 3.4). Transgenic events fit a linear model for both visual injury and PCG.

At 14 dat, transgenic plants for all four events showed minimal injury at all rates (Table 3.1). The control, ‘Penn A-4’, showed injury above 40% at 200 g ai ha<sup>-1</sup> and was killed at 800 g ai ha<sup>-1</sup>. PCG at 21 dat was reduced to five percent at 400 g ai ha<sup>-1</sup> for ‘Penn A-4’. PCG for transgenic events was reduced by 16 – 28 % at 3200 g ai ha<sup>-1</sup>. At 21 dat, ‘Penn A-4’ injury was severe at 100 g ai ha<sup>-1</sup> and PCG was reduced to 18%. Transgenic events 2 – 4 survived to 3200 g ai ha<sup>-1</sup> with ~50 % injury and PCG was reduced by 50%. By 28 dat, transgenic plants with injury showed some recovery. For example, percent injury for event four was reduced from 21 to 16% (Figure 3.5 and 3.6). Control plants were almost completely killed at 200 g ai ha<sup>-1</sup> and showed severe injury at 100 g ai ha<sup>-1</sup>. Event 4, the best performing transgenic event, only had 27% injury and a reduction of 7 PCG at 800 g ai ha<sup>-1</sup>.

## Discussion

The bentgrass ACCase cDNA sequence was compared to ACCase genes from corn, rice, sorghum, and wheat. The bentgrass ACCase coding sequence was highly conserved, as is the case with other grass species (Kawabe and Miyashita, 2003, Zhang et al., 2001, Zhang et al., 2012). Using nuclear codon bias, the gene was optimized for nuclear expression while simultaneously adding many synonymous mutations. DNA 2.0 further optimized gene design by excluding rare codons during back-translation. Removal of rare codons is important as rare codons can cause ribosomal stalling, resulting in an altered folding pattern that may change the enzyme’s activity (Carlini and Stephan, 2003, Saunders and Deane, 2010, Tsai et al., 2008).

Thirteen percent of hygromycin-resistant events from calli inoculated with the modified *ACCase* with the 1781 LEU mutation were sethoxydim-resistant. In vitro selection of HR paspalum (Heckart et al., 2010) and bentgrass(Chapter 2) produced events at a much lower rate. For paspalum, 20,250 calli went through the selection process, and two regenerable events appeared during the third round of selection; the efficiency of selection was 0.0098 %. In vitro selection for sethoxydim resistance in creeping bentgrass produced four resistant calli per 13,725 calli that were originally placed on selection medium, yielding an efficiency of 0.029 %. As seen with paspalum, HR bentgrass events did not appear until the third round of selection. Given that originally 500 calli were inoculated in the present experiment, the overall efficiency is 1.2 %. Sethoxydim-resistant creeping bentgrass was obtained through OE of a modified *ACCase* at 121 and 41 times higher frequency than with in vitro selection of seashore paspalum and creeping bentgrass, respectively. As previously reported in paspalum, it is estimated that the chance of obtaining an A to T mutation at the first position of the 1781 codon is one in 1.3 billion. In addition, paspalum and bentgrass in vitro events appeared during the third round of selection and transgenic events appeared during the first round of selection, reducing the time in culture by almost 2 months.

Sethoxydim E-Pro<sup>®</sup> is labeled for use on centipedegrass (*Ermochloa ophiuroides* Munro.) and fine fescue (*Festuca ovina* L.), because these grasses are naturally resistant. Rates for seedling and well established stands of centipedegrass are 210 g ai ha<sup>-1</sup> and 315 g ai ha<sup>-1</sup>, respectively (Anonymous, 2011). The creeping bentgrass used in this study was regenerated in vitro then grown in soil for two weeks prior dose response experiments and closely resemble seedling plants. Injury was only 16% at 28 dat for event four and reduction of PCG was only 6% at 400 g ai ha<sup>-1</sup>, approximately double the rate recommended for seedling centipedegrass.

No sethoxydim-resistant events were recovered from calli inoculated with the bentgrass ACCase transgenes or the modified ACCase without the 1781 mutation. If gene silencing occurred with cells containing the bentgrass ACCase transgenes, down-regulation of the native gene would be lethal and could explain why no events were recovered. Overexpression of the modified gene without the 1781 mutation could cause cells to be resistant due to an increased number of active sites as seen with Palmer amaranth (*Amaranthus palmeri* S.Wats) and *Kochia scoparia* (L.) (Gaines et al., 2010, Wiersma et al., 2014). The relative gene copy increase seen in *P. amaranth* was 5 -160. *K. scoparia* had a three to ten-fold increase in EPSPS copy number. Dinelli et al., (2008) observed a doubling of EPSPS mRNA in resistant *Conyza bonariensis* (L.) without an increase in gene copy number. To our knowledge, ACCase copy number increase has not yet been shown to cause resistance to ACCase inhibitors. We did not study the hygromycin-resistant and sethoxydim-susceptible events for transcription, although it may have given an indication to the extent of gene-silencing.

In summary, the present study demonstrates that a synthetic ACCase gene with synonymous mutations was able to bypass PTGS, while maintaining amino acid identity. Furthermore, the introduction of a SNP known to cause resistance to ACCase inhibitors made it possible to use the synthetic gene to recover sethoxydim-tolerant bentgrass. Apart from herbicide resistance, OE of native enzymes has many possible applications to improve a breeding program and this study shows that this method of gene design is possible.

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Table 3.1. ACCase genes used to create the alignment for primer design.

Accession number	Species	Length (bp)
AY312171	<i>Zea mays</i>	7324
ZMU19183	<i>Zea mays</i>	7464
NM001111903	<i>Zea mays</i>	7464
AY312172	<i>Zea mays</i>	7327
AF294805	<i>Setaria italica</i>	7630
AY219174	<i>Setaria italica</i>	7271
AY219175	<i>Setaria italica</i>	7446
HQ395758	<i>Echinochloa crus-galli</i>	7527
HQ395759	<i>Echinochloa crus-galli</i>	7527
AJ310767	<i>Alopecurus myosuroides</i>	7589
AF029895	<i>Triticum aestivum</i>	6993
AF359516	<i>Lolium rigidum</i>	3215
Os05g22940	<i>Oryza sativa</i>	7245
Os10g21910	<i>Oryza sativa</i>	6843
XM003581327	<i>Brachypodium distachyon</i>	7553

Table 3.2. Primer list.

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5'UTR1	TGTCCAAAGGGAGGACGATG
BG750R	ACCAACCTTGTCACCTAGTGCGTT
BG10F	TTGAGGTGGCTCAGCTATGTT
3'UTR1	CATTGTGTTGGATGTGTTGGTATCAT
BGSTARTF	TCATTTGAGGACCTTCTTGACTTCC
BGSTOPR	TCATTTGAGGACCTTCTTGACTTCC
PV2-SBFI-IF-F	CATGATTACGAATTCCTGCAGGAAGCCAACTAAACA
PVUBI2R	CTGCAAAAGAGAACCAGACAACAG
SynACF1IF-F	TCTCTTTTGCAGAGGCCTATG
SynACF1IF-R	ATGCTCGTAGCCGGCCAG
SynACF2IF-F	GGCTACGAGCATCCGGTCAAC
SynACF2IF-R	CCATGGATGTTCTCCACCCCC
SynACF2LEUIF-R	GAGCCATGGAGGTTCTCC
SynACF3IF-R	AATGTTTGAACGAGGCCTTCA
SynACF3-IF-F	GGAGAACATCCATGGCTCCGC
SynACF3LEUIF-F	GAACCTCCATGGCTCCGC
NOS AMP F	CGTTCAAACATTTGGCAATAAAGTTTC
NOS-MAUBI-IF-R	ACATGACACCGCGCGGATAATTTATCCTAGTTTG
BGSTART-IF-F	TCTCTTTTGCAGGATATCATGGGTTCCACACTTGCCC
BGSTOP-IF-R	AATGTTTGAACGAGGCCTTCATTTGAGGACCTTCTTGAC
BG1781LEUF	AGAACTTACAGGGAAGTGCTGCTATTG
BG1781LEUR	TTCCCTGTAAGTTCTCCACACCTAGTC
SYNACF	GAGGGGCTTCAGCTACATCTACCTGGGG
SYNACR	TGAACAGTGGGAGGCCCT
VIRGF	AGGTGAGCCGTTGAAACACG
VIRGR	TCACCTGCCGTAAGTTTCACCT
BG2921F	TTGAGCCTCTGATGAGCCTAC
BG3300R	GTTCCCTTGCAATGCTTGTGCG
BG4880F	TGGGGCACTCCTATAATTCCTATG
BG6300R	CTCCCTGGAGTGCTGCTTTCA
HYG370F	GCGGCCGATCTTAGCCAGACG
HYG720R	GCCAACCACGGCCTCCAGAAG

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Table 3.3. Hygromycin- and sethoxydim-resistant creeping bentgrass events selected.

