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RESEARCH

Molecular Characterization of cDNA Encoding Resistance Gene-Like Sequences in Buchlo ' dactyloides

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Abstract

Current knowledge of resistance (*R*) genes and their use for genetic improvement in buffalograss (*Buchlo' dactyloides* [Nutt.] Engelm.) lag behind most crop plants. This study was conducted to clone and characterize cDNA encoding *R* gene-like (RGL) sequences in buffalograss. This report is the first to clone and characterize of buffalograss RGLs. Amplified fragments of expected size were isolated and cloned using degenerate primers designed from the conserved motifs. Sequence analysis of cDNA clones and analysis of putative translation products revealed that most encoded amino acid sequences shared the similar conserved motifs found in the cloned plant disease resistance genes *RPS2*, *MLA6*, *L6*, *RPM1*, and *Xa1*. These results indicated diversity of the *R* gene candidate sequences in buffalograss. Analysis of 5' rapid amplification of cDNA ends (RACE), applied to investigate upstream of RGLs, indicated that regulatory sequences such as TATA box were conserved among the RGLs identified. The cloned RGL in this study will further enhance our knowledge on organization, function, and evolution of *R* gene family in buffalograss. With the sequences of the primers and sizes of the markers provided, these RGL markers are readily available for use in a genomics-assisted selection in buffalograss.

Index Entries: Buffalograss; cDNA; molecular evolution; resistance genes; RACE.

1. Introduction

Buffalograss (*Buchlo' dactyloides* [Nutt.] Engelm.) is a perennial, warm season, and sod-forming grass that is native to the shortgrass prairies of North America ***(1)***. It is an alternative turfgrass for home lawns, road sides, roughs, cemeteries, sport fields, and rangelands ***(2)***. Buffalograss has received attention since the early 1980s for use as a turfgrass species because of its drought resistance and relatively low maintenance requirement ***(3)***.

Chinch bugs (Hemiptera: Lygaeidae) *Blissus occiduus* Barber, has emerged as an important insect pest of buffalograss in Nebraska ***(4)***. In addition, mealybug (Homoptera: Pseudococcidae) (*Tridiscus sporoboli* and *Trionymous* spp.) insect pests are the most economically important pests of buffalograss ***(5)***. There are environmental and economical concerns about the increased use of pesticides and fungicides for buffalograss maintenance. The availability of insect-resistant transgenic plants is expanding rapidly, with research activity in the public and private sectors. However, target insects of transgenics belong to the orders Coleoptera, Lepidoptera, Homoptera, and Orthoptera ***(6)***. Transgenic plants have not been reported for insects belonging to the order Hemiptera. Therefore, enhancing disease and insect-resistance

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genotypes is a high priority for most buffalograss breeding and genetics programs ***(7)***.

Plant disease resistance often results from the presence of a specific *R* gene in the plant and a corresponding avirulence (*avr*) gene in the pathogen ***(8–11)***. There are a number of *R* genes conferring resistance to the major classes of pathogens, including bacteria, viruses, and fungi. It was reported that 75% of the cloned *R* genes were from *NBS/LRR* gene family ***(12)***. Some of these *R* genes are induced and some are constitutive ***(13)***. The *R* genes have been isolated from a wide range of plant species by map-based cloning on transposon tagging approaches, and were mostly determined by a single dominant gene ***(14,15)***. Sequence analysis of the predicted proteins of *R* genes revealed that common motifs occur in *R* genes from diverse origins and pathogen specificity ***(12)***.

The majority of *R* genes encode proteins with specific domains. The first key domain contains a conservative nucleotide-binding site (NBS), which is necessary for ATP-/GTP-binding proteins ***(16)***, and leucine-rich repeat (LRR) region at the C-terminus. The NBS domain comprises of kinase 1a, kinase 2, and kinase 3a motifs ***(17)***. The second domain, LRR domain, contains a number of repeated motifs of amino acids with leucine or other hydrophobic amino acids spaced at a regular distances ***(18,19)***. Others include those with an LRR domain and a serine–theronine protein kinase domain with no NBS domain, and a protein kinase domain without an NBS domain or an LRR domain or a coiled coil (CC) domain with a putative signal anchor at the N-terminus ***(20)***. The proteins of NBS/LRR domain can be differentiated based on their amino-terminal sequence, where they have either an N-terminal CC, or toll-interleukin receptor-like (TIR) domains ***(21–23)***. Homologs of *R* genes have been isolated and characterized by polymerase chain reaction (PCR)-based degenerate primers ***(7,24–26)***.

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Breeding for resistance based on inoculation and selection for phenotypic reaction is a challenging process, especially if *R* genes are tightly linked to undesirable traits. Molecular analyses of *R* gene sequences in plant species have increased our knowledge of the structure of complex *R* gene clusters, and enhanced our understanding of the possible mechanisms involved in their evolution ***(12)***. There is a lack of knowledge regarding *R* gene families and their evolution in buffalograsses. A better understanding of the molecular and genetic basis of genes conferring resistance to disease and insect would enhance the effectiveness of buffalograss breeding and genetics program.

In buffalograss breeding and genetics programs, map-based cloning or transposon-tagging methods have not been used for cloning *R* genes with distinct phenotype. The complex organization of buffalograss genome might make map-based gene cloning difficult. It comprises a polyploid series of diploid, tetraploid, pentaploid, and hexaploid with a basic chromosome number of 10 ***(27–32)***. However, effective use of these *R* genes (e.g., as molecular markers and elucidating organization and evolution of buffalograss genome complex) requires isolation and characterization of these genes. In this study, we report on the cloning, molecular characterization, and a better understanding evolution of a set of *R* gene candidate sequences in buffalograss using degenerate primers, based on conserved domains.

2. Materials and Methods

2.1. Plant Materials

A bulk leaf sample of "Bowie" buffalograss was used for isolation of cDNA encoding kinase 1a type *R* gene-like (RGL) sequences. The genotypes were planted in 15-cm diameter pots containing a soil mixture of 35% peat, 32% vermiculite, 9% soil, and 24% sand (v/v). A nutrient solution (21N–1.5P–12.5K) containing 200 mg/L nitrogen was applied biweekly until the soil was saturated. The greenhouse was maintained at 25° ± 1°C with supplemental light supplied by metal halide lamps on a 15/9-h photoperiod (Sylvania Co., Danver, MA).

2.2. Degenerate Primer Design

Five oligonucleotides were designed based on highly conserved motifs, kinase 1a and hydrophobic (GLPL) domains of NBS/LRR type *R* genes (**Table 1**) for PCR amplification and cloning of

Table 1

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Table 1
Degenerate Primer Sequences Used for Amplification of Buffalograss

Primers	Domain sequence	Primer sequence[a]	Reference
RGL1	GGVGKTT	5'-ggA ggg gTT ggg AAR AcA Ac-3'	
	GLPLAL	5'-ccH Acg ccR ATg gAW gAc c-3'	
RGL2	GSGKTT	5'-ggR AcT ggN AAR AcN Ac-3'	
	LPLGL	5'-AAA Agc ccA AAN ggg AAA-3'	
RGL3	GSGKTT	5'-ggI TcN ggN AAg AcN Ac-3'	This study
	LPALGL	5'-AAA gRc cNA AgN ggN AAg-3'	
RGL4	GGIGKTT	5'-ggg AcgTg ggg AAg Acg Ac-3'	
	GLPFAL	5'-Agg gcT Agg ggg Agg ccc gcc-3'	
RGL5	GSGKTT	5'-ggT AcT ggN AAR cNA Ac-3'	
	LPLGL	5'-gAA gNc cNA AgN ggN AAg Ac-3'	
LM638	GGVGKTT	5'-ggI ggI gTIg gIA AIA cIA c-3'	*25*
LM637	GLPLAL	5'-A(A/g)I gcT A(A/g)I ggI A(A/g)I cc-3'	

[a]H = A, C, T; N = A, C, G, T; R = A, G; W = A, T; I; Inosine.

Au: EST = expressed sequence tag?

RGL sequences from buffalograss. Kinase 1a and hydrophobic peptide sequences were obtained from known *R* genes and expressed sequence tag (EST) databases of rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and barley (*Hordeum vulgare* L.). These sequences were aligned and the most conserved domain sequences were determined. Codon preferences from the EST sequences were identified where domains had identical peptide sequences.

2.3. RNA Isolation

Without adding liquid nitrogen, 200 mg of leaf tissue was ground with 1.5 mL of Trizol® reagent. Using a wide-bore pipet tip, 1 mL of liquid was poured into an Eppendorf tube, which was kept on ice while processing the other samples. After processing all the samples, the tubes were incubated at room temperature for 10 min; 0.4 mL of chloroform was added, and the tubes were shaken and let stand at room temperature for 5 min. Then, the samples were centrifuged at 12,000 rpm for 15 min at 4°C (Eppendorf, 5415D). The RNA was in the upper layer, which was transferred to a fresh tube. After chloroform extraction, 0.5 mL of isopropanol was added to precipitate the RNA. Samples were then incubated at room temperature for 10 min and spun at 12,000 rpm for 10 min at 4°C. The RNA pellet was washed with 1 mL of 75% ethanol after centrifugation. Samples were mixed by vortexing and spun at 7500 rpm for 5 min at 4°C. The RNA pellet was dried at room temperature for 10 min and placed in 20–50 µL of formamide, depending on the size of the pellet, and allowed to sit in the 55°C water bath for 1 h to improve suspension.

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2.4. cDNA Cloning and Sequencing

Total RNA from the bulk leaf sample was reverse transcribed using an Omniscript RT kit (Qiagen). First-strand cDNA was synthesized from the poly(A)+ RNA with Moloney murine leukemia virus (MMLV) reverse transcriptase. Degenerate primers designed for the sites of kinase 1a (GVGKTT) and hydrophobic domain (GLPLAL) (**Table 1**) were used to amplify and clone the cDNA. A diagram of the organization of the *R* genes and region aiming to clone are depicted in **Fig. 1**. Amplifications were carried out as combinations of kinase 1a primers versus hydrophobic domain. Amplification of cDNA fragments was performed in 20-µL PCR reactions. Each reaction mixture contained 2 µL of first-strand cDNA, 2 µL of 10× PCR buffer without $MgCl_2$, 2.5 m*M* $MgCl_2$, 200 µ*M* dNTP mix, 1 µ*M* of forward primer, 1 µ*M* of reverse primer, 1.25 U *Taq* DNA polymerase. Amplifications were carried out using an MJ Research PTC-100 thermocycler

Fig 1

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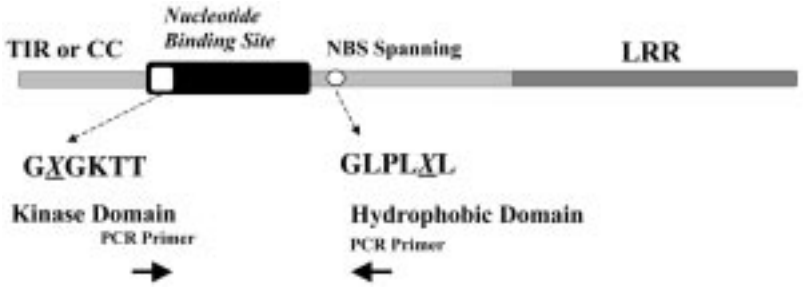

Fig. 1. A diagram for predicted protein motifs of NBS-LRR-type resistance genes. Arrows point to the region designed primers to clone. GXGKTT and GLPLXL are highly conserved domains.

programmed for 32 cycles of 1 min at 94°C; 1 min at 50°C; 1 min at 72°C; followed by a final extension at 72°C for 5 min before cooling to 24°C. The PCR products obtained from first-strand cDNAs synthesized from total RNA of plants were size-fractioned in 12% polyacrilamide gel using a Hoefer vertical-gel apparatus (SE600).

Amplified fragments were excised from gels and gel extraction was performed using a Qiaquick® Gel Extraction Kit (Qiangen), inserted into PGEM®-T Easy Vector System I (Promega) and propagated in *Escherchia coli* grown in Luria Bertani (LB) medium containing 100 µg/mL ampicillin (LBA). Three clones per fragment were selected for the sequencing.

2.5. Rapid Amplification of cDNAs

To amplify the corresponding full-length cDNA, 5'prime end of cDNA were obtained by geneRacer kit (Invitrogen). Gene-specific primers and nested gene-specific primers were designed based on the sequence information obtained from cDNAs. The rapid amplification of cDNA ends (RACE) reactions were carried out according to the manufacturer's protocol. Each amplification reaction was cloned into BlueSCript II KS (+), (Stratagene, LaJolla, CA) for sequence analyses.

2.6. cDNA Sequence Analysis

The cloned cDNA sequences were first exposed to a VecScreen algorithm (http://www.ncbi.nlmn.nih.gov/) to remove vector contamination. Sequences were then grouped at 90% nucleotide identity level and the longest sequence from each group was used for further analysis. The BLAST algorithm ***(33)*** was used to analyze the DNA sequences (http://www.ncbi.nlm.nih.gov). The identified RGL sequences were compared with the known *R* genes to show their structural resemblance. The known *R* genes belonging to NBS/LRR type were presented in **Table 2**. The ClustalW algorithm (Vector NTI version 9.0) was used to identify the motifs. Exon prediction was conducted using gene finding programs GENESCAN (http://genes.mit.edu/GENSCAN.html**)** and GENEMARK (http://opal.biology.gatech.edu/GeneMark/eukhmm.cgi) as outlined by Budak et al. ***(34)***. All sequences were run against *Arabidopsis thaliana*.

Table 2

Table 2
Source of Known NBS-LRR-Type *R* Genes Used for Comparisons
With cDNA Clones Identified From Buffalograss

Plant species	*R* genes	Protein identity	References
Wheat	CRE3	AAC05834	*46*
Barley	MLA6	CAC29241	*47*
Rice	XA1	BAA25068	*48*
	RPR1	BAA75812	*49*
Arabidopsis	RPP1	AAA21874	*50*
	RPS2	AAA21874	*51*
	RPS5	AAC26126	*52*
	RPP8	AAC83165	*53*
	RPM1	CAA61131	*54*
Flax	L6	AAD25965	*55*

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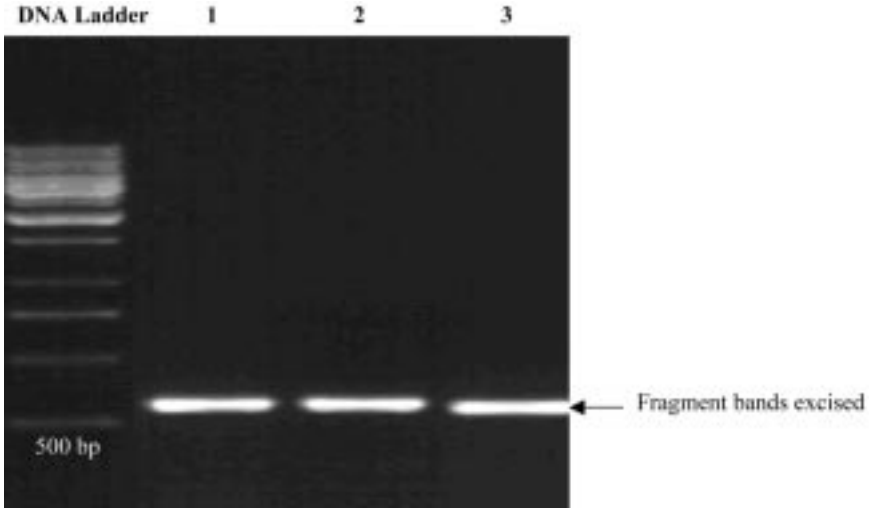

Fig. 2. Amplified cDNA PCR products with designed degenerate primer RGL1 from (lane 1) Bowie, (lane 2) NE95-2, and (lane 3) Tatanka. Arrow points to the band excised and cloned.

3. Results and Discussion

The combinations of degenerate primers (**Table 1**) were used for cDNA amplifications of RGL sequences. The resulting sizes of the PCR products were excised from the gel depicted in **Fig. 2**. The 30 clones were isolated and sequenced. A GenBank search, performed with BLASTX algorithm ***(33)***, resulted in homology with the known or putatively annotated NBS/LRR type of *R* genes that had been cloned from other plant species with E values of no more than 10–21 to no more than 10–9. There were also NBS/LRR-type cloned *R* genes, pathogen-related (proteases and kinases), ATP- and GTP-, and also stress-related kinases. Seven sequences found to be structurally similar to kinase-1a-type genes were considered for further analysis. The remaining sequences with no motif showed similarity to pathogen-related (proteases, phosphates, peroxidase, transcription factor, and kinases) sequences; these were not analyzed further. One should be cautious about the number of RGL sequences matches, because nucleotide sequences of clones were first translated into protein and searched against both translated and protein databases, which increases the probability of error in the search. Some of the sequences isolated did not match to plant NBS/LRR *R* genes in intervening regions. This response would be expected because intervening sequences are highly variable compared to kinase 1a domain.

Multiple alignments of predicted amino acid sequences using ClustalW (default values) algorithm were performed with seven sequences of RGL (GenBank accession nos. AY970295, AY970294, AY966896, AY966895, AY966893, AY966892, and AY971604) and known *R* gene sequences (**Fig. 3**). Of the 30 clones, 7 had partial or overall matches to kinase 1a type *R* genes at mostly conserved domains. Kinase 1a motif was shared by other protein families ***(35)*** and contains 12 different subfamilies representing unique structure ***(36)***. AY970294 and AY971604 had consensus motif residues of GIGKTT for kinase 1a domain and were similar, as found in the *R* genes. However, because of the relatively low number of primer combinations used in this study, the RGL sequences isolated may represent only a subset of the NBS/LRR-type sequences found in buffalograss. This indicates that different kinase 1a primers designed for the 5' site with the combination of various gene specific primers for the 3' site increase the number of different clones.

To predict their functionality, the sequences of all cloned RGLs were further analyzed by GENESCAN (http://genes.mit.edu/GENSCAN.

Fig 2

Fig 3

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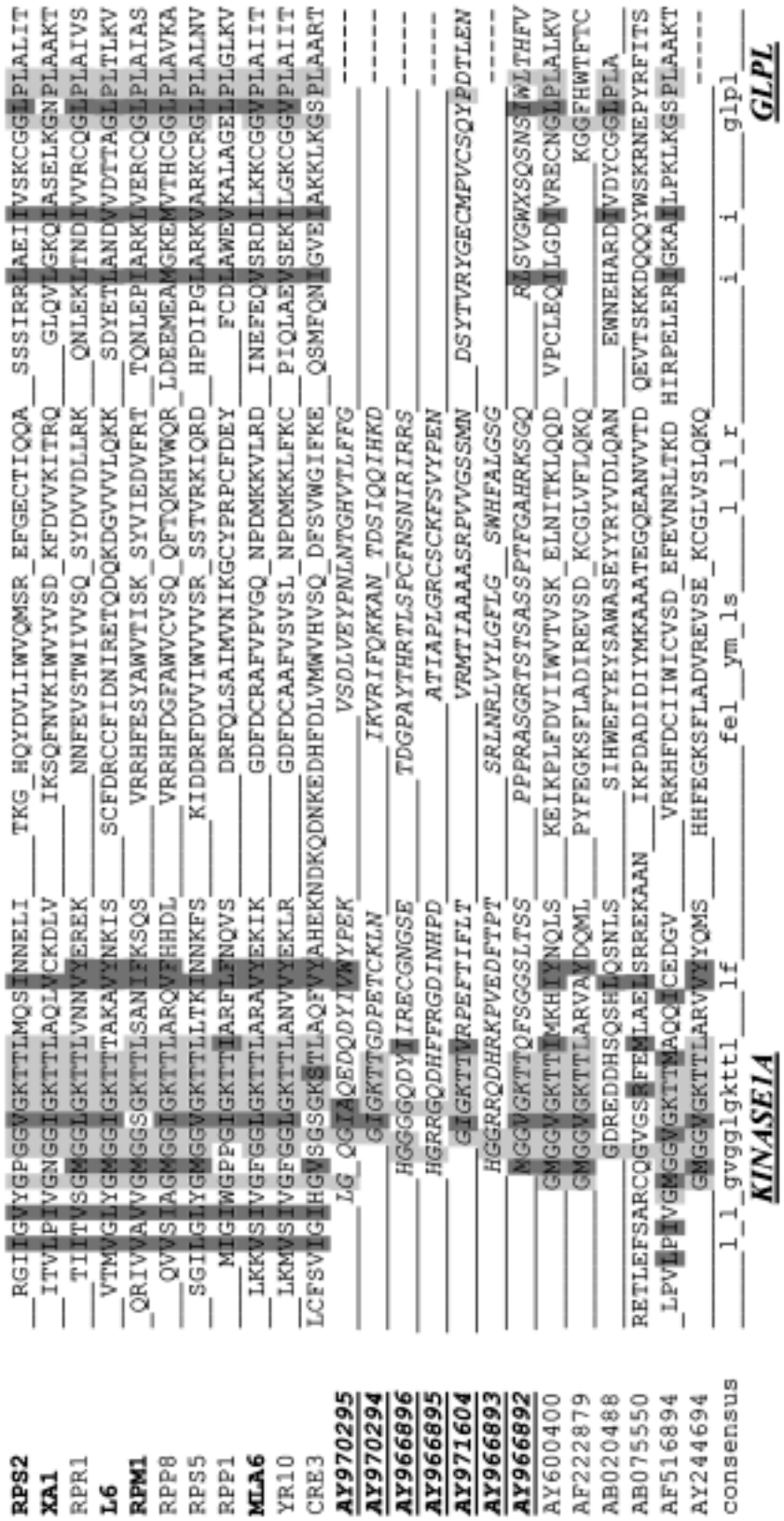

Fig. 3. Protein alignment of resistance genes and previously identified proteins. The underlined sequences belong to buffalograss.

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html) and GENEMARK (http://opal.biology.gatech.edu/GeneMark/eukhmm.cgi) gene prediction programs ***(34)***. Both programs showed that the two of them had no open reading frame, indicating that it might be the result of frame shift mutations or premature stop codons. It might also be the result of the effect of both stop codons and shift mutations. In this study, approx 29% of RGL are likely to be pseudogenes. The pseudogenes ratio was 10% in *Arabidopsis* ***(37)***, 11% in soybean ***(25)***, and 9.3% in tomato ***(22)***. Ploidy and genome organization are also important factors that effect the accumulation of pseudogenes in *Buchlo'*. A diploid genotypes ($2n = 2x = 20$) used in this study had one pseudogene. However, tetraploid, pentaploid, and hexaploid ($4n = 4x = 40$, $4n = 5x = 50$, and $6n = 6x = 60$, respectively) had three, three, and five pseudogenes. This might indicate that the RGL subfamilