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Synonymous mutation gene design to overexpress ACCase in creeping bentgrass to obtain resistance to ACCase-inhibiting herbicides

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Abstract 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 mutations that do not cause amino acid changes, known as 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 (WT) 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 possible.

Keywords Acetyl coenzyme A carboxylase (ACCase) · Aryloxyphenoxypropionate (APP) · Cyclohexandione (CHD) · Herbicide-resistance · Posttranscriptional gene silencing · Sethoxydim resistant · Synonymous mutation · Synthetic gene design

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Introduction

Gene overexpression has potential to improve numerous traits in crop species. Some examples include

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modification of cell wall composition for improved biofuel production (Li et al. 2014), improved phytoremediation (Antosiewicz et al. 2014), improved nitrogen use efficiency (Thomsen et al. 2014), improved tolerance to abiotic stresses (Cabello et al. 2014; Delorge et al. 2014; Shi and Chan 2014) including salt tolerance (Yamaguchi and Blumwald 2005; Shi et al. 2003), and anaerobic stress (Vartapetian et al. 2014).

Arguably, the most well-known attempt to overexpress a gene was the introduction of an extra chalcone synthase gene in petunia so as to darken the flower color (Napoli et al. 1990). Instead of darker flowers, however, the flowers or sectors of flowers were white, due to a phenomenon that was later termed cosuppression (Metzlaff et al. 1997). Cosuppression is now understood to be a form of sense-post-transcriptional gene silencing (S-PTGS) (Jorgensen et al. 2006) and has been a hurdle for reliable overexpression of native genes.

Silencing results from processing 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 $\sim$21 bp of contiguous identity are required between native and introduced genes for silencing mechanisms to degrade transcripts from both. Thus, to obtain reliable expression of an introduced gene, the sequence identity of the introduced gene to any native gene must be low.

Transgenic expression of heterologous genes is commonly used because the chosen transgene and native gene sequence similarity is below the threshold needed for S-PTGS. Nevertheless, the use of native genes modified to have a desired mutation could have advantages. Introducing heterologous genes may circumvent S-PTGS mechanisms; however, there may be altered enzymatic function (Fang et al. 2010). Transgenic expression of viral suppressors of PTGS has been used to bypass PTGS by down-regulating silencing mechanisms; however, plants became susceptible to viral infection (Anandalakshmi et al. 1998) and commonly show developmental abnormalities (Chapman et al. 2004; Alvarez et al. 2008). Thus alternative strategies must be used.

Since cosuppression depends on sequence similarity between a transgene and native gene, modifying transgenes with synonymous mutations that eliminate sequence identity while preserving amino acid identity has been attempted to bypass S-PTGS. Tomato transformed with a tomato *phytoene synthase* modified with synonymous mutations did not show silencing (Drake et al. 2001). Kumar et al. (2006) used a similar method to rescue silenced phenotypes. The gene of interest, *salicylic acid-binding protein*, was previously silenced using a hairpin construct. The phenotype was rescued by expressing the same protein with a gene sequence modified with synonymous mutations. These examples show that PTGS mechanisms can be avoided by addition of synonymous mutations.

The use of the synonymous mutation gene design can be especially useful for herbicide resistance. Amino acid substitutions in native enzymes have been found to confer resistance to herbicides that include acetyl coenzyme A carboxylase (ACCase) inhibitors, acetohydroxyacid synthase inhibitors, photosystem II inhibitors, and an EPSPS inhibitor (Powles and Yu 2010). Although only one or two amino acid substitutions are all that is needed to obtain resistance, development of herbicide-resistant crops has largely relied upon transformation with heterologous genes to obtain the necessary mutations, as was the case for glyphosate-tolerant crops (Barry et al. 1992). Based on herbicide resistance seen in weed species, it may be possible to transform plants with a native gene modified with a known point mutation that confers resistance.

In the present study, the purpose was to express a modified version of a native gene that incorporates mutations designed to confer herbicide resistance. Accordingly, the use of synonymous codons to bypass silencing as a strategy to overexpress a modified native enzyme was tested using a synthetic *ACCase* in creeping bentgrass to obtain plants resistant to ACCase inhibitors, while avoiding gene silencing. The ACCase family of herbicides is able to control important grassy weeds, and can provide a versatile management tool for weed control.

Materials and methods

Cloning bentgrass ACCase

The bentgrass ACCase sequence had to be known prior to designing a synthetic gene to ensure that native

and synthetic gene sequences do not have identical stretches 21 bp or longer. In addition, the native amino acid sequence was needed to make sure the synthetic gene coded for the same sequence. 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 1) were aligned using Geneious® (Biomatters Ltd. Auckland, NZ), and highly conserved regions throughout the gene were used for primer design. All primer sequences are in Table 2. 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® (Ambion® Life Technologies, Grand Island, NY). First strand cDNA synthesis was performed with RNA to cDNA EcoDry™ Premix Oligo dT kit (Clonetech 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® proofreading polymerase (New England Biolabs, Ipswich, MA). 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® 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. The resulting cDNA sequence was 6.88 kb in length and has been deposited in GenBank (accession number KP126512).