Construct	Inoculated Calli	Hygromycin events	Sethoxydim Events <sup>†</sup>
		----- # -----	
Bentgrass ACCase	500	45	0
Bentgrass ACCase w/1781LEU	500	63	0
Modified ACCase	500	39	0
Modified ACCase w/1781LEU	500	46	6
Pcambia 1305.2	500	37	0

<sup>†</sup>Seth. events were selected from Hyg. events.

Table 3.4. Percent injury and percent green cover of transgenic events and control ‘Penn A-4’ in response to various concentrations of sethoxydim.

Genotype	Penn A4	Event 1	Event 2	Event 3	Event 4	Penn A4	Event 1	Event 2	Event 3	Event 4
g a.i. ha <sup>-1</sup>	Injury (%) 14 DAT					Green Cover (%) 14 DAT				
0	0	4	1	0	0	58	62	67	64	66
0	6	5	4	2	1	40	59	64	66	62
100	27	8	6	5	4	30	53	56	53	60
200	41	12	9	5	4	25	47	50	49	59
400	69	15	10	6	6	5	43	46	50	52
800	100	14	9	9	9	0	41	42	44	57
1600	100	13	9	6	5	0	42	46	51	47
3200	100	22	12	10	8	0	34	50	48	45
g a.i. ha <sup>-1</sup>	Injury (%) 21 DAT					Green Cover (%) 21 DAT				
0	10	14	5	5	8	59	64	66	68	71
50	31	14	7	6	6	39	62	59	66	63
100	68	22	20	10	9	18	56	59	63	63
200	84	15	19	33	17	17	60	48	48	59
400	100	39	34	40	21	0	44	47	43	52
800	100	52	45	55	41	0	32	32	32	46
1600	100	49	57	47	46	0	31	36	39	34
3200	100	94	54	49	52	0	10	35	33	33
g a.i. ha <sup>-1</sup>	Injury (%) 28 DAT					Green Cover (%) 28 DAT				
0	3	15	1	1	3	62	60	71	79	68
50	26	10	2	2	1	46	57	66	73	73
100	72	20	2	14	5	17	53	67	71	68
200	89	17	14	26	8	13	60	61	59	64
400	100	28	24	25	16	0	50	53	55	62
800	100	34	43	56	27	0	45	43	41	61
1600	100	48	54	59	57	0	40	38	39	39
3200	100	97	66	65	63	0	3	35	23	34

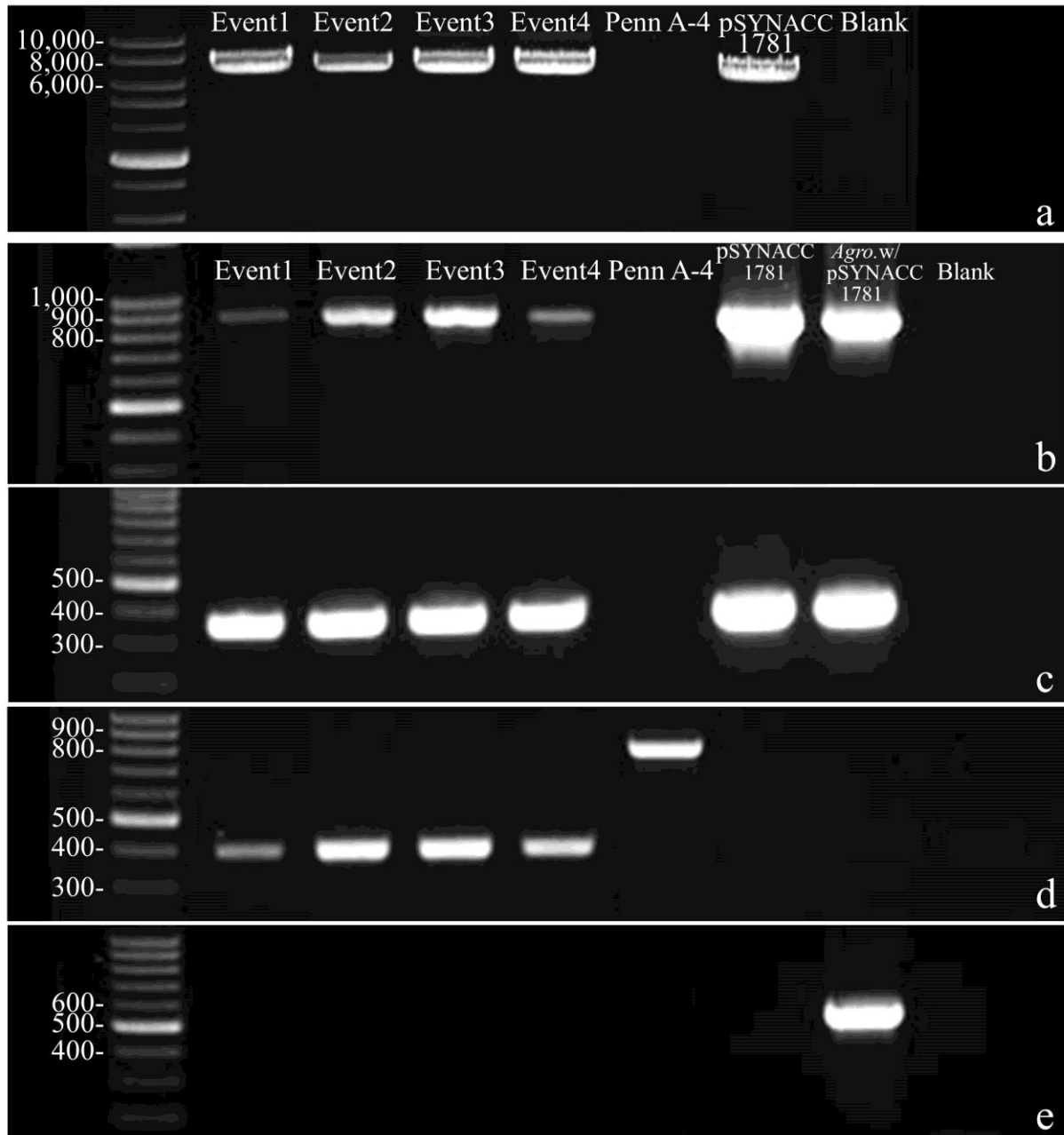


Figure 3.1 PCR analyses of transgenic events. PCR on genomic DNA from transgenic events show the transgene is intact (a). Reverse transcriptase PCR shows transcription of the modified transgene (b) and the hygromycin-resistance gene (c). Reactions looking for *Agrobacterium* and genomic DNA contamination were negative (d) and (e), respectively. The plasmid used for transformation (pSYNACC1781) and *Agrobacterium* with the plasmid were used for both positive and negative controls. Genomic DNA from the control ('Penn A-4') was used as a positive and negative control.

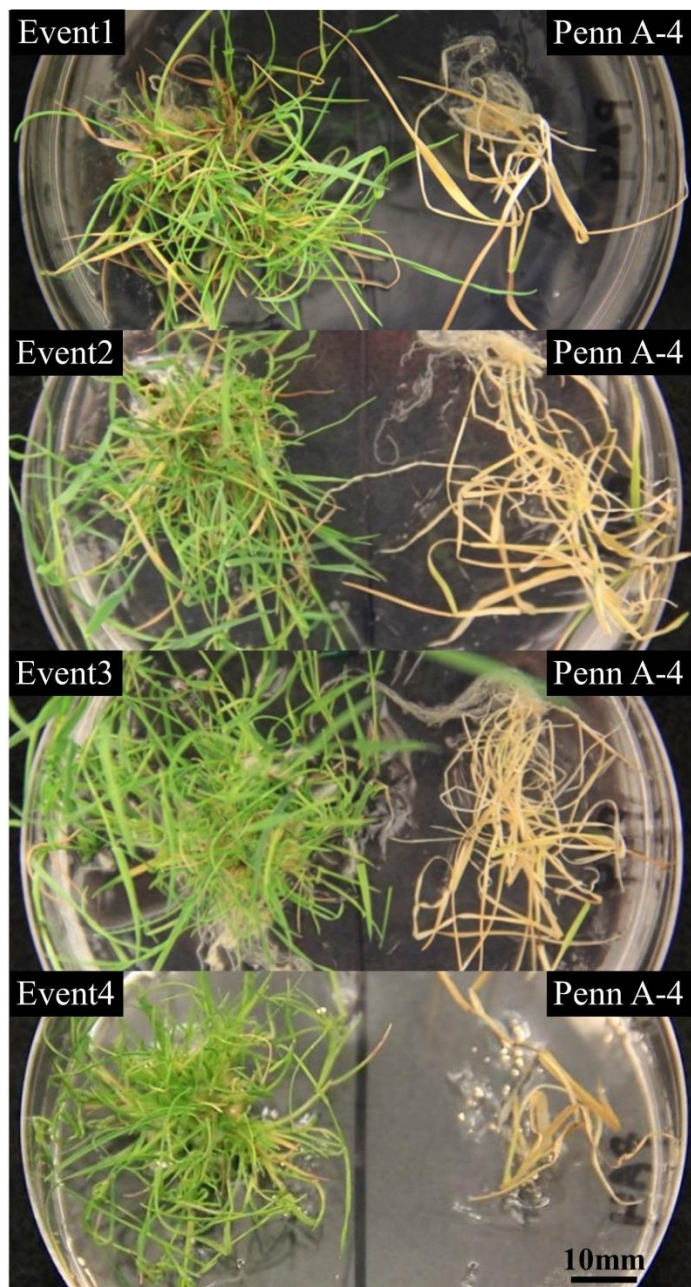


Figure 3.2. In vitro response of transgenic events to sethoxydim compared to 'Penn A-4' control. Transgenic events 1-4 were placed on MS basal medium containing 0.5  $\mu$ M sethoxydim. The untransformed 'Penn A-4' was placed on the same plate for comparison. Plants were evaluated after three weeks.

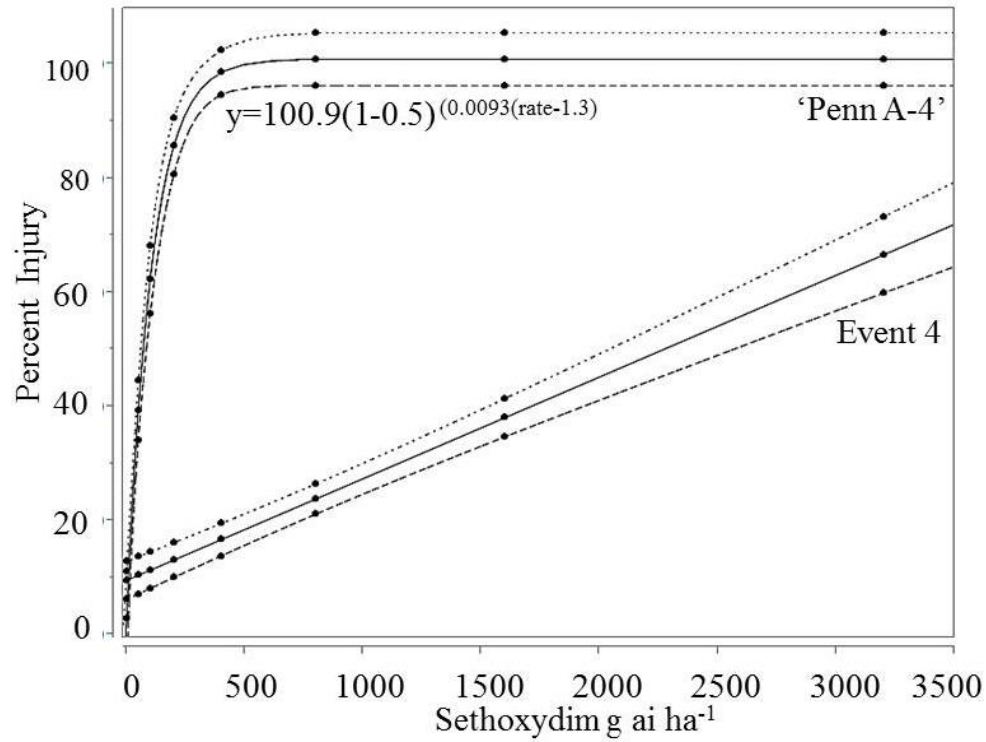


Figure 3.3. Percent Injury of plants from 'Penn A-4' and transgenic event four in response to foliar applied sethoxydim 28 days after treatment. Five replicate plants were visually assessed for injury for each genotype at each rate. Data for 'Penn A-4' was fitted to a Misticich model and data for event four was fitted to a linear model.

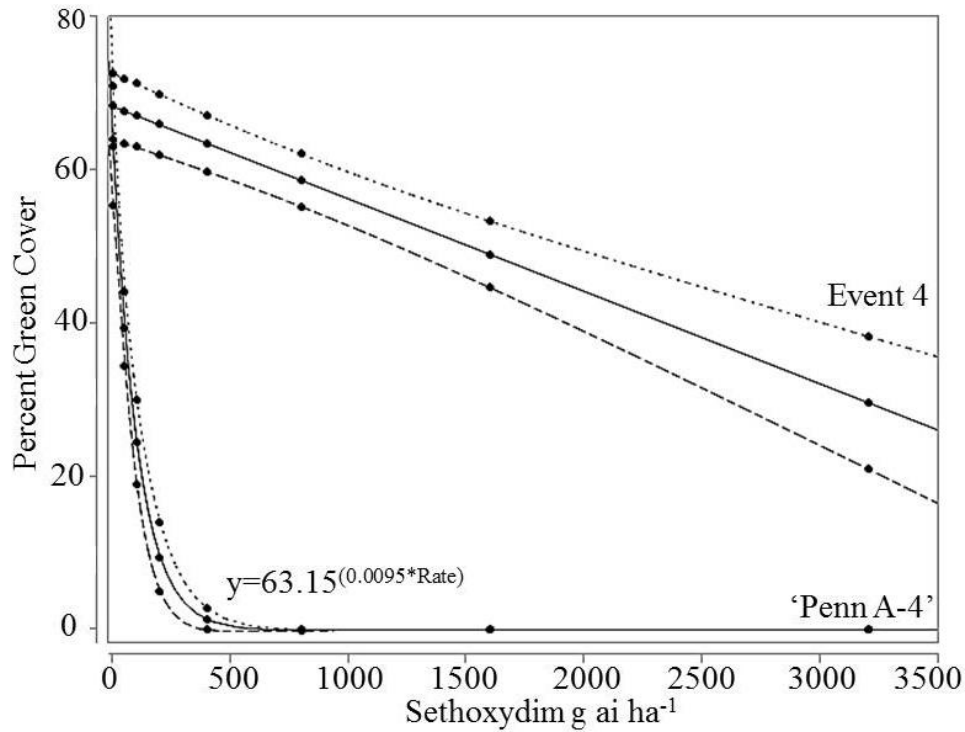


Figure 3.4. Percent green cover of plants from 'Penn A-4' and transgenic event four in response to foliar applied sethoxydim 28 days after treatment. Photos of five replicate pots for each genotype at each rate were analyzed SigmScan Pro 5.0 for percent green cover. Data for 'Penn A-4' was fitted to a negative exponential decay model and data for event four was fitted to a linear model.

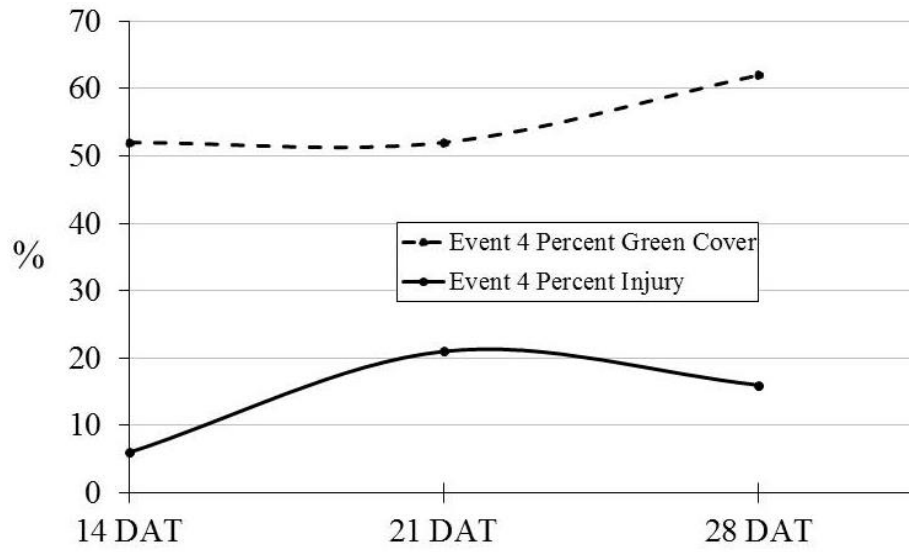


Figure 3.5. Recovery of percent green cover and injury for event four following foliar application of sethoxydim. Five replicate plates were analyzed 14, 21, and 28 dat. Plants were treated with 400 ga ai ha<sup>-1</sup>.

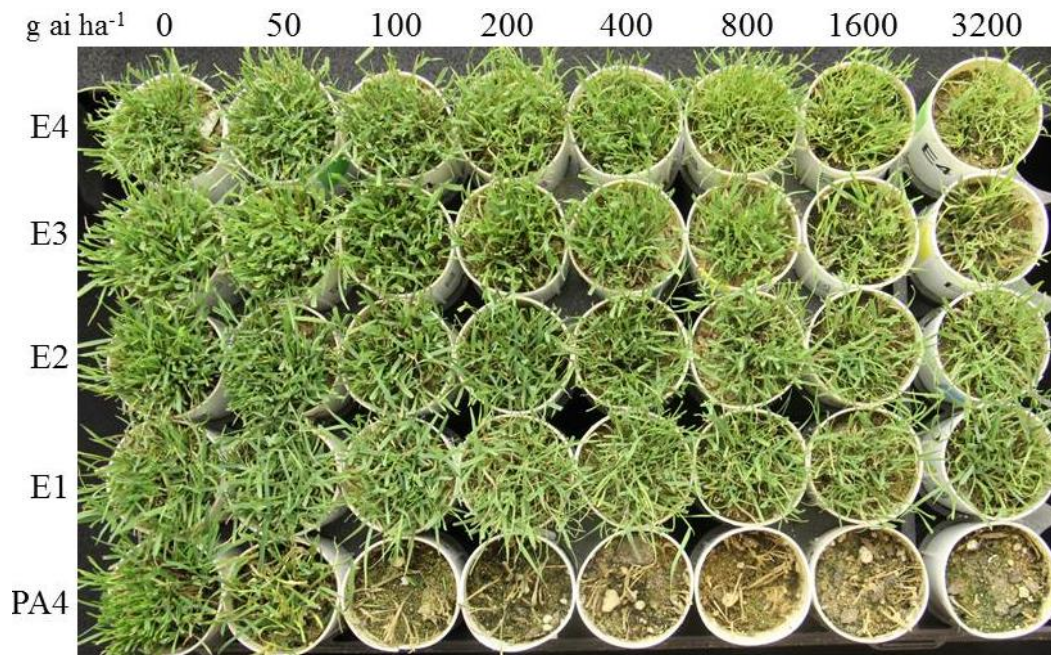


Figure 3.6. Response of transgenic events to varying rates of sethoxydim compared parental type 'Penn A-4' (PA4) 28 days after treatment. Transgenic events have the ACCase transgene modified with synonymous mutations and the 1781 isoleucine to leucine herbicide-resistance mutation.

CHAPTER 4  
EVALUATION OF PLANT-DERIVED TRANSCRIPTIONAL PROMOTERS AND  
TERMINATORS FOR TRANSGENE EXPRESSION<sup>4</sup>

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<sup>4</sup>Heckart, D.L., P.R. Lafayette, R.K. Dawe and W.A. Parrott. To be submitted to *Transgenic Research*.

## **Abstract**

The long terminal repeats (LTR) from the retrotransposons CRM1 and CRM2, and the centromere repeat element CentC were cloned from maize (*Zea mays* L.) and evaluated for promoter activity. The CentC and the CRM LTRs were placed upstream of the GUSPlus reporter gene in forward and reverse orientations. Constructs contained the luciferase reporter gene as an internal standard for transient expression. LTRs and CentC were compared to a 35S positive control, *maize ubiquitin promoter (Zmubi)* promoter as a positive control, and a randomly generated nucleotide sequence of equal length to CentC as a negative control. Possible transcription terminator sequences were cloned from switchgrass (*Panicum virgatum* L.) *Pvubi2*, foxtail millet (*Setaria italica* L.) *acetyl coenzyme A carboxylase (ACCase)*, wheat (*Triticum aestivum* L.) *ACCase*, maize *ACCase*, and rice (*Oryza sativa* L.)  $\beta$ -*tubulin* and *catalase*. Sequences replaced the NOS terminator (T-NOS), controlling GUSPlus transcription. Promoter and terminator sequences were evaluated by bombardment of rice callus tissue. Transient assays were performed by measuring MUG concentration and correcting luciferase expression. When CRM1 and CRM2 were used as promoters, GUS expression was 49% and 57% when compared to 35S, respectively. Surprisingly, promoter activity of the centromeric repeat element, CentC, was 87% of 35S. Among the terminator sequences evaluated, GUS expression was highest with the switchgrass *Pvubi2* 3'UTR and was not significantly different from T-NOS.

## **Introduction**

The centromere was once thought to be transcriptionally silent; however, it is now known that sequences in the centromere are actively transcribed (Topp et al., 2004). The centromere is predominantly made up of non-coding sequences that are necessary for proper cell division. One non-coding sequence, CentC, is a tandem repeat element in maize (*Zea mays* L.) that forms long

arrays interspersed with retrotransposons, specifically CRM (Ananiev et al., 1998, Zhong et al., 2002). Centromeric proteins not only interact with genomic sequence of CentC and CRMs, but with the RNA sequences from these elements as well (Topp et al., 2004).

Retrotransposons are classified as long terminal repeat (LTR), including CRMs, or non-LTR retrotransposons. LTR retrotransposons have terminal repeats that can range from a few hundred base pairs to 5 kb (Kumar and Bennetzen, 1999). Non-centromeric LTRs are known to contain promoters and terminators responsible for transcription of proteins needed for transposition (Kumar and Bennetzen, 1999). Topp et al (2004) suggested that CRM retrotransposons may have their own promoter elements and may cause read-through transcription of CentC, and suggested that the activity was in the LTR themselves. In the present study, functionality of the LTR region from maize retrotransposons, CRM1 and CRM2, was evaluated for promoter activity. Additionally, the CentC sequence was analyzed for promoter activity because transcripts have been found with CRM transcripts.

Transcription terminator sequences have been shown to have a significant impact on levels of gene expression (Ingelbrecht et al., 1989, Mitsuhashi et al., 1996, Nagaya et al., 2010). The 3' untranslated region (3'UTR) possesses *cis* elements that are required for stabilization of the mRNA molecule, processing into mature mRNA, efficient translation, and transport from the nucleus to the cytoplasm. Plant 3'UTR structure is highly variable and *in silico* identification of terminators is difficult (Loke et al., 2005), making functional analysis necessary. Nagaya et al. (2010) found a heat shock protein (HSP) terminator from Arabidopsis led to 2.5-fold higher reporter gene expression levels relative to T-NOS. The HSP terminator increased transcription with multiple promoters and reporter genes, indicating function of the terminator was independent of other sequences.

The second objective of the present study was to identify plant-derived terminators able to increase promoter effectiveness. Possible terminator sequences were cloned from maize, foxtail millet (*Setaria italica*), switchgrass (*Panicum virgatum*), wheat (*Triticum aestivum*), and rice (*Oryza sativa*). Functionality of each sequence was determined by analyzing their effect on expression of the GUSPlus™ (Cambia, Canberra, AU) reporter gene in rice tissue using a transient assay system.

## **Methods and materials**

### ***Plant material***

Seeds of greenhouse-grown Rice cv Taipei 309 were dehusked and surface-sterilized with 70% ethanol for 2 min then placed in 60% v/v Clorox with several drops of tween 20, rinsed three times with sterile water, and placed on modified NB medium with 500 mg L<sup>-1</sup> (Chen et al., 1998). The media used in this experiment were solidified with 2.5 g L<sup>-1</sup> Gelzan™ (Caisson Laboratories, North Logan, UT) and brought to pH 5.8. Plates were sealed in Petri dishes with 3M Micropore™ tape (St. Paul, MN) and callus tissue was transferred every three weeks. The calli were placed in a dark growth chamber at 25 °C.

### ***Vector construction***

Transient expression vectors were constructed by replacing the hygromycin resistance gene, *hptII*, in pCambia vector 1305.2 (Cambia, Canberra, AU) with the firefly luciferase (LUC) gene (Figure 4.1). *LUC* is used as an internal control for normalization of gene expression (Leckie et al., 1994). The vector was first digested with the *XhoI* to release *hptII*. *LUC* was amplified from pHLucGWgus (Mann et al., 2011) with the primer set 1305:luc-F/ 1305:luc-R (Table 4.1). In-Fusion™ (Clontech Laboratories Inc., Mountain View, CA) cloning methodology was used to insert *LUC* in place of *hptII*. LTR sequences were cloned from *Zea*

*mays* genotype *HI II*. DNA was extracted using the CTAB method as described by Lassner et al. (1989). Phusion<sup>®</sup> (New England Biolabs, Inc. Ipswich, MA) proofreading DNA polymerase was used to amplify all fragments used in this study. CentC and CRM sequences were amplified using primers listed in supplementary Table 4.1. CentC and CRM sequences were compared in forward and reverse orientation to determine directionality of promoter activity. When amplifying forward and reverse CentC and CRM fragments, primers were amplifying similar sequences that differ by a few SNPs (data not shown). To ensure that forward and reverse oriented sequences were identical; sequences were first amplified in forward orientation then used as a template for reverse oriented sequences. The 35S promoter driving GUSPlus<sup>™</sup> was replaced with CentC, a CRM, maize ubiquitin promoter (*Zmubi*) or a randomly generated sequence 926 bp in length, equal to the length of CentC. The random sequence was synthesized by Genart (Life Technologies, Grand Island, NY). *Zmubi* was used as an additional positive control as it has been shown to drive high levels of GUSPlus expression in rice (Mann et al., 2011). *Zmubi* was cloned from pAHC25 (Christensen and Quail, 1996). pCambia1305.2:LUC was digested with *HindIII* and *NcoI* and the CRMs and CentC were inserted using In-Fusion<sup>™</sup> cloning. An initial experiment was performed to determine if the CRMs and CentC had promoter activity and if promoter activity was dependent on orientation. The CRM and CentC constructs were re-bombarded with terminator analysis constructs and the additional positive control promoter, *Zmubi*.

The 3' UTRs chosen for analysis are from sequences listed in Table 4.1. mRNA 3'UTRs from rice *catalase* (265 bp) and  *$\beta$ -tubulin* (197 bp) were compared to 500 bp of the corresponding genomic sequence. The genomic fragment had the same sequence as the mRNA 3'UTR with additional sequence to 500 bp. DNA extraction and fragment amplification followed

procedures as described earlier for LTR amplification. Additionally, 3'UTRs from switchgrass *ubiquitin 2*, wheat *acetyl coenzyme A carboxylase (ACCase)*, foxtail millet *ACCase*, and maize *ACCase* were cloned to characterize their ability to act as a transcription terminator.

Due to a lack of restriction sites available to remove the T-NOS terminator for insertion of possible terminators, the LUC/GUSPlus construct was removed without T-NOS and inserted into pBluescript II KS(+) (Alting-Mees and Short, 1989). Next, p1305.2:LUC was digested with *SacII* and *BstEII*. pBluescript II KS(+) was digested with *SacII* and *ApaI* to insert LUC/GUSPlus fragment. The fragment was cloned into the vector using T4 ligase (Thermo Scientific, Pittsburgh, PA) as per the manufacturer's protocol. Insertion of the putative terminator sequences occurred between the *PmlI* and *ApaI* restriction sites. Digestion with *PmlI* results in the loss of the last six base pairs of the GUSPlus™ gene. InFusion™ cloning was used for insertion of the terminator sequences and the six base pairs lost were added between the required 15-bp overhang and beginning of the terminator sequence (Table 4.1). Additional constructs were made with a combination of promoters and terminators. Either *S. italica* ACCase 3' UTR or Pvubi2 3'UTR were used with CRM2 and Zmubi promoter sequences. The *S. italica* ACCase UTR and Pvubi2 UTR constructs were modified by removing the 35S and replacing it with *Zmubi* or CRM2 using the previously described procedures.

### ***Transient expression assays***

Biolistic transformation and transient expression assays followed procedures as described by Mann et al. (2011). Data were analyzed from three bombardments of each construct. Gene expression was analyzed by quantifying GUS activity by measuring MUG concentration and normalized by measuring luciferase activity. For each bombardment, ~10 calli were analyzed by histochemical staining for GUS as described by Jefferson (1987). Calli were placed in a 1.5 mL

microcentrifuge tube with 150  $\mu$ L GUS staining solution followed by 30 min of vacuum infiltration. Data for relative expression were tested for normal distribution using JMP<sup>®</sup> PRO (Version 11.0.0 SAS Institute Inc., Cary, NC). Data were transformed with a square root transformation prior to ANOVA. Differences between samples within each experiment were determined using a protected Fischer's Least Significant Difference (LSD) test ( $p = 0.05$ ).

## Results

The initial experiments comparing CentC, CRM1, and CRM2 in sense and antisense orientations showed promoter activity in the sense orientation (Figure 4.2). In the antisense orientation, promoter activity of the CentC, CRM1, and CRM2 LTRs was not significantly different from the random sequence. Surprisingly, the centromeric repeat, CentC, drove expression of GUSPlus at 61% of the expression level of 35S. Expression controlled by CentC and CRM1 was approximately the same, and both were significantly lower than that of 35S. The LTR regions from CRM2 drove GUSPlus expression at 26% of GUSPlus expression driven by 35S. CRM LTRs and CentC in the forward orientation were re-bombarded with terminator analysis constructs, and the additional positive control *Zmubi*, so that statistical comparison could be made. Results differed slightly from the initial experiment to determine directionality (Figure 4.3). In the second experiment, CentC drove expression of the GUSPlus reporter gene at 85% of the expression level of 35S. CentC expression level was not significantly different from 35S, but was significantly lower compared to *Zmubi*. In contrast, GUSPlus expression driven by the CRM1 and CRM2 were just 49% and 57% of 35S. Gus expression driven by the random sequence was 20% of the 35S promoter, representing the background levels.

Evaluation the Pvubi2 3'UTR as a transcription terminator revealed that it was not significantly different from the NOS terminator (Figure 4.4). The-190 bp 3'UTR cloned from

rice *β-tubulin* was compared to 500 bp of the genomic 3'UTR of *β-tubulin* and there were no significant differences between the two (Figure 4.4, 4.5). The *β-tubulin* UTRs were not significantly different from the T-NOS and Pvubi2 terminators. Similarly, the 265 bp 3'UTR from the rice catalase mRNA sequence was compared to 500 bp of the genomic 3'UTR. Both constructs with catalase UTRs expressed GUSPlus at 73% of the T-NOS control. Expression from all three of the 3'UTRs from ACCase mRNA sequences from foxtail millet, maize, and wheat were significantly lower than that of T-NOS at ~40-50%.

To explore if promoter and terminator activity are independent of each other, two promoters and two terminators were used in combination. The Zmubi promoter and CRM2 LTR driving GUSPlus expression were used with either the *S. italica* ACCase 3'UTR or the Pvubi2 3'UTR. Zmubi with *S. italica* ACCase 3'UTR expressed GUSPlus approximately 30% lower than the constructs with the T-NOS or the Pvubi2 3'UTR; yet there were no significant differences statistically (Figure 4.6, 4.7). In contrast, GUSPlus expression was significantly lower when the CRM2 LTR and the *S. italica* ACCase 3'UTR were used together. GUSPlus expression driven by CRM2 was not significantly different with either the T-NOS or the Pvubi2 3'UTR.

## **Discussion**

The idea that LTR regions from LTR retrotransposons have promoter activity began with the observations of White et al. (1994). Since then, promoter activity of the LTR has been shown in several species. Recently, it was observed that blood oranges show increased anthocyanin production due to promoter activity of LTR retrotransposons (Butelli et al., 2012). Three blood orange accessions were found to have an LTR insertion in the inactive promoter of the *Ruby* transcription factor, up-regulating anthocyanin production. The cultivar, Moro, only has the 3'

LTR portion of the Tsc1 retrotransposon inserted into the promoter region, whereas the cultivar Jingxian had the complete retrotransposon in the antisense orientation. These observations indicate that not only do LTRs have promoter activity, but appear to be bidirectional. The first experiment showed that the CRM LTRs do have promoter activity; however, when placed in the antisense orientation, expression was not higher than that of the randomly generated sequence. Based on previous reports on promoter activity of non-centromeric LTR retrotransposons, it is reasonable to expect that centromeric LTRs would exhibit promoter activity as well. However, observing promoter activity in CentC was not expected, as Topp et al. (2004) hypothesized that CentC transcription was likely due to read-through transcription from a CRM LTR. The present study showed CentC drove higher expression than either LTR at ~80 % of the 35S promoter strength.

Analysis of terminator sequences revealed that 3'UTR from Pvubi2 permitted the highest level of expression of the terminators compared; yet was not significantly different from the NOS terminator. In contrast, all the ACCase 3'UTRs from the three different species resulted in expression significantly lower than that of T-NOS. As discussed earlier, the variable nature of cis elements in plant 3'UTRs makes *in silico* analysis unreliable; therefore, the mRNA 3'UTRs from two rice genes were compared to extended genomic sequences. GUSPlus expression was essentially the same between mRNA 3'UTRs and corresponding 500 bp genomic sequences for both  *$\beta$ -tubulin* and *catalase*. For these examples, the shorter mRNA 3'UTR is sufficient to act as a terminator.

To summarize, expression driven by the strongest promoter, Zmubi, was not significantly affected by strong or weak terminators. In contrast, expression driven by the weak promoter, CRM2, was significantly affected by the use of a weak promoter. These examples provide

evidence that promoter/terminator combinations should be functionally characterized prior to using for transgene expression.

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Table 4.1. Primers for amplification and cloning of promoter and terminator sequences.

CENTCF IF F	CENTC –forward orientation w/InFusion ends	GCAGGCATGCAAGCTTTGATGAGGAC
CENTCF IF R		TTAGTAGTAGCCATGGTGATAAACCC
CENTCR IF F	CENTC-reverse orientation w/InFusion ends	GCAGGCATGCAAGCTTTGATAAACCC
CENTCR IF R		TTAGTAGTAGCCATGGTGATGAGGAC
CRM1F IF F	CRM1-forward orientation w/InFusion ends	CAGGCATGCAAGCTTTGATGC
CRM1F IF R		TGATGTAGCTGAGGTGCCCGA
CRM1R IF F	CRM1-reverse orientation w/InFusion ends	TGATGCGGACATCAACACCAA
CRM1R IF R		CAGGCATGCAAGCTTTGATGT
CRM2F IF F	CRM2-forward orientation w/InFusion ends	GCAGGCATGCAAGCTTTGATGAAGAC
CRM2F IF R		TTAGTAGTAGCCATGGTGATGTGCAC
CRM2R IF F	CRM2-reverse orientation w/InFusion ends	GCAGGCATGCAAGCTTTGATGTGCAC
CRM2R IF R		TTAGTAGTAGCCATGGTGATGAAGAC
RAND IF F	Randomly generated sequence w/InFusion ends	ACCTGCAGGCATGCAAGCTTGCTCATTCCCGACGTCTGAGAG
RAND IF R		CTTAGTAGTAGCCATGGAGTAGTCGACTCGCCGTTC
PV2- PML1-IF -F	Pvubi2 3'UTR w/InFusion ends	CACCATCACCATCACGTGTGAGCCATCGGTCACCGAAGCTGC
PV2-STUI-IF-R		AATGTTTGAACGAGGCCTCTGCAAAAGAGAACCAGACAACAG
ZM-AC-UTR-F	Maize ACCase 3'UTR	TGATACCAACACATCCAACACAATG
ZM-AC-UTR-R		GTATCCGCATTAUACACCCUAAAAGUAC
ZM-PML1-IF-F	Maize ACCase 3'UTR w/InFusion ends	CACCATCACCATCACGTGTGAUGAUACCAACACAUCCAACACAATG
ZM-AC-UTR-IF-R		AAAAGCTGGGTACCGGGCCCTCGAATGCTAATTCAACAACCTTTATTTCAC
TA-AC-UTR-F	Wheat ACCase 3'UTR	TCAGATGATACCAACGCATCCAATTCAG
TA-AC-UTR-R		AGAGGTAATGCACGAGACCATATCCAAGAGC
TA-PML1-IF-F	Wheat ACCase 3'UTR w/InFusion ends	CACCATCACCATCACGTGTGATCAGATGATACCAACGCATCCAATTCAG
TA-AC-UTR-IF-R2		AAAAGCTGGGTACCGGGCCCAGAGGTAATGCACGAGACCATATCCAAGAG
SI-AC-UTR-F	<i>S. italica</i> ACCase 3'UTR	TGAATACCAACACATTCAACACAAT
SI-AC-UTR-R		TAGACTGCTAATTCAACAAGTTTATTACAC
SI- PML1-IF -F	<i>S. italica</i> ACCase 3'UTR w/InFusion ends	CACCATCACCATCACGTGTGATGAATACCAACACATTCAACACAAT
SI-AC-UTR-IF-R		AAAAGCTGGGTACCGGGCCCTAGACTGCTAATTCAACAAGTTTATTACAC
OS-BTUB-IF-F	Rice $\beta$ -tubulin 3'UTR	CACCATCACCATCACGTGTGAGCCACCTCTCTGCATGTT
OS-BTUB-190-IF-R	Rice $\beta$ -tubulin mRNA 3'UTR (190 bp)	CTGGGTACCGGGCCCAATCCACCTCAAGACAATAATGTT
OS-BTUB-500-IF-R	Rice $\beta$ -tubulin gDNA 3'UTR (500 bp)	CTGGGTACCGGGCCCATATAAAGAATCGGATGATTACAGAACTAATCT
OS-CAT-IF-F	Rice catalase 3'UTR	CACCATCACCATCACGTGTGAGGAGATCGATCAGCGTTGCAA
OS-CAT-265-IF-R	Rice catalase mRNA 3'UTR (265 bp)	GCTGGGTACCGGGCCCAATAATTAATTTACTGTATATAAATTATTGAT
OS-CAT-500-IF-R	Rice catalase gDNA 3'UTR (500 bp)	GCTGGGTACCGGGCCCTATGTGGGATCTATGTGGGTCC
1305:luc-F	Luciferase w/ InFusion ends	ATCTATCTCTCTCGAGTGATAACAGCATGGAAGACGC
1305:luc-R		TCTGCGAAAGCTCGAGATGCTTACACGGCGATCTTTC



Figure 4.1. Transcription regulatory sequence evaluation construct. Sequences evaluated for promoter replaced the 35S promoter driving GUSPlus™ expression. Sequences analyzed for terminator efficiency replaced the GUSPlus NOS terminator.

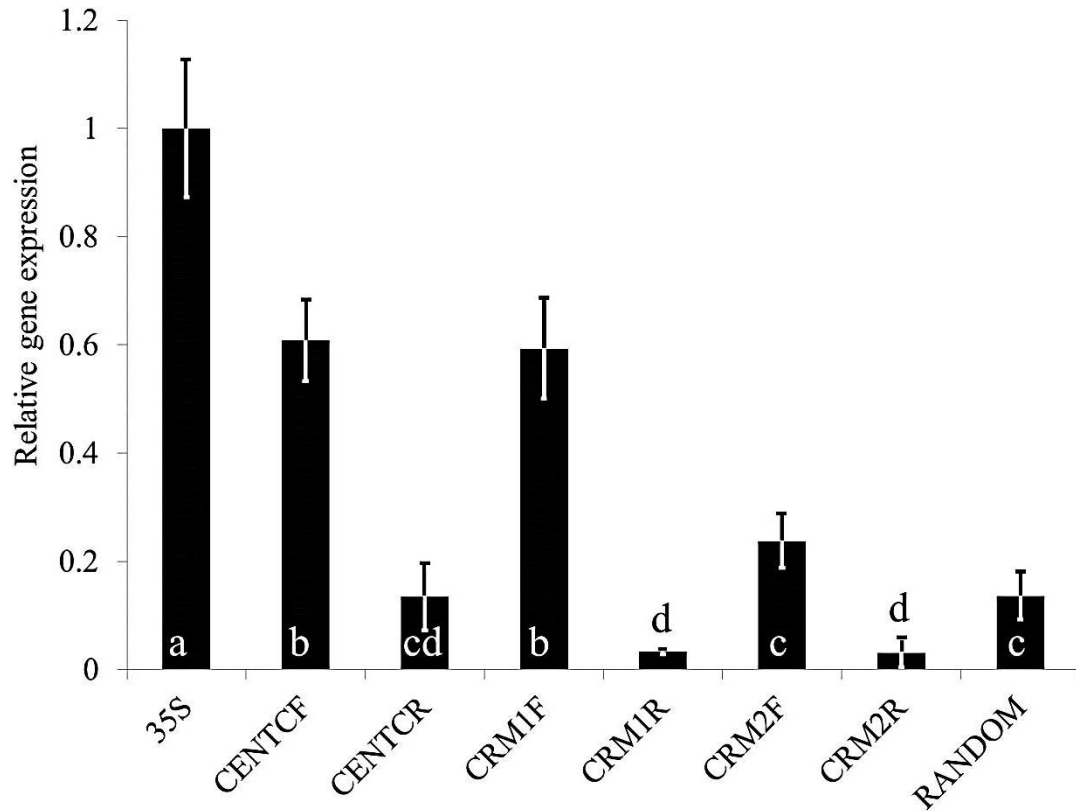


Figure 4.2. Directional promoter activity of CRM LTRs and CentC in rice callus tissue compared to the 35S. Gus expression values for each construct were normalized by the luciferase internal control. Gene expression is relative to the *CaMV* 35S control. Bars represent the mean for three independent replicates. Error bars represent standard error. Treatments that share the same letter are not significantly different as calculated by LSD ( $P \leq 0.05$ ).

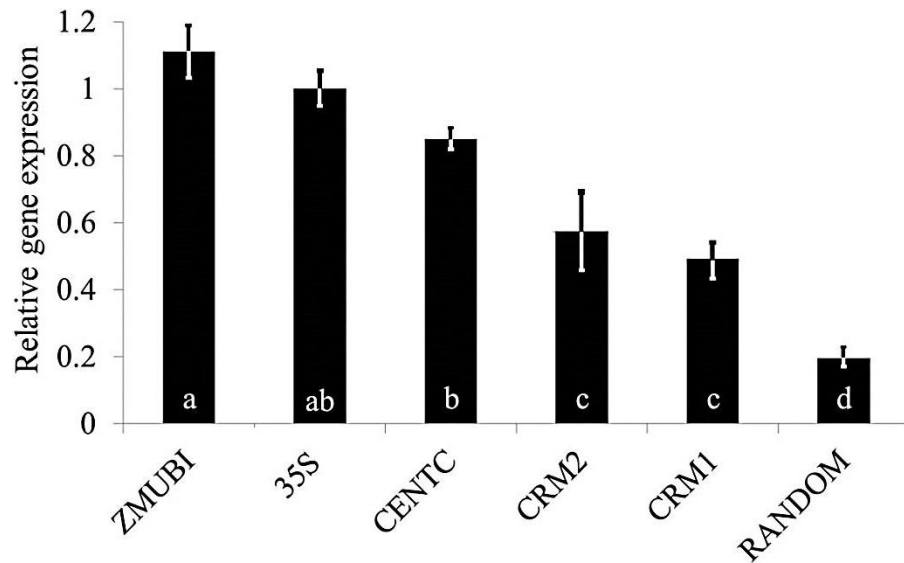


Figure 4.3. Analysis of promoter activity of CRM LTRs and centromeric repeat CentC in rice callus tissue compared to *Zmubi* and *35S*. Gus expression values for each construct were normalized by the luciferase internal control. Gene expression is relative to the *CaMV 35S* control. Bars represent the mean for three independent replicates. Error bars represent standard error. Treatments that share the same letter are not significantly different as calculated by LSD ( $P \leq 0.05$ ).

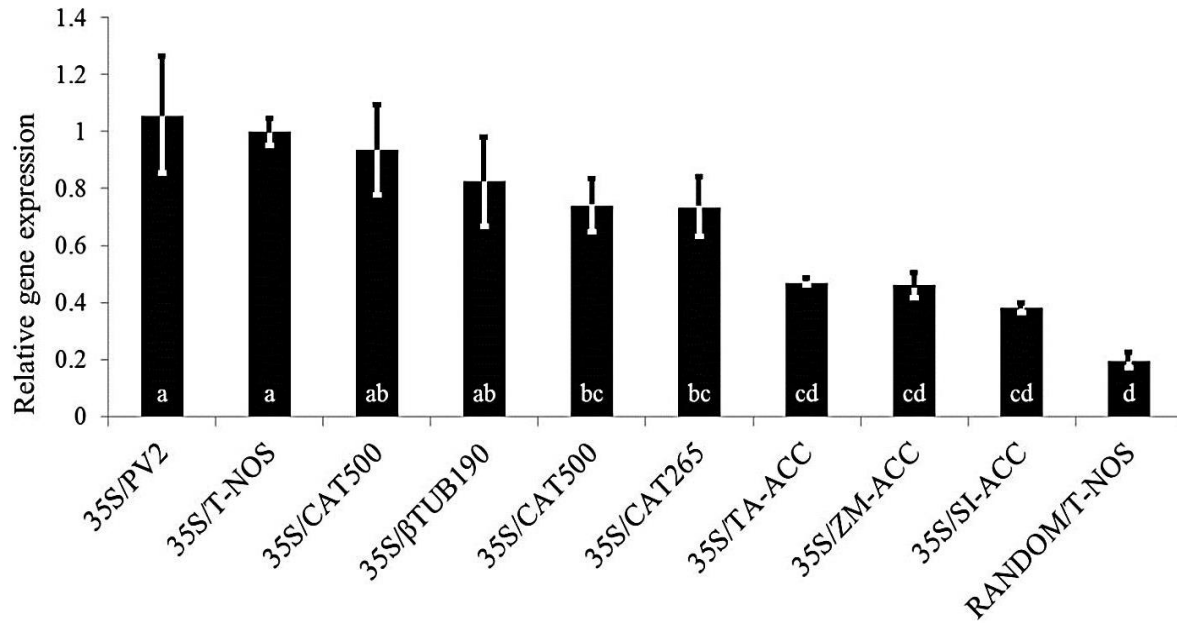


Figure 4.4. Analysis of putative terminators in rice callus tissue compared to T-NOS. Gus expression values for each construct were normalized by the luciferase internal control. Gene expression is relative to the T-NOS control. Bars represent the mean for three independent replicates. Error bars represent standard error. Treatments that share the same letter are not significantly different as calculated by LSD ( $P \leq 0.05$ ).

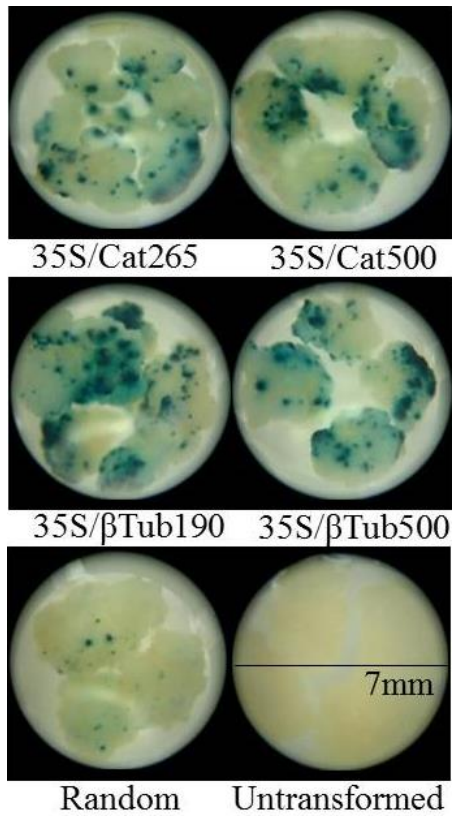


Figure 4.5. Histochemical staining of GUS in rice callus tissue of ‘Taipei 309, comparing expression levels controlled by mRNA or extended 3’UTRs. Rice catalase 265 bp 3’UTR from mRNA (Cat265) was compared to the corresponding genomic sequence extended to 500 bp (Cat500). The rice  $\beta$ -tubulin mRNA 190 bp 3’UTR ( $\beta$ Tub190) was compared to the corresponding 500 bp genomic sequence extended to 500 bp ( $\beta$ Tub500). Terminators were compared to a negative control with GUSPlus™ driven by a randomly generated sequence 926 bp long and untransformed calli.

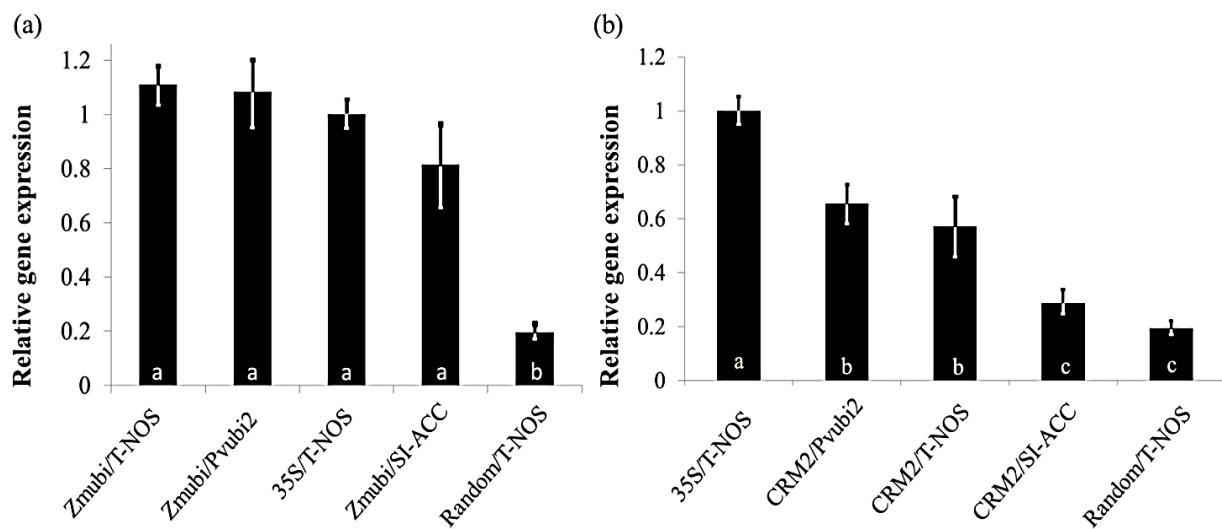


Figure 4.6. Comparison of the Zmubi and CRM2 promoters with different terminator sequences. Gus expression values for each construct were normalized by the luciferase internal control. Gene expression is relative to the T-NOS control. Bars represent the mean for three independent replicates. Error bars represent standard error. Treatments that share the same letter are not significantly different as calculated by LSD ( $P \leq 0.05$ ).

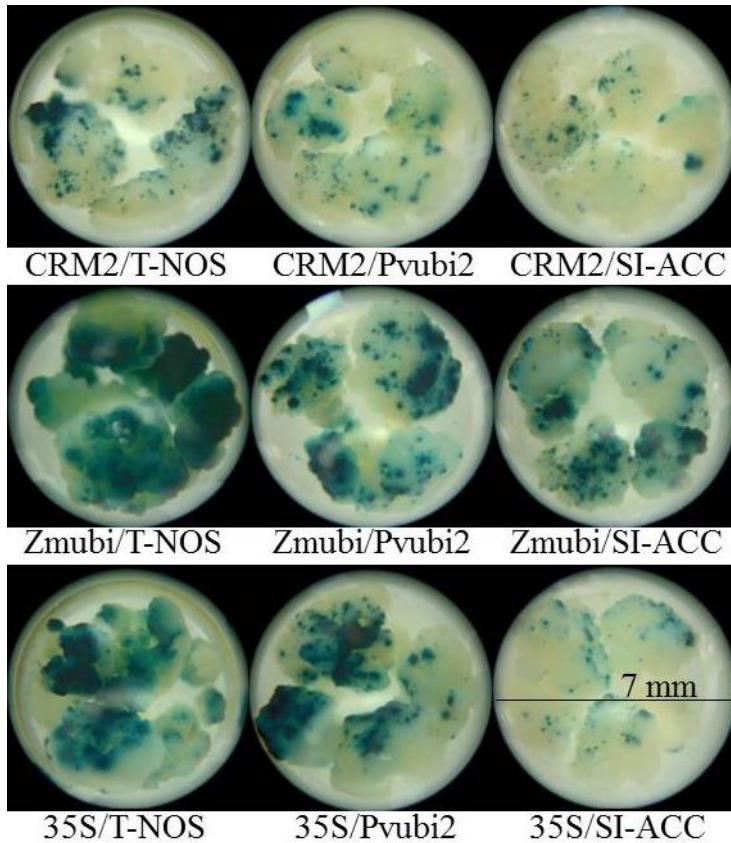


Figure 4.7. Histochemical staining of GUS in rice callus tissue comparing terminator efficiency in strong and weak promoters. The strong promoter, the maize (*Zea mays* L.) ubiquitin promoter (*Zmubi*) was paired with the strong terminator from the switchgrass (*Panicum virgatum* L.) ubiquitin 2 terminator (*Zmubi/Pvubi2*) or the weak terminator from the foxtail millet (*Setaria italica* L.) acetyl coenzyme A carboxylase (*ACCase*) (*Zmubi/SI-ACC*). The weak promoter, the LTR region from the maize retrotransposon CRM2, was paired with the strong terminator (*CRM2/Pvubi2*) and the weak terminator (*CRM2/SI-ACC*). Constructs were compared to control constructs with the *Zmubi* or CRM2 with the nopaline synthase terminator (*Zmubi/T-NOS*)(*CRM2/T-NOS*) and constructs with the 35S promoter with the *Pvubi2* or *SI-ACC* terminator (*35S/Pvubi2*)(*35S/SI-ACC*).

## CHAPTER 6

### CONCLUSION

Post-transcriptional gene silencing (PTGS) mechanisms prevent reliable overexpression of genes that share a high level of identity with a native gene. There are many possibilities for improving crop plants if overexpression of native enzymes was possible. Since PTGS mechanisms rely on sequence identity between the transgene and introduced gene, modification with synonymous mutations appears to be a promising method to overexpress a native gene while reducing identity to the native gene sequence, yet there are few examples of using synonymous gene for this use. The examples that do exist do not show OE of a native enzyme. Thus, the purpose of this research is to OE a native enzyme to obtain herbicide resistance (HR) in creeping bentgrass.

In grasses, specific point mutations in *acetyl coenzyme A carboxylase* (*ACCase*) confer resistance to ACCase-inhibiting herbicides. Ten known mutations confer resistance to two groups of ACCase inhibitors differentially. If ACCase could be overexpressed, two HR mutations could be included and transgenic plants could be resistant to two groups of herbicides by transformation with a single enzyme. A proof-of-concept experiment was designed to OE ACCase in creeping bentgrass by inclusion of synonymous mutations and a single HR mutation. Prior to designing an ACCase with synonymous mutations, an in vitro selection experiment was conducted to select ACCase-inhibitor resistance to compare to transgenic events developed (Chapter 3). Selection occurred on medium containing the ACCase inhibitor, sethoxydim. Four resistant events were obtained and sequencing showed that each of the events possessed a

mutation in the 1781 amino acid (aa) codon that has previously been shown to confer resistance to ACCase-inhibitors. The whole plant dose response showed resistance to high levels of sethoxydim and showed cross resistance to other ACCase-inhibiting herbicides.

Following in vitro selection of sethoxydim-resistant events, a creeping bentgrass gene was designed with synonymous mutations and OE in creeping bentgrass (Chapter 4). The native sequence was cloned to use as a template for synonymous mutation gene design. The 6.88 kb sequence was cloned by designing primers based on alignments of ACCase genes from other species. Four constructs were made and included the wild-type (WT) gene and gene modified with synonymous mutations with or without the 1781 mutation conferring. Six sethoxydim-resistant-transgenic events were obtained and were only from calli transformed with the modified gene with the 1781 aa mutation. PCR reactions on cDNA from transgenic events showed the transgene was transcribed. A dose response was conducted on plants from the first four events regenerated and each line showed high levels of resistance compared to the untransformed controls. Each event was sequenced to ensure that resistance did not originate from a mutation in a native gene. This experiment shows that OE of a native enzyme is possible with introduction of synonymous mutations. In addition, OE of ACCase with the HR mutation is a novel HR trait.

It is possible that a transgenic event with a native gene modified with synonymous mutations would not be considered as a regulated event by government agencies. Regulation on this type of gene design may be debatable; however, there are more transgene components to consider. Commonly used transcription regulatory sequences include the *cauliflower mosaic virus* (*CaMv*) 35S promoter and nopaline synthase (T-NOS) terminator. Both sequences are from plant pathogens and transgenic plants with these sequences are regulated. Grass-derived sequences were evaluated for promoter and terminator activity to replace commonly used plant

pathogen sequences (Chapter 5). Long terminal repeats (LTRs) from maize (*Zea mays* L.) centromere-specific CRM LTR retrotransposons were evaluated for promoter activity by fusion to the GUSPlus™ reporter gene. The luciferase gene was used as an internal control. The centromeric repeat, CentC, was also tested for promoter activity because transcripts have been found with CRM transcripts. Surprisingly, the CentC element showed the highest level of promoter activity ~87% compared to the 35S promoter, where the CRMs were ~60% of 35S promoter activity. This experiment showed that CentC may be used as a promoter because it was not statistically different from 35S. Possible transcription terminator sequences were tested to replace T-NOS. Sequences were cloned from switchgrass (*Panicum virgatum* L.), foxtail millet (*Setaria italica* L.), wheat (*Triticum aestivum* L.), and rice (*Oryza sativa* L.). The Pvubi2 3'UTR from *P. virgatum* was the only 3'UTR that was not significantly different from T-NOS and could be used as a replacement.

In summary, OE of native enzymes has been shown to work with the OE of ACCase in creeping bentgrass. ACCase was overexpressed with a mutation conferring resistance to ACCase inhibitors. Transgenic bentgrass and in vitro selected HR plants both showed high levels of resistance to the ACCase inhibiting herbicides. Plant-derived TRS sequences were also identified that may be used to replace regulated-plant-pathogen-derived TRS.