

Expression of Leaf Spot Induced Resistance Gene-Like Sequences in Buffalograss

Bimal S. Amaradasa¹ and Keenan Amundsen¹

Abstract

American buffalograss [*Buchloë dactyloides*(Nutt.)Engelm. syn. *Bouteloua dactyloides* (Nutt.) Columbus] is a drought, heat and cold tolerant United States native turfgrass species. Leaf spot disease caused by *Curvularia inaequalis* negatively impacts buffalograss visual quality. Seven candidate resistance gene-like sequences have been previously identified in buffalograss based on similarity to known resistance genes. Here, the expression of these seven genes were evaluated in two leaf spot susceptible (Prestige and NE-BFG-7-3453-50) and two resistant (95-55 and NE-BFG-7-3459-17) buffalograss lines when challenged with *C. inaequalis*. Real-time quantitative PCR assays were used to detect expression of the sequences on each buffalograss line. Four of the sequences were expressed in all buffalograss lines. One sequence had higher expression in both resistant lines and another had higher expression in 95-55 when challenged with the pathogen. Compared to uninoculated controls, inoculated susceptible lines did not show a change in expression of resistance gene-like sequences. Inoculated resistant lines had higher expression of the tested resistance gene-like sequences than the susceptible lines. Expression of these genes was elevated in uninoculated plants and may contribute to the innate immunity to leaf spot disease of the resistant lines.

Keywords: resistance gene-like sequences, qRT-PCR, disease resistance, buffalograss

¹ PhD, Department of Agronomy and Horticulture, University of Nebraska-Lincoln, NE 68503, USA.
Phone: 402-472-8390, E-mail: kamundsen2@unl.edu

Introduction

Buffalograss [*Buchloë dactyloides* (Nutt.) Engelm. syn. *Bouteloua dactyloides* (Nutt.) Columbus] is a prairie shortgrass native to the Great Plains of North America. It is a warm-season grass with exceptional drought, heat, and cold tolerance (Beetle, 1950; Reeder, 1971). These qualities make turf-type buffalograss ideally suited for use as a low-input turfgrass species. Replacing high-maintenance turfgrasses with buffalograss can conserve resources such as water, pesticides, and fertilizer. Although buffalograss generally tolerates many diseases, leaf spot can cause death or decline of buffalograss turf. Leaf spots caused by a species complex consisting of *Curvularia*, *Bipolaris* and *Cercospora* species (Smiley *et al.*, 2005; Smith *et al.*, 1989). In Nebraska, *Curvularia inaequalis* (Shear) Boedijn and *B. spicifera* (Bainier) Subram (teleomorph: *Cochliobolus spicifer* Nelson) are commonly isolated from buffalograss with leaf spot symptoms (Amaradasa and Amundsen, 2013). On lawns, leaf spot initiates as dark brown leaf spots followed by leaf tip dieback and eventual blighting of entire tillers. As the disease progresses, patches of leaf decline and canopy thinning occur. Leaf spot symptoms of both causal organisms are identical and therefore it is not possible to distinguish the causal pathogen by disease symptoms alone.

Disease activity of the two pathogens is observed at temperatures between 22°C and 32°C. Disease severity increases when buffalograss is under stress by adverse weather conditions such as high temperatures, high humidity, drought, excess rain, and cloud cover. Since buffalograss is often considered a low-maintenance turfgrass, the use of fungicides to control disease is usually discouraged. Incorporating host resistance to leaf spot disease through plant breeding is one of the best ways to combat leaf spot disease in the future. Conventional breeding for disease resistance is a difficult and time consuming process, based on inoculation, rating for occurrence and severity of disease, and selection of resistant genotypes. Identification of genes that confer leaf spot resistance would pave the way for molecular-based strategies to enhance the efficiency of breeding for resistant genotypes.

Plant resistance to pathogens can be broadly divided into two phases. In phase 1, pathogen- or microbe-associated molecular patterns (PAMP/MAMP) such as chitin or flagellin are recognized by pattern-recognition receptors (PPR) located in the plant cell membranes (Balmer *et al.*, 2012). These PPRs then confer the first level of defense against disease. This is also referred to as innate immunity and largely responsible for preventing a pathogen from causing disease in a non-host plant.

PAMP-triggered immunity (PTI) can halt further colonization of the pathogen due to cell-wall appositions and anti-microbial metabolites at the infection site, but no hypersensitive cell death response (HR) is observed (Jones and Dangl, 2006). Host-adapted pathogens such as bacteria, fungi, and oomycetes can manipulate the initial phase of the plant defense system located in the cell membrane by delivering effector molecules and entering the cytoplasm of host cells (Balmer *et al.*, 2012). In response to effector molecules encoded by pathogen avirulence (*Avr*) genes, plants have a second level defense system initiated by *R* genes.

The *R* genes most often encode nucleotide-binding site leucine rich repeat (NBS-LRR) proteins capable of recognizing pathogen-derived effector molecules (Balmer *et al.*, 2012; Elmore *et al.*, 2011). Effector molecule recognition by resistant plant *R* genes results in effector-triggered immunity (ETI). Often, resistance to a plant disease is conferred by a single *R* gene in the plant that interacts with a corresponding *Avr* gene in the pathogen (Budak *et al.*, 2006; Nirmala *et al.*, 2011) which leads to HR (Jones and Dangl, 2006). NBS-LRR proteins usually consists of an N-terminal TIR (Toll/Interleukin-1 Receptor) domain or coiled-coil (CC) motif. Genes encoding NBS-LRR proteins represent one of the largest and widely conserved gene families in plants, with the majority of sequenced plants having more than 100 NBS-LRR family members (Balmer *et al.*, 2006). For instance, approximately 125 NBS-LRR type proteins have been identified in *Arabidopsis* Col-0 genome (Jones and Dangl, 2006). Genes encoding TIR-NBS-LRR homologues are rare in monocots, CC-NBS-LRR homologues are more common in these plants than dicots (Balmer *et al.*, 2006).

Budak *et al.* (2006) designed degenerate PCR primers targeting highly conserved kinase and hydrophobic domains of known NBS-LRR proteins to amplify candidate RGL sequences from buffalograss. Budak *et al.* (2006) identified sequences similar to NBS-LRR-type *R* genes, seven of which shared partial or overall similarity to kinase 1a type *R* genes of buffalograss. The level of gene expression of these candidate *R* genes in response to disease was not tested. In the present study, leaf spot resistant and susceptible buffalograss lines were challenged with *C. inaequalis* to quantify the activity of the seven RGL sequences identified by Budak *et al.* (2006).

Materials and Methods

Greenhouse screening of buffalograss for leaf spot resistance and selection of experimental lines

Eighty experimental lines and seven named varieties of buffalograss were used for a preliminary greenhouse resistance screening against *Curvularia inaequalis*. Stolons of each buffalograss line were planted in 7-cm-diameter plastic pots filled with Fafard® 3B Mix potting medium. Pots were kept in a greenhouse with a 16 h day and 8 h night photoperiod. The average daytime and nighttime temperature of the greenhouse was maintained at 30°C and 22°C, respectively. Plants were watered daily, fertilized biweekly with 20-20-20 to provide approximately an annual rate of 10g N m⁻², and clipped with scissors regularly to a height of 6 to 7 cm to promote prostrate growth and pot coverage. After 12 wk of growth, plants were arranged in a randomized complete block design (RCBD) with three replications. Single-spore *C. inaequalis* strain 4L-SS01 was used to prepare a spore culture of 1 x 10⁶ spores ml⁻¹ according to the method described by Brecht *et al.*(2007).

Each pot was sprayed with 15 ml of the spore solution. Following inoculation, each pot was covered with a clear plastic bag to increase humidity and encourage disease development. Every other day, bags were kept open for a few hours and plants were sprayed lightly with water. Disease development was monitored and disease severity visually rated 10 d and 13 d after inoculation on a scale of 0-10, where zero is no disease and 10 is 100% diseased. Both leaf tip die-back and chlorotic leaf lesions were used to determine disease severity. Data were analyzed by averaging disease severity ratings collected at 10 d and 13 d and then performing one-way analysis of variance (ANOVA) with level of significance at $P \leq 0.05$ using JMP® version 10 (SAS Institute, Cary, NC). Student's t-test was performed for all possible comparisons using JMP option Compare Means, Each Pair, Student's t.

Based on the results of this preliminary disease susceptibility screen (Supplementary Table1), the two susceptible lines Prestige and NE-BFG-7-3453-50 and the two resistant lines 95-55 and NE-BFG-7-3459-17, were selected for further studies. The inoculation with *C. inaequalis* was repeated twice for these four lines and disease severity recorded to validate the level of susceptibility of these lines to *C. inaequalis*.

On both occasions, average disease ratings of Prestige and NE-BFG-7-3453-50 were above five while 95-55 and NE-BFG-7-3459-17 rated below 2.5 (data not shown) supporting the results of the initial screening experiment and the classification of these lines as either susceptible or resistant, respectively. Another set of these four lines was inoculated again using the same methodology described above. Untreated controls were also included with three replicates and sprayed with water in place of the spore solution. The experiment was designed as a completely randomized design with three replications per treatment. After 10 days, when susceptible lines were exhibiting distinct disease symptoms, leaf tissue from both inoculated and uninoculated pots was harvested into separate freezer bags and immediately frozen in liquid nitrogen. Samples were kept at -80°C for later use.

Real-Time PCR Primers, Probes and Conditions

The resistant (95-55 and NE-BFG-7-3459-17) and susceptible (Prestige and NE-BFG-7-3453-50) buffalograss lines were homogenized in liquid nitrogen using a mortar and pestle and RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA samples were qualitatively analyzed by agarose gel electrophoresis, and quantified using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific Inc, Wilmington, Delaware). Real-time quantitative reverse transcription PCR (qRT-PCR) assays were performed to detect the expression of seven previously reported RGL sequences (Budaket *et al.*, 2006) with the following GenBank

(<http://www.ncbi.nlm.nih.gov/genbank/>) accession numbers: AY966892 (P6), AY966893 (P5), AY966895 (P3), AY966896 (P4), AY970294 (P2), AY970295 (P1), and AY971604 (P7).

P1 through P7 refers to the identifiers used in this study for the amplified products derived from these template sequences. The expression level of ubiquitin conjugating enzyme (UCE) gene was used as an endogenous control to normalize the experimental results (Czechowski *et al.*, 2005; Gutsche *et al.*, 2009). The primer and the probe sequences were designed using the Custom TaqMan[®] Assay Design Tool (<https://www5.invitrogen.com/custom-genomic-products/tools/gene-expression/>).

The qRT-PCR primers used for amplifying the above sequences are as follows, AY966892_F: CCCAAGCCTGCGCTACA, AY966892_R: ACTGAGCATAGAGCCCTTCCAT and AY966892_probe: FAM-ACAGCATCCAAAAGTG; AY966893_F: GTCCGCTGGATGACTCGTTT, AY966893_R: AAGATCCAAGGTAAAAGAGGCGATTT, and AY966893_probe: FAM-CAGCATCCCAACTTC; AY966895_F: ATCCCGATGCCACCATAGC, AY966895_R: TGTTTTTCAGGATATACACTTTAGAACTTGCA, and AY966895_probe: FAM- TTGGCCGGTGTTCGTG; AY966896_F: TCAGTTCGGAATGAATGCTATACTTTTGA, AY966896_R: CCCGTGTCAACTACTCATTTCTGTT, and AY966896_probe: FAM-TCGTTCCCTCGAAATCC; AY970294_F: CCTCTATTTATTTTCGGGTTCTAGCCTAA, AY970294_R: AAACCACTGTCTGCTGTAGTAACAG, and AY970294_probe: FAM-TCCTCGCCTAAAGTTC; AY970295_F: GGCTCAACTAAACGAAACAATGCT, AY970295_R: GATGCTTTCCCCTCTCTCTTAAAT, and AY970295_probe: FAM-CCTCCTGCCCTCTTTC; and AY971604_F: CATTAAAGTATCTTACATCTTTCTCGTGAGGTT; AY971604_R: AGTATACGAGTCACTGCAGTTCTCT, and AY971604_R: FAM-CCTGCCTTCGGTTCTT. The UCE gene was amplified by a forward primer GACCCGCCTACATCATGCA and a reverse primer TTGCTTGCCAGTGAAACATGTC. The probe sequence of the UCE gene was CTCACCAGCAGGCCCT. All of the above sequences are in the 5' to 3' orientation. Primers and probes were synthesized by Applied Biosystems Inc (Foster City, CA).

The qRT-PCR assays for detecting RGL expression levels were performed using an Applied Biosystems TaqMan® RNA-to-CT™ 1-Step Kit. Each assay was carried out in a 20 µl reaction mixture containing 40 ng template RNA, 900 nM each primer, 250 nM TaqMan probe, 10 µl of TaqMan RT-PCR Mix (2×) and 0.5 µl of TaqMan RT Enzyme Mix (40×). Each sample was run as three technical replicates and average expression values were taken for the analysis. Probes were tagged with a FAM fluorophore and a TAMRA quencher. Amplifications were carried out on an Applied Biosystems 7500 Fast Real-Time PCR System with an initial cDNA synthesis step at 48°C for 15 min, followed by an initial denaturing step at 95°C for 10 min and then 40 quantification cycles, each consisting of 95°C for 15 s and 60°C for 60 s. Threshold cycle numbers (CT) were obtained during the logarithmic phase of amplification where reaction efficiency was close to 100%.

Relative expression levels were calculated using the CT of each inoculated and uninoculated tissue sample using the formula $2^{-(\Delta CT_{\text{sample}})}$ and the method described by Benn *et al.* (2008) and Schmittgen and Livak (2008). The ΔCT represents the difference in CT values between the target and UCE amplicons.

Validation of Selected RGL Sequences in Uninoculated Buffalograss Lines

The preliminary RGL sequence expression study indicated elevated expression of P1, P2 and P6 gene products in the uninoculated resistant lines compared to the uninoculated susceptible lines. Gene expression in uninoculated lines represents the basal expression levels and the initial results indicated that the above three gene products are expressed more constitutively in healthy resistant plants. To validate this further, we tested the expression of P1, P2, and P6 in a total of eight uninoculated buffalograss lines growing in the greenhouse and field. Leaf samples were taken in triplicate from greenhouse pots of resistant lines 609 and 11-3625, and susceptible lines 378 and 5-3009. These pots were arranged in a completely randomized design (CRD) with three replicates.

Resistant and susceptible lines were identified from the preliminary greenhouse leaf spot disease susceptibility screen (Supplementary Table 1). Field samples were collected in mid-July 2013 from experimental lines maintained at the University of Nebraska John Seaton Anderson Turfgrass Research Facility, Mead, NE. The weather conditions at the time samples were collected was favorable for leaf spot development and most of the susceptible lines growing in the field had disease symptoms to a varying degree. Little to no leaf spot symptoms were observed in the buffalograss lines NE-BFG-5-2973, NE-BFG-5-2964, and NE-BFG-5-2974 suggesting these lines are resistant to the disease. Healthy leaves of these three lines were sampled from a field study arranged as an RCBD with three replications. These three lines were not included in the initial greenhouse disease screening. Leaf samples of the susceptible line Prestige from disease free areas were also taken from field plots to test consistency of greenhouse and field expression levels. Total RNA was extracted from healthy leaves to reflect the uninoculated status of the plants, and qRT-PCR was performed on all lines as described above.

Statistical analysis qRT-PCR data

For the preliminary study, technical replicates of each biological replicate of leaf spot susceptible Prestige and resistant 95-55 was run together in a 96-well-plate. Similarly, each replicate of NE-BFG-7-3453-50 and NE-BFG-7-3459-17 was run in a single plate. Since a separate 96-well-plate was used for each replicate, this experimental design resembles an RCBD with each plate representing a block. Gene expression of inoculated and uninoculated lines was compared individually using a t-test. The relationships between the expression levels in resistant and susceptible buffalograss lines (i.e. Prestige and 95-55 together, and NE-BFG-7-3453-50 and NE-BFG-7-3459-17 together) were also evaluated using the Dunnett's Probability test. The uninoculated susceptible lines were used as control samples for this test. Validation of the expression of P1, P2, and P6 in eight uninoculated buffalograss lines was carried out in a similar RCBD arrangement as above, and a one-way ANOVA was performed to test for differences in expression. Expression data were evaluated using JMP after log₂-transformation for normalization. P-values less than 0.05 were considered significant.

Results

In the preliminary leaf spot disease susceptibility screen, 14 of the 87 lines tested had an average disease rating of > 5 , indicating these lines were susceptible. Due to the presence of necrotic leaf tissue and detrimental effect to plant health, a disease severity rating of at least five was required for a plant to be categorized as susceptible. Buffalograss accessions having a disease rating of ≤ 2.5 were considered resistant (Supplementary Table 1). The average disease ratings for Prestige and NE-BFG-7-3453-50 were 6.33 and 5.83, respectively while 95-55 and NE-BFG-7-3459-17 had disease ratings of 2.5 and 2.33, respectively. The ratings for these susceptible and resistant lines were different based on Fisher's LSD at $P = 0.05$ (Supplementary Table 1).

Supplementary Table 1: Results of the Student's t-test for mean disease ratings of 87 buffalograss accessions. LSD: least significant difference

Variety	Mean	Variety	Mean	Variety	Mean
NE-BFG-11-362 ⁵	1.33	NE-BFG-07-346 ²	3.67	NE-BFG-07-346 ⁷	4.83
NE-BFG-05-316 ⁴	2.17	NE-BFG-07-345 ⁴	3.33	NE-BFG-07-346 ⁶	4.83
NE-BFG-07-346 ⁴	3.00	315	3.17	NE-BFG-07-346 ⁶	4.50
NE-BFG-07-346 ²	2.50	NE-BFG-11-361 ⁴	3.50	NE-BFG-07-346 ⁴	4.17
NE-BFG-07-345 ³	2.33	NE-BFG-11-360 ⁹	3.50	NE-BFG-07-346 ³	4.17
609	2.17	NE-BFG-07-345 ⁹	4.00	NE-BFG-07-345 ⁶	4.67
95-55	2.50	NE-BFG-07-345 ⁹	3.00	NE-BFG-07-345 ³	5.00
NE-BFG-11-361 ⁰	2.67	NE-BFG-05-326 ⁷	4.00	NE-BFG-C-2781	5.17
NE-BFG-07-346 ⁵	3.00	NE-BFG-05-306 ⁸	3.33	N.P. Buffalograss	5.33
NE-BFG-07-345 ³	2.33	NE-BFG-11-361 ⁷	4.17	NE-BFG-11-362 ³	5.17
NE-BFG-07-345 ³	2.33	NE-BFG-11-361 ⁶	3.83	NE-BFG-11-361 ¹	4.83
NE-BFG-05-300 ⁰	2.67	NE-BFG-11-361 ⁵	4.33	NE-BFG-07-346 ⁶	5.00
NE-BFG-11-362 ²	2.83	NE-BFG-07-346 ⁷	4.17	NE-BFG-07-346 ⁵	5.00
NE-BFG-11-361 ³	3.00	NE-BFG-07-346 ⁷	3.83	NE-BFG-05-302 ¹	4.33
NE-BFG-07-346 ⁴	3.17	NE-BFG-07-346 ⁷	3.50	NE-BFG-05-298 ⁵	4.67
NE-BFG-07-346 ²	2.67	NE-BFG-07-346 ⁶	4.33	NE-BFG-07-346 ³	5.50
NE-BFG-07-345 ⁵	2.67	NE-BFG-07-346 ²	4.50	NE-BFG-07-345 ⁴	5.50
NE-BFG-07-345 ³	2.50	NE-BFG-07-346 ²	4.67	378	5.00
Density	3.17	NE-BFG-07-345 ⁴	4.17	NE-BFG-09-356 ⁵	5.50
NE-BFG-11-362 ¹	3.83	NE-BFG-07-345 ⁴	4.00	NE-BFG-07-346 ⁶	5.17
NE-BFG-11-362 ⁰	3.50	NE-BFG-05-301 ⁰	4.50	NE-BFG-07-345 ³	5.83
NE-BFG-11-361 ⁹	3.67	NE-BFG-07-346 ⁵	4.17	NE-BFG-05-304 ⁶	5.50
NE-BFG-11-361 ²	3.00	NE-BFG-07-346 ⁴	4.00	NE-BFG-05-297 ⁹	5.50
NE-BFG-07-346 ⁹	3.00	NE-BFG-07-346 ²	4.00	NE-BFG-07-345 ³	6.00
NE-BFG-07-345 ³	3.33	NE-BFG-07-345 ⁵	3.83	NE-BFG-05-300 ⁹	6.17
NE-BFG-11-360 ⁸	3.67	NE-BFG-07-345 ³	4.50	Prestige	6.33
NE-BFG-09-350 ²	3.67	NE-BFG-05-296 ⁴	4.67		
NE-BFG-07-347 ⁰	3.83	Legacy	4.00		
NE-BFG-07-346 ⁹	3.67	NE-BFG-11-362 ⁴	5.17	LSD (P=0.05)	1.93

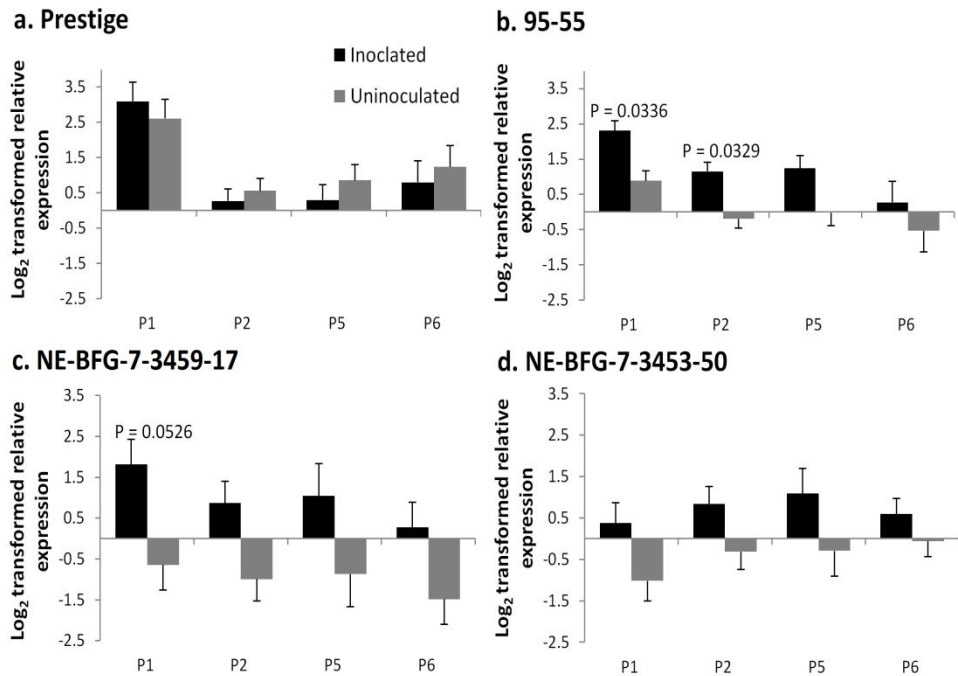


Figure 1:Relative expression of defense related genes P1, P2, P5, and P6 following infection by *C. inaequalis*. Prestige (a), and NE-BFG-7-3453-50 (d) are susceptible to leaf spot. 95-55 (b), and NE-BFG-7-3459-17 (c) are leaf spot resistant lines. P1 and P2 exhibited significantly higher expression levels in pathogen infected 95-55. P1 amplicon in inoculated NE-BFG-7-3459-17 showed a trend of higher expression at $P = 0.0526$ compared to the uninoculated. Susceptible lines Prestige and NE-BFG-7-3453-50 did not show differential expression for any of the genes. The expression level of ubiquitin conjugating enzyme (UCE) gene was used as an endogenous control to normalize the experimental results

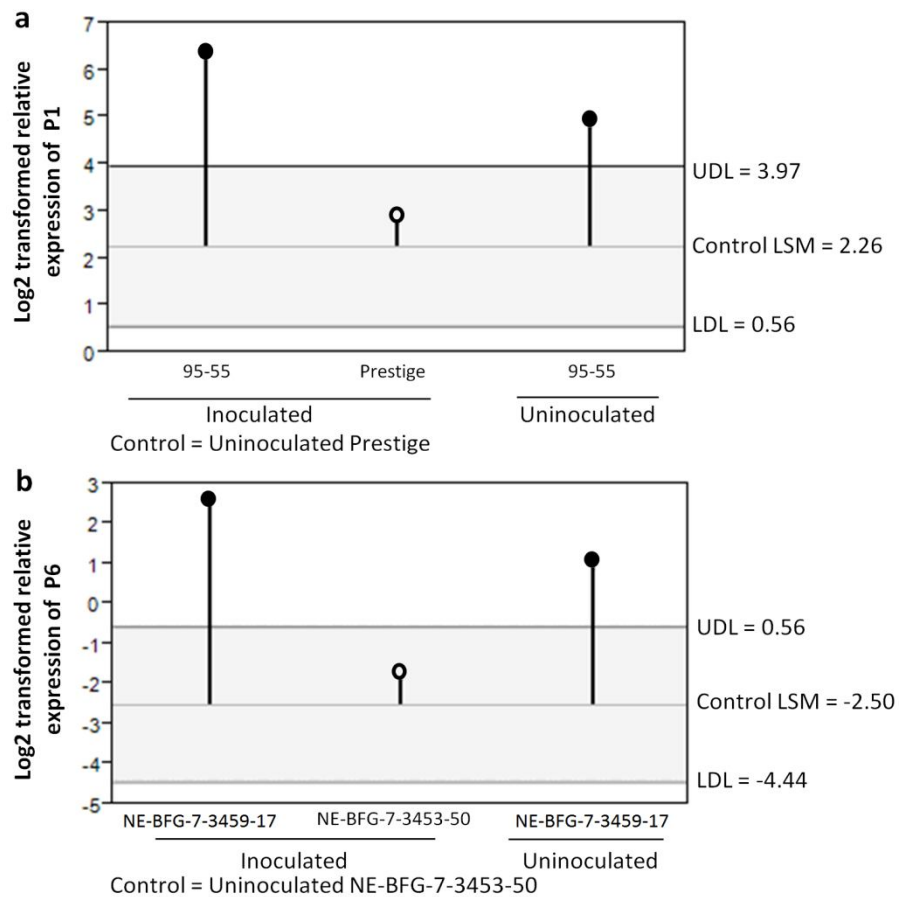


Figure 2: Comparison of relative expression of defense related genes in leaf spot resistant and susceptible lines infected with *C. inaequalis*. a: Expression of P1 in inoculated and healthy 95-55 (leaf spot resistant) and Prestige (susceptible). b: Expression of P6 in inoculated and healthy NE-BFG-7-3459-17 (leaf spot resistant) and NE-BFG-7-7359-50 (susceptible). In both a and b expression levels are compared to the uninoculated susceptible line using the Dunnett's test. Both inoculated and uninoculated resistant lines exhibit higher levels of P1 and P6 compared to the uninoculated control in susceptible lines. UDL = upper decision limit, LDL = lower decision limit. The expression level of ubiquitin conjugating enzyme (UCE) gene was used as an endogenous control to normalize the experimental results

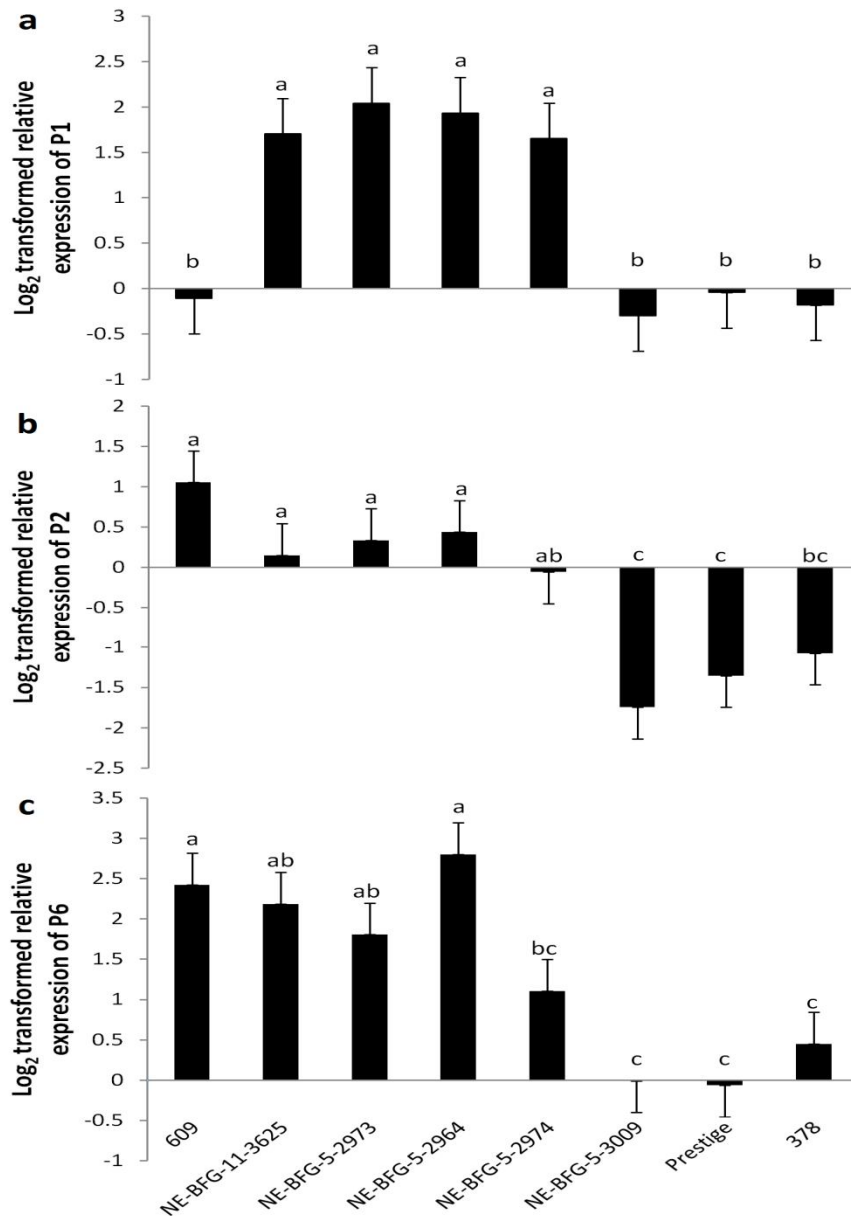
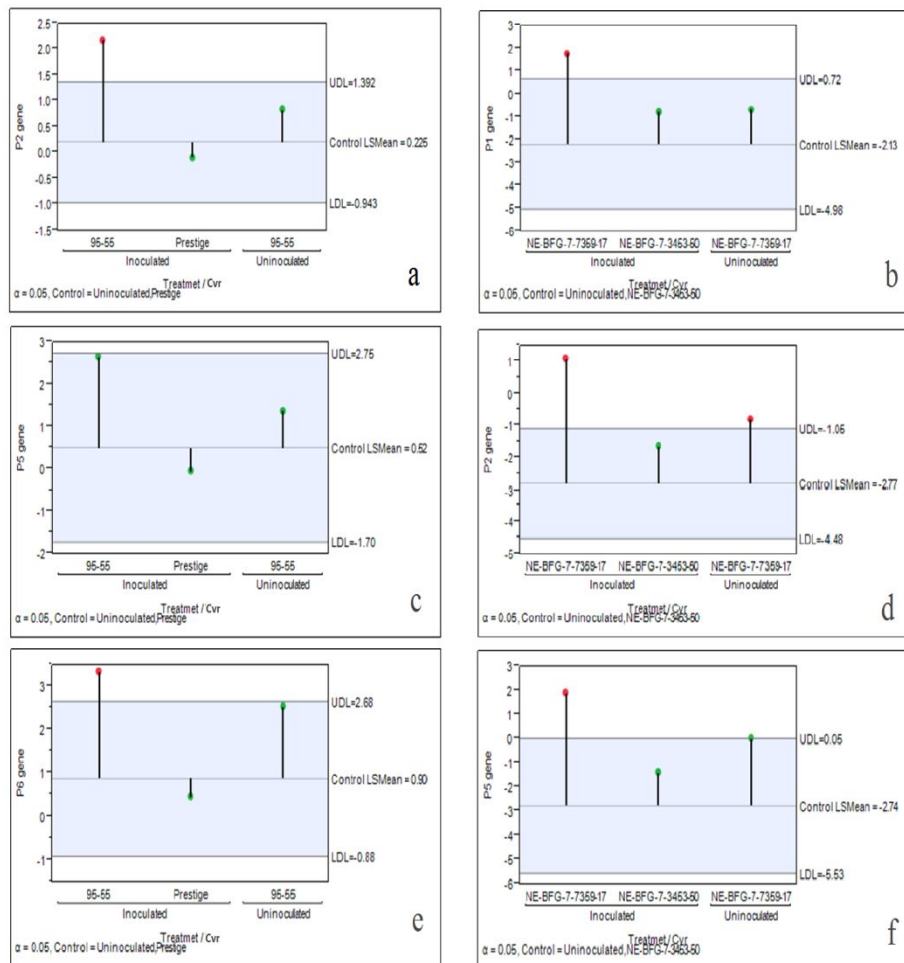


Figure 3 Basal expression of defense related genes P1, P2 and P6 in eight buffalograss lines. Genotypes 609, NE-BFG-11-3625, NE-BFG-5-2973, NE-BFG-5-2964, and NE-BFG-5-2974 are resistant to leaf spot. Prestige, NE-BFG-5-3009, and 378 are susceptible lines. The expression level of ubiquitin conjugating enzyme (UCE) gene was used as an endogenous control to normalize the experimental results.



Supplementary Figure 2: Comparison of expression levels of defense related genes in 95-55 and Prestige, and NE-BFG-7-3459-17 and NE-BFG-7-3453-50 challenged with *C. inaequalis*. Prestige, and NE-BFG-7-3453-50 are susceptible to leaf spot. 95-55, and NE-BFG-7-3459-17 are leaf spot resistant lines. The expression levels are compared to uninoculated susceptible lines using the Dunnett's test.

Initial tests conducted with uninoculated Prestige did not amplify a PCR product for P3, P4 and P7 primers. Therefore, relative expression levels were quantitated for P1, P2, P5 and P6 products only. Expression of these four products when influenced by *C. inaequalis* did not change in susceptible lines Prestige and NE-BFG-7-3453-50 (Figures 1a and 1d). Resistant line 95-55 had higher levels of P1 and P2 transcripts in inoculated samples (Figure 1b).

Although, outside the statistical limit of $P = 0.05$, P1 transcripts had higher expression in inoculated NE-BFG-7-3459-17 compared to the uninoculated control ($P = 0.0526$; Figure 1c). Since 95-55 (leaf spot resistant) and Prestige (leaf spot susceptible) samples were run on the same plate we could analyze them together. Also, NE-BFG-7-3459-17 and NE-BFG-7-3453-50 had a similar experimental set up and analyzed in the same fashion. The two-way ANOVA performed on 95-55 and Prestige, and NE-BFG-7-3459-17 and NE-BFG-7-3453-50 showed significant results for cultivar and treatment (data not shown). There was no cultivar \times treatment interaction. The mean comparison by Dunnett's test showed higher expression of P1 in both inoculated and uninoculated resistant line 95-55 compared to the uninoculated susceptible line Prestige (Figure 2a). Similarly, inoculated and uninoculated NE-BFG-7-3459-17 exhibited higher levels of P2 (Supplementary Figure 2d) and P6 (Figure 2b) in comparison to the uninoculated NE-BFG-7-3453-50. Mean comparison by Fisher's LSD also indicated higher expression of all RGL sequences in inoculated resistant lines compared to inoculated and uninoculated susceptible lines (data not shown).

Healthy leaves of all of the resistant lines NE-BFG-11-3625, NE-BFG-5-2973, NE-BFG-5-2964, and NE-BFG-5-2974, with the exception of 609, showed higher expression of P1 transcripts (Figure 3a) compared to the susceptible lines. P2 expression was higher in all of the resistant cultivars except for NE-BFG-5-2974 (Figure 3b). NE-BFG-5-2974 grouped with the resistant lines and 378, a susceptible line. Expression of P2 in NE-BFG-5-2974 was higher than in the other two susceptible lines, Prestige and NE-BFG-5-3009. P6 expression was similar to P2 with each of the resistant cultivars except for NE-BFG-5-2974, exhibiting higher level of expression compared to the susceptible lines (Figure 3c). The expression of P6 in NE-BFG-5-2974 was similar to resistant lines NE-BFG-11-3625 and NE-BFG-5-2964 but lower than 609 and NE-BFG-5-2964 (Figure 3c).

Discussion

In this study, we performed transcriptional analysis of NBS-LRR-type candidate resistance genes in resistant and susceptible buffalograss in response to the leaf spot fungus *C. inaequalis*. The expression data was analyzed according to a randomized complete block (RCBD) design and each 96-well plate was considered as a statistical block represented by a biological sample of each line.

The RCBD design is more suitable over CRD since qRT-PCR is a sensitive method and the analysis can be influenced by plate-to-plate variation and slight differences in reaction mixture due to other factors, such as pipetting errors. Although there were expression differences among biological replicates, we did not observe extreme deviations among them. Detection of gene expression was carried out using TaqMan® assays since specific hybridization between probe and target is required to generate fluorescent signal which significantly reduces background noise and false positives. This eliminates the post-PCR processing of generating a melt curve as used for SYBR® Green detection dye chemistry. One of the assumptions in the comparative CT method is that efficiency of the PCR is close to one (1) and the PCR efficiency of the target genes is similar to the internal control gene (Livak and Schmittgen, 2001). Since it was not practical to calculate the efficiency of all samples by plotting graphs for different concentrations of the templates, the PCR efficiency was approximated by the shape of the logarithmic PCR amplification plot (Schmittgen and Livak, 2008). All of our reactions produced near identical shaped amplification graphs demonstrating similar PCR efficiency.

The two susceptible lines Prestige and NE-BFG-7-3453-50 did not exhibit a change in expression for the tested RGL amplicons P1, P2, P5, and P6, in response to the pathogen (Figure 1). However, expression levels in both inoculated resistant lines (95-55 and NE-BFG-7-3459-17) exhibited higher expression of P1 when challenged by *C. inequalis* (Figure 1). Additionally, P2 was upregulated in 95-55 in response to the pathogen. These results indicate the possibility of an incompatibility reaction taking place between the resistant plants and the pathogen. In resistant plants, pathogen recognition triggers a complex host defense response at infection sites including the production of reactive oxygen intermediates, nitric oxide (NO) and salicylic acid, cell-wall modifications, production of antimicrobial metabolites/proteins, and programmed cell death (Jones and Dangl, 2006; Nimchuket *et al.*, 2003; Radwan, 2009). Though further studies are necessary, in our study, one or more of these activities mediated by RGL sequences may have conferred leaf spot resistance in buffalograss. Jones and Dangl (2006) explained the ultimate amplitude of disease resistance or susceptibility by the equation $[PTI - ETS + ETI]$. Where PTI is the immunity gained by plants in response to PAMPs in pathogens, which is the first stage of immunity. ETS and ETI are effector triggered susceptibility and effector triggered immunity, respectively.

This is the second phase of immunity plants show in response to effector molecules of pathogens that can overcome phase one resistance of plants mediated by PTI. Activity of NBS-LRR proteins plays a greater role during this second phase of plant defense. Though little is known about the regulation of the plant genes that encode NBS-LRRs, basal level of these proteins in healthy plants may predispose the plants for resistance. Many NBS-LRR genes are constitutively expressed at low levels in healthy, unchallenged tissue, therefore, primary mRNA amounts, in addition to the rate, and the level of induction may contribute to the rapid response to pathogen attack (McHale *et al.*, 2006; Zarandi *et al.*, 2011).

Since the expression of RGL sequences in different buffalograss lines (Figures 1a to 1d) are independent of each other, and therefore, it is not possible to compare the basal expression level (i.e. the expression level of uninoculated plants), we performed a two-way ANOVA for the factors disease resistance or susceptibility and inoculated or uninoculated with one resistant and one susceptible cultivar as warranted by the experimental design. This was followed by mean separation using the Dunnett's test with the uninoculated susceptible lines used as the control (Figure 2). These preliminary comparisons indicate higher basal expression of P1 in 95-55 and higher expression of P2 and P6 in NE-BFG-7-3459-17 resistant lines compared to the susceptible lines (Figure 2 and Supplementary Figure 2). Similar results have been reported from varieties of wheat resistant to *Fusarium* head blight caused by *F. graminearum* (Subramaniam *et al.*, 2009). In wheat, the basal expression levels of a few receptor-like kinases (RLK) and NBS-LRR genes were significantly higher in resistant than in susceptible plants, possibly contributing to resistance.

To validate the higher basal expression levels of P1, P2, and P6 further in buffalograss, we tested the expression levels of these genes in healthy, uninoculated leaves of eight lines previously identified as being either resistant or susceptible to leaf spot. None of the amplicons consistently had higher expression in all of the resistant lines compared to the susceptible lines, but a clear trend of elevated expression of these genes were observed in the resistant germplasm (Figure 3). For example, 609, a resistant line, grouped with susceptible lines (Figure 3a) when P1 expression was assayed. It is interesting that under uninoculated conditions P1, P2, and P6 had higher expression in 80% (4/5) of the resistant lines (Figures 3a, 3b and 3c). Interestingly, if data from all three primer pairs tested were grouped together, the level of leaf spot resistance could be predicted based on the expression of these genes.

The use of these genes as a tool for predicting resistance/susceptibility needs further study since association based on eight resistant and susceptible individuals is insufficient for determining if any marker is diagnostic or predictive of a trait. Additionally, the NBS-LRR gene family consists of hundreds of members, including these tested RGLs, and it is therefore unlikely that these few gene products are the route of leaf spot resistance. However, if further testing reveals that these genes can distinguish leaf spot resistant and susceptible buffalograss genotypes, it could be used as a tool to accelerate breeding eliminating the need for time-consuming pathogenicity tests. It is important to note that prior to this study, the level of leaf spot resistance was not known for the experimental buffalograss lines NE-BFG-5-2973, NE-BFG-5-2964, and NE-BFG-5-2974. Based on the field evaluations and the results from the gene expression study, we now have confidence that these lines are resistant to leaf spot disease and they will be incorporated as germplasm into the buffalograss breeding program.

Our preliminary greenhouse leaf spot screen of 87 buffalograss lines showed different levels of resistance with the majority of lines falling in the moderately resistant category. It would be interesting to determine the level of P1, P2, and P6 expression in moderately resistant lines compared to highly resistant and susceptible lines. This test would provide more information on how successful these genes are at predicting leaf spot resistance. It would also be important to test temporal expression of these RGL sequences at different times following inoculation. In similar studies, chickpea genotypes resistant to *Fusarium* (Zarandiet *al.*, 2011), and sunflower lines resistant to rust and downy mildew (Radwan, 2009) exhibited peak expression levels of defense related genes a few days post inoculation of the relevant pathogen, and then decreased to low levels after the incompatible reaction. In our study, we tested expression of RGL sequences 10 days post inoculation and found that only P1 and P2 exhibited high levels of transcript abundance in the resistant line 95-55, while NE-BFG-7-3459-17 showed high expression levels of P1 only. It is possible that the expression of other RGLs studied already reached peak expression and were decreasing before transcript levels were measured.

Buffalograss leaf spot disease is caused by several species. In Nebraska, *C. inaequalis* and *B. spicifera* are the most common causal organisms (Amaradasa and Amundsen, 2013) while occasionally, *B. cynodontis* and *Alternaria* species also have been isolated from diseased leaves.

All of these species are closely related members of the Pleosporaceae family. In the present study we tested how seven previously described *R* genes behave in response to *C. inaequalis*, but it would be beneficial to study if these RGL sequences express differently when challenged with other leaf spot causal organisms. This information will help us understand if RGL sequences P1, P2, P5, and P6 are species specific or if they have broad activity against many pathogens. The experiments described here showed that transcription of P1 and P2 was induced during the incompatible interaction between the tested resistant buffalograss lines and *C. inaequalis*. The function of *R* gene products as receptors interacting with pathogen elicitors in host defense signaling has been suggested (Jones and Dangl 2006; Nimchuk *et al.*, 2003). Most NBS-LRR type gene expression studies have been done in economically important crops such as rice (Jia *et al.*, 2000) and sunflower (Radwan, 2009). Our study is a first and preliminary expression analysis of NBS-LRR type genes in buffalograss, an important amenity grass. Accurate identification of leaf spot resistant plants would accelerate our ability to develop leaf spot resistant buffalograss cultivars. This study is the foundation for future marker assisted selection of leaf spot resistance in buffalograss.

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