Table 1 ACCase genes used to create the alignment for primer design

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

ACCase gene design with synonymous codons

The cDNA sequence was translated into an amino acid sequence using Geneious® 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 the closest relative to creeping bentgrass (Edwards et al. 2011) with known codon usage at the time, and was therefore used to determine codon frequencies for back-translation. An alignment was made between the cDNA sequence and the modified sequence to find regions of identity of 21 bp or greater. 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) and deposited in GenBank (accession KP859588).

Vector construction

Four genes were used in this study: (1) wild-type (WT) *ACCase* with no mutations (GenBank accession KP126517), (2) wild-type *ACCase* with an A to C mutation in the first position of the 1781 codon (GenBank accession KP859587), (3) modified ACCase with no mutations (4) modified *ACCase* with an A to C mutation in the first position of the 1781 codon (GenBank accession KP859589). Plasmid Cambia 1305.2 (Cambia, Canberra, AU) was digested with *Mau*BI and *Sbf*I to remove the 35S promoter, GUSPlus™ reporter gene, and a portion of the NOS

Table 2 Primer list

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	ACATGACACCGCGCGCGATAATTTATCCTAGTTTG
BGSTART-IF-F	TCTCTTTTGCAGGATATCATGGGTTCCACACACTTGCCC
BGSTOP-IF-R	AATGTTTGAACGAGGCCTTCATTTGAGGACCTTCTTGAC
BG1781LEUF	AGAACTTACATGGAAGTGCTGCTATTG
BG1781LEUR	TTCCATGTAAGTTCTCCACACCTAGTC
SYNACF	GAGGGGCTTCAGCTACATCTACCTGGGG
SYNACR	TGAACAGTGGGAGGCCCT
VIRGF	AGGTGAGCCGTTGAAACACG
VIRGR	TCACCTGCCGTAAGTTTCACCT
BG2921F	TTGAGCCTCTGATGAGCCTAC
BG3300R	GTTCCTTGCAATGCTTGTGCG
BG4880F	TGGGGCACTCCTATAATTCCTATG
BG6300R	CTCCCTGGAGTGCTGCTTTCA
HYG370F	GCGGCCGATCTTAGCCAGACG
HYG720R	GCCAACCACGGCCTCCAGAAG

terminator (T-NOS). Vectors were assembled with In-Fusion® cloning (Clonetech Laboratories Inc. Mountain View, CA) (Fig. S1). The ACCase genes were under control of the switchgrass (*Panicum virgatum* L.) *PvUbi*2 promoter (Mann et al. 2011) and the *nos* terminator. The *PvUbi*2 promoter was shown to be a strong monocot promoter and was chosen to replace the 35S promoter (Mann et al. 2011). *PvUbi2*, T-NOS, and the modified gene were amplified with In-Fusion primers. 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 herbicide-resistance (HR) SNP in the 1781 codon. An A to C mutation was included in the first position of the 1781 codon, causing an isoleucine to leucine substitution. The amino acid substitution prevents the binding of ACCase inhibitors while allowing normal enzymatic function (Delye et al. 2005). 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).

Two WT bentgrass ACCase gene controls were used to compare to the modified genes to ACCase genes with synonymous mutations. The WT controls included the unmodified bentgrass ACCase and the bentgrass ACCase modified only with the HR mutation at the 1781 aa position. 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 In-Fusion ends to facilitate cloning into the vector. For the bentgrass ACCase gene with the HR mutation, the gene was amplified in two fragments overlapping by 15 bp to allow In-Fusion cloning. The HR 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^{-1} Gelzan™ (Caisson, North Logan, UT). Plates were sealed with Micropore™ tape (3 M, St. Paul, MN). *Agrobacterium* LBA4404 containing each construct was maintained in a freeze stocks and streaked on solid YM plates containing 50 mg L^{-1} kanamycin. Cultures were grown for 3 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^{-1} hygromycin to select for transgenic events and 150 mg L^{-1} timentin. Hygromycin-resistant events were transferred to medium supplemented with the ACCase-inhibiting herbicide, sethoxydim (Chemservice Inc. West Chester, PA). Sethoxydim was diluted with methanol to a concentration of 1 mg mL^{-1} and added to the medium following autoclavation to a concentration of 10 μM. Differences in the number of events selected for each construct with hygromycin and sethoxydim were determined using Fisher's Least Significant Difference (LSD) (SAS Institute, Inc. 2008. SAS OnlineDoc® 9.2. Cary, NC). 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^{-2} s^{-1}. Regenerating plants were placed on rooting medium consisting of solidified MS basal medium (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® and cDNA was produced using SuperSript® III reverse transcriptase (Life Technologies Grand Island, NY). 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 hygromycin phosphotransferase (*hph*) gene. In a previous experiment, sethoxydim-resistant creeping bentgrass obtained through in vitro selection possessed an A to C mutation in the first position of the 1781 codon, causing the isoleucine to leucine substitution (Heckart et al. in press). 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. Prior to in vitro analysis of transgenic events, 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 μM sethoxydim. Rooted Penn A-4 plants were placed on medium in 100 × 20 mm Petri dishes (Bioexpress, Kaysville, UT) in a 3 × 3 grid with five replications per concentration. Plant health was evaluated after 3 weeks. Transgenic events were then evaluated in vitro on medium containing 0.5 μM sethoxydim as determined by the dose response. 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™ measuring 4 × 14 cm (Stuewe and Sons Inc. Corvallis, Oregon) containing a 1:1 mix of Fafard® 3B (Sun Gro Horticulture, Agawam, MS) mix and sand. Plants were transferred to a greenhouse held at ~30 °C and placed under sodium lights with a 16-h photoperiod. Leaf blades 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 ™ (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^{-1}. Sethoxydim was applied at a spray volume of 187 L ha^{-1} 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 days after treatment (dat). Evaluation of plant injury was performed using a scale of 0–100, 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 (PGC) in each Cone-Tainer. Photos of each Cone-Tainer were taken with a GoPro® Hero3 Black edition (GoPro, San Mateo, CA) 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 ~4 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® Pro (Systat Software, San Jose, CA). Prior to image analysis, PGC was adjusted by removing the black background outside of the Cone-Tainer in PowerPoint® 2010 v10 (Microsoft, Redmond, WA). Hue was adjusted to 50–150 and brightness was 5–99. SigmaScan® forced a PGC reading for pots where plants were completely dead; therefore, these pots were set to a PGC of zero.

Data for visual injury at 28 dat were fitted to a Mitscherlich model (Grey et al. 2006), using nonlinear regression, and the resulting equation was $y = \beta_0(1 - 0.5e_1^{(-\beta(\text{rate}-\chi p))}) = 101(1 - 0.5e^{(-0.0093(\text{rate}-1.3))})$. PGC was modeled using nonlinear regression to an exponential decay model, and the resulting equation was $\beta_0(e_1^{(-\beta\chi)}) = y = 63.15(e^{(-0.0095\chi)})$. Differences among genotype means at each herbicide rate for PGC and visual injury estimates were determined using a

Table 3 Hygromycin- and sethoxydim-resistant creeping bentgrass events obtained

Construct	Inoculated Calli	Hygromycin events‡	Sethoxydim events†
Bentgrass ACCase	500	45b	0b
Bentgrass ACCase w/1781LEU	500	63a	0b
Modified ACCase	500	39b	0b
Modified ACCase w/1781LEU	500	46ab	6a
pCAMBIA 1305.2	500	37b	0b

† Sethoxydim resistant events were selected from the hygromycin-resistant events

‡ Means on the same column followed by the same letter are not significantly different at 0.05 according to a Fischer's protected least significant difference

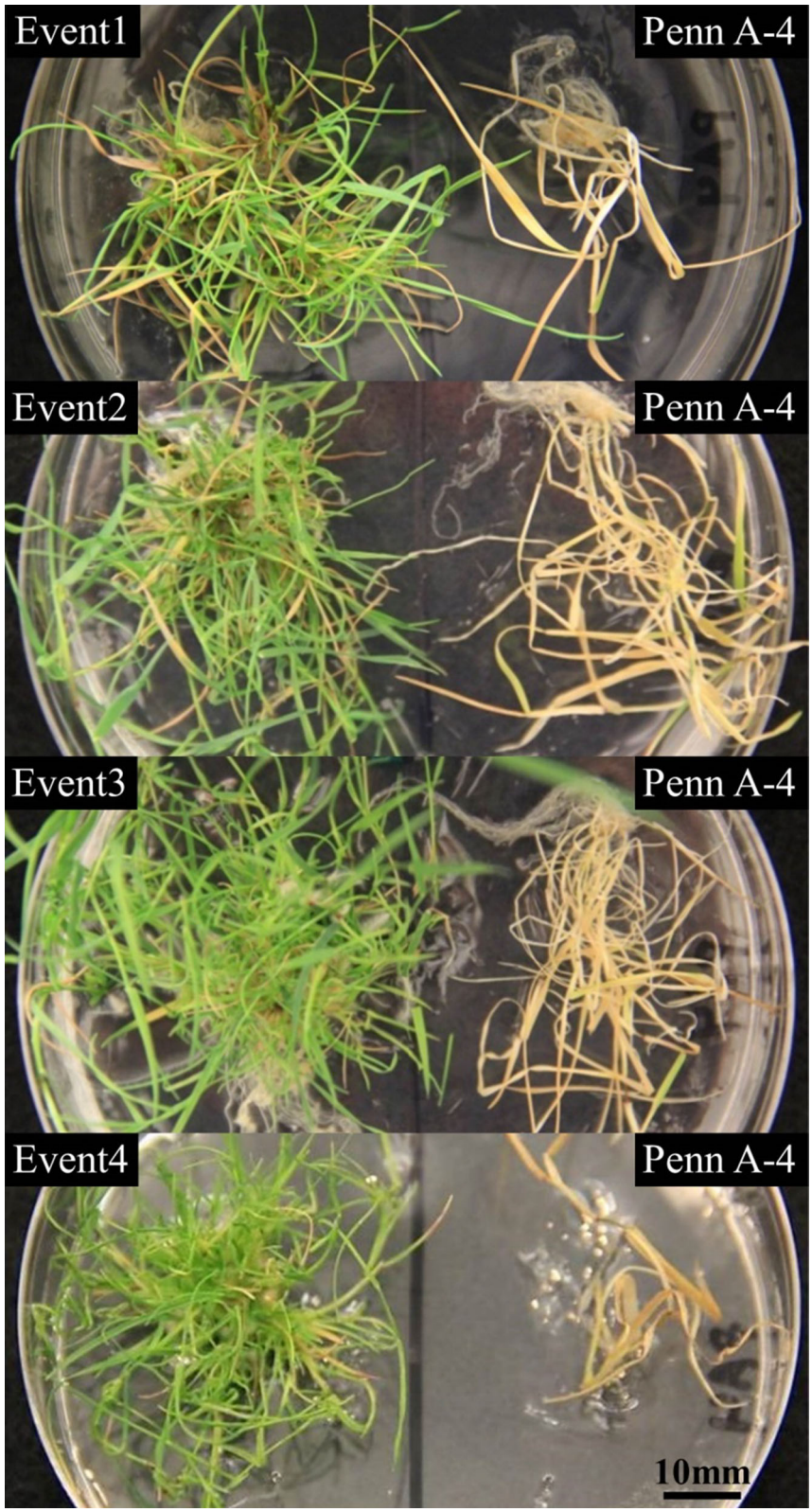

Fig. 1 In vitro response of transgenic events to sethoxydim compared to the Penn A-4 control. Transgenic events 1–4 were placed on MS basal medium containing 0.5 μM sethoxydim. The untransformed Penn A-4 was placed on the same plate for comparison. Plants were evaluated after 3 weeks

protected Fisher's LSD (SAS Institute, Inc. 2008. SAS OnlineDoc® 9.2. Cary, NC).

Results

In all, 500 calli were inoculated for each of the five constructs, including the pCAMBIA 1305.2 control. Hygromycin selection produced 37–60 resistant events per construct (Table 3). Six sethoxydim-resistant events were recovered from calli inoculated with the *ACCase* modified with the 1781 substitution, but none were obtained from any of the other constructs. All events were PCR-positive for the complete synthetic gene (Fig. S2a). All events showed expression of the ACCase transgene and *hph* (Fig. S2b and c, respectively). The events were negative for *Agrobacterium* and genomic DNA contamination (Fig. S2d 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 1781–2096 codons.

The in vitro dose response experiment showed that all doses were lethal to Penn A-4. Therefore, the lowest dose of 0.5 μM sethoxydim was used to test transgenic events. Due to availability of tissue, the first four events were selected for further analysis. All four transgenic events tested showed high levels of sethoxydim resistance compared to the Penn A-4 controls (Fig. 1). Penn A-4 plants were completely killed, but all transgenic events survived and showed vigorous growth at 0.5 μM sethoxydim.

Transgenic events fit a linear model for both visual injury (Fig. 2) and PGC (Fig. 3). At 14 dat, transgenic plants for all four events showed minimal injury at all rates (Table 4). The control, Penn A-4, showed injury above 40 % at 200 g ai ha^{-1}, which is approximately the 1X labeled rate for the naturally ACCase-resistant centipedegrass (*Ermochloa ophiuroides* Munro.) (Anonymous 2011). Penn A-4 was killed at a 4X rate. By 21 dat, PGC was reduced to five percent at a 2X rate for Penn A-4. PGC for transgenic events was reduced by 16–28 % at a 15X rate. At 28 dat, Penn A-4 injury was severe at a 0.5X rate, and PGC was reduced to 18 %. Transgenic events 2–4 survived to 15X with just ~50 % injury and with PGC 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 % (Figs. 4, 5).

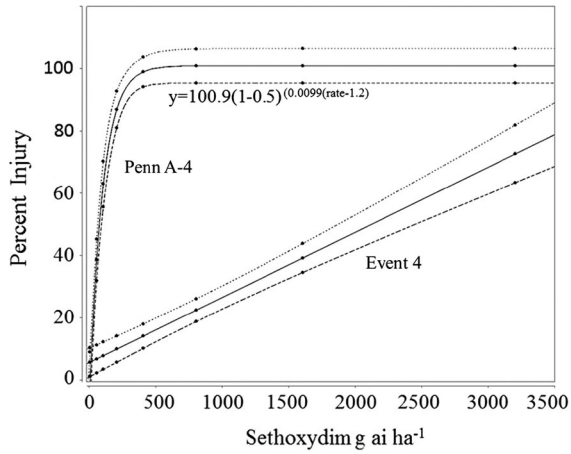

Fig. 2 Percent Injury of plants from Penn A-4 and transgenic event four in response to foliar applied sethoxydim 28 days after treatment. Five replicate plants were visually assessed for injury for each genotype at each rate. Data for Penn A-4 were fitted to a Misterlich model and data for event four were fitted to a linear model

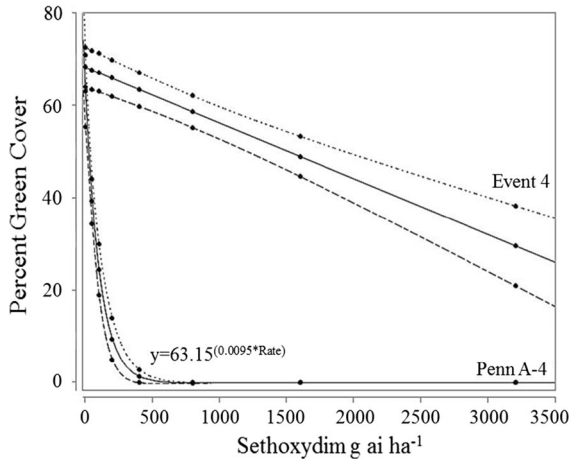

Fig. 3 Percent green cover of plants from Penn A-4 and transgenic event four in response to foliar applied sethoxydim 28 days after treatment. Photos of five replicate pots for each genotype at each rate were analyzed SigmaScan Pro 5.0 for percent green cover. Data for 'Penn A-4' were fitted to a negative exponential decay model and data for event four were fitted to a linear model

Discussion

It was not possible to recover events that expressed an extra copy of the native gene, with or without an amino acid substitution. In contrast, the use of a synthetic gene strategy incorporating a desired mutation was

Table 4 Percent injury and percent green cover of transgenic events and control Penn-A4 in response to various concentrations of sethoxydim

g ai ha^{-1}	Genotype					Genotype				
	Penn A4	Event 1	Event 2	Event 3	Event 4	Penn A4	Event 1	Event 2	Event 3	Event 4
	Percent injury 14 dat[†]					*Percent green cover 14 dat*				
0	0b[‡]	4ab	1b	0b	0b	58b	62ab	67a	64a	66a
50	6b	5b	4b	2b	1b	40b	59a	64a	66a	62a
100	27b	8bc	6c	5c	4c	30b	53a	56a	53a	60a
200	41a	12b	9b	5b	4b	25c	47b	50b	49b	59a
400	69a	15bc	10c	6c	6c	5b	43a	46a	50a	52a
800	100a	14b	9b	9b	9b	0c	41b	42ab	44ab	57ab
1600	100a	13b	9b	6b	5b	0c	42ab	46a	51a	47a
3200	100a	22b	12c	10c	8c	0c	34b	50a	48a	45a
	Percent injury 21 dat					*Percent green cover 21 dat*				
0	10a	14a	5a	5a	8a	59ab	64ab	66a	68a	71a
50	31b	14bc	7c	6c	6c	39b	62a	59a	66a	63a
100	68a	22b	20b	10b	9b	18b	56a	59a	63a	63a
200	84a	15b	19bc	33d	17bc	17d	60a	48c	48bc	59ab
400	100a	39b	34b	40b	21b	0c	44a	47a	43a	52a
800	100a	52b	45b	55ab	41b	0c	32b	32b	32b	46a
1600	100a	49b	57b	47b	46b	0c	31ab	36a	39a	34a
3200	100a	94a	54b	49b	52b	0c	10b	35a	33a	33a
	Percent injury 28dat					*Percent green cover 28dat*				
0	3b	15a	1b	1b	3b	62b	60b	71ab	79a	68ab
50	26a	10b	2b	2b	1b	46c	57b	66ab	73a	73a
100	72a	20b	2b	14b	5b	17c	53b	67a	71a	68a
200	89a	17bc	14bc	26b	8c	13b	60a	61a	59a	64a
400	100a	28b	24b	25b	16b	0b	50a	53a	55a	62a
800	100a	34c	43bc	56b	27c	0c	45b	43b	41b	61a
1600	100a	48b	54b	59b	57b	0b	40a	38a	39a	39a
3200	100a	97a	66b	65b	63b	0c	3c	35a	23b	34ab

[†] Days after treatment

[‡] Means on the same row (herbicide rate) followed by the same letter are not significantly different at 0.05 according to a Fischer's protected least significant difference

successful at achieving the goal, namely, the expression of a native enzyme differing from the native version by just one amino acid, while avoiding gene silencing.

The bentgrass sequence was highly conserved, as is the case with other grass species (Kawabe and Miyashita 2003; Zhang et al. 2001, 2012). The synthetic gene was designed to exclude rare codons during back-translation. Removal of such codons is important, as these can cause ribosomal stalling, resulting in an altered folding pattern that may change the enzyme's activity (Carlini and Stephan 2003; Tsai et al. 2008; Saunders and Deane 2010).

Thirteen percent of the hygromycin-resistant events from calli inoculated with the modified *ACCase* with the 1781 LEU mutation were also sethoxydim-resistant. In vitro selection of HR paspalum (Heckart et al. 2010) and bentgrass (Heckart et al. in press) can produce resistant events due to spontaneous mutations, though at a much lower rate. Using hygromycin for the initial selection phase helped ensure that sethoxydim resistant plants were due to presence of

the synthetic gene, rather than to a mutation for resistance.

The use of sequential selection on hygromycin and sethoxydim also made it possible to monitor the transformation efficiency and ensure that the lack of transgenics with some of the constructs was not due to problems with transformation. Although transformation efficiency was similar for all four constructs as evidenced by the recovery of hygromycin-resistant calli (Table 3), no sethoxydim-resistant events were recovered from calli inoculated with the native bentgrass ACCase transgene or the synthetic 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 for these constructs.

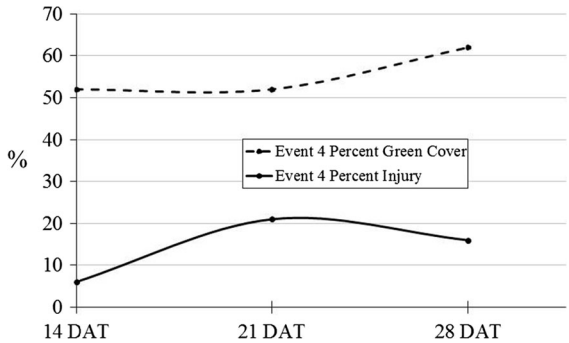

Fig. 4 Recovery of percent green cover (PGC) and injury for event four following foliar application of sethoxydim. Five replicate plates were analyzed at 14, 21, and 28 dat. Plants were treated with 400 ga ai ha^{-1}

The use of the synthetic gene was more efficient than recovering spontaneous mutations via in vitro selection for resistance. For paspalum, 20,250 calli went through the selection process, and two regenerable events appeared during the third round of selection resulting in an efficiency of selection of 0.0098 % (Heckart et al. 2010). In vitro selection for sethoxydim resistance in creeping bentgrass produced four resistant calli out of the 13,725 calli that were originally placed on selection medium, yielding an efficiency of 0.029 % (Heckart et al. in press). In contrast, sethoxydim-resistant creeping bentgrass was obtained through overexpression 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 a spontaneous A to T mutation at the first position of the 1781 codon is one in 1.3 billion (Heckart et al. 2010).

The effectiveness of the synthetic gene strategy also is evidenced by the level of resistance in the resulting plants. Sethoxydim E-Pro® is labeled for use on centipedegrass and fine fescue (*Festuca ovina* L.), because these grasses are naturally resistant. Rates for seedling and well-established stands of centipedegrass are 210 and 315 g ai ha^{-1}, respectively (Anonymous

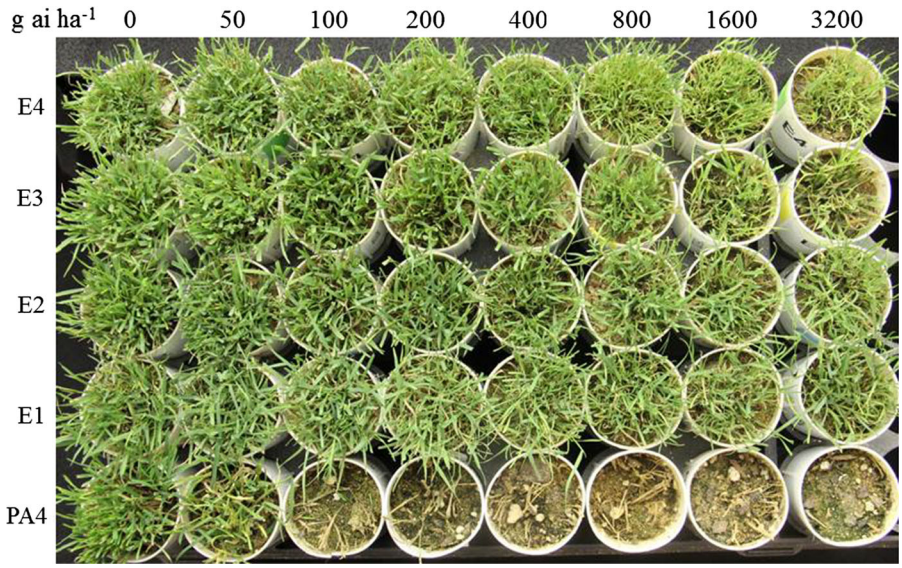

Fig. 5 Response of transgenic events to varying rates of sethoxydim compared parental type Penn A-4 28 days after treatment. Transgenic events have the ACCase transgene modified with synonymous mutations and the 1781 isoleucine to leucine herbicide-resistance mutation. The X axis represents transgenic events 1–4 and Penn A-4 control. The Y axis represents concentration of sethoxydim applied

2011). The creeping bentgrass used in this study was regenerated in vitro then grown in soil for 2 weeks prior to dose response experiments and closely resembled seedling plants. Injury was only 16 % at 28 dat for event four and reduction of PGC was only 6 % at approximately a 2X rate recommended for seedling centipedegrass.

As explained previously, the use of antibiotic resistance for selection was to measure the number of events that had useful levels of resistance as a percent of all transformants. With proof of concept in place, herbicide resistance itself could be used for selection. Furthermore, if pathogen-derived DNA elements are avoided in the construct and the events are engineered biolistically, the resulting plants would be exempt from USDA regulations as they currently stand.

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, overexpression of native enzymes has many possible applications and this study shows that this method of gene design is possible.

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References

Alvarez ML, Pinyerd HL, Topal E, Cardineau GA (2008) P19-dependent and P19-independent reversion of F1-V gene silencing in tomato. Plant Mol Biol 68:61–79. doi:10.1007/s11103-008-9352-2

Anandalakshmi R, Pruss GJ, Ge X, Marathe R, Mallory AC, Smith TH, Vance TH (1998) A viral suppressor of gene silencing in plants. Proc Natl Acad Sci USA 95:13079–13084. doi:10.1073/pnas.95.22.13079

Anonymous (2011) Herbicide label for sethoxydim E-Pro label. Etigra LLC. www.wplawinc.com/LiteratureRetrieve.aspx?ID=73109. Accessed 1 Nov 2015)

Antosiewicz DM, Barabasz A, Siemianowski O (2014) Phenotypic and molecular consequences of overexpression of metal-homeostasis genes. Frontiers Plant Sci. 5:1–7. doi:10.3389/fpls.2014.00080

Barry G, Kishore G, Padgette S, Taylor M, Kolacz K, Weldon M et al (1992) Inhibitors of amino acid biosynthesis: strategies for imparting glyphosate tolerance to crop plants. Curr Topics Plant Physiol 7:139–145

Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNAinterference. Nature 409:363–365. doi:10.1038/35053110

Cabello JV, Lodeyro AF, Zurbriggen MD (2014) Novel perspectives for the engineering of abiotic stress tolerance in plants. Curr Opin Biotechnol 26:62–70. doi:10.1016/j.copbio.2013.09.011

Carlini DB, Stephan W (2003) In vivo introduction of unpreferred synonymous codons into the *Drosophila* ADH gene results in reduced levels of ADH protein. Genetics 163:239–243

Chapman EJ, Prokhnevsky AI, Gopinath K, Dolja VV, Carrington JC (2004) Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. Genes Dev 18:1179–1186. doi:10.1101/gad.1201204

Delorge I, Janiak M, Carpentier S, Van Dijck P (2014) Fine tuning of trehalose biosynthesis and hydrolysis as novel tools for the generation of abiotic stress tolerant plants. Frontiers Plant Sci. 5:1–9. doi:10.3389/fpls.2014.00147

Delye C, Zhang XQ, Michel S, Matejicek A, Powles SB (2005) Molecular bases for sensitivity to acetyl-coenzyme A carboxylase inhibitors in black-grass. Plant Physiol 137:794–806. doi:10.1104/pp.104.046144

Drake C, Bird C, Schuch W (2001) Enhancement of tomato phytoene synthase gene expression with a modified DNA. US patent 6239331:B1

Edwards E, Aliscioni S, Bell H et al (2011) New grass phylogeny resolves deep evolutionary relationships and discovers C4 origins. New Phytol 193:304–312. doi:10.1111/j.1469-8137.2011.03972.x

Fang G, Bhardwaj N, Robilotto R, Gerstein MB (2010) Getting started in gene orthology and functional analysis. Comput Biol 6:e1000703. doi:10.1371/journal.pcbi.1000703

Grey T, Bridges D, Raymer P, Davis J (2006) Imazethapyr rate responses for wild radish, conventional canola, and imidazolinone-tolerant canola. Plant Health Progress. doi:10.1094/PHP-2006-1018-01-RS

Hamilton A, Baulcombe D (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286:950–952. doi:10.1126/science.286.5441.950

Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. Science 293:1146–1150. doi:10.1126/science.1064023

Heckart DL, Parrott WA, Raymer PL (2010) Obtaining sethoxydim resistance in seashore paspalum. Crop Sci 50:2632–2640. doi:10.2135/cropsci2010.02.0080

Jorgensen R, Doetsch N, Müller A, Que Q, Gendler K, Napoli C (2006) A paragenetic perspective on integration of RNA silencing into the epigenome and its role in the biology of higher plants. Cold Spring Harbor Symp Quant Biol 71:481–485. doi:10.1101/sqb.2006.71.023

Kawabe A, Miyashita N (2003) Patterns of codon usage bias in three dicot and four monocot plant species. Genes Genet Syst 78:343–352. doi:10.1266/ggs.78.343

Kumar D, Gustafsson C, Klessig D (2006) Validation of RNAi silencing specificity using synthetic genes: salicylic Acid Binding Protein 2 is required for innate immunity in plants. Plant J 45:863–868. doi:10.1111/j.1365-313X.2005.02645.x

Lassner M, Peterson P, Yoder J (1989) Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. Plant Mol Biol Rep 7:116–128. doi:10.1007/BF02669627

Li Q, Song J, Peng S, Wang JP, Qu GZ, Sederoff RR, Chiang VL (2014) Plant biotechnology for lignocellulosic biofuel production. Plant Biotechnol J 12:1174–1192. doi:10.1111/pbi.12273

Luo H, Hu Q, Nelson K, Longo C, Kausch A, Chandlee J, Wipff J, Fricker C (2004) *Agrobacterium tumefaciens*-mediated creeping bentgrass (*Agrostis stolonifera* L.) transformation using phosphinothricin selection results in a high frequency of single-copy transgene integration. Plant Cell Rep 22:645–652. doi:10.1007/s00299-003-0734-2

Mann D, King Z, Liu W, Joyce B, Percifield R, Hawkins J, LaFayette P, Artelt B, Burris JN, Mazarei M (2011) Switchgrass (*Panicum virgatum* L.) polyubiquitin gene (Pvubi1 and Pvubi2) promoters for use in plant transformation. BMC Biotechnol 11:74. doi:10.1186/1472-6750-11-74

Metzlaff M, O'Dell M, Cluster P, Flavell R (1997) RNA-mediated RNA degradation and chalcone synthase A silencing in petunia. Cell 88:845–854. doi:10.1016/S0092-8674(00)81930-3

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell 2:279–289. doi:10.1105/tpc.2.4.279

Powles SB, Yu Q (2010) Evolution in action: plants resistant to herbicides. Annu Rev Plant Biol 61:317–347. doi:10.1146/annurev-arplant-042809-112119

Saunders R, Deane CM (2010) Synonymous codon usage influences the local protein structure observed. Nucleic Acids Res 38:6719–6728. doi:10.1093/nar/gkq495

Shi H, Chan Z (2014) Improvement of plant abiotic stress tolerance through modulation of the polyamine pathway. J Integr Plant Biol 56:114–121. doi:10.1111/jipb.12128

Shi H, Lee BH, Wu SJ, Zhu JK (2003) Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. Nat Biotechnol 21:81–85. doi:10.1038/nbt766

Thomsen HC, Eriksson D, Møller IS, Schjoerring JK (2014) Cytosolic glutamine synthetase: a target for improvement of crop nitrogen use efficiency? Trends Plant Sci 19:656–663. doi:10.1016/j.tplants.2014.06.002

Tsai C, Sauna Z, Kimchi-Sarfaty C, Ambudkar S, Gottesman M, Nussinov R (2008) Synonymous mutations and ribosome stalling can lead to altered folding pathways and distinct minima. J Mol Biol 383:281–291. doi:10.1016/j.jmb.2008.08.012

Vartapetian BY, Dolgikh YI, Polyakova LI, Chichkova NV, Vartapetian AB (2014) Biotechnological approaches to creation of hypoxia and anoxia tolerant plants. Acta naturae 6:19–30

Yamaguchi T, Blumwald E (2005) Developing salt-tolerant crop plants: challenges and opportunities. Trends Plant Sci 10:615–620. doi:10.1016/j.tplants.2005.10.002

Zamore PD, Tuschl T, Sharp PA, Bartel DP (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21–23 nucleotide intervals. Cell 101:25–33. doi:10.1016/S0092-8674(00)80620-0

Zhang L, Pond SK, Gaut BS (2001) A survey of the molecular evolutionary dynamics of twenty-five multigene families from four grass taxa. J Mol Evol 52:144–156. doi:10.1007/s002390010143

Zhang Y, Nie X, Jia X, Zhao C, Biradar SS, Wang L, Du X, Weining S (2012) Analysis of codon usage patterns of the chloroplast genomes in the Poaceae family. Aust J Bot 60:461–470. doi:10.1071/BT12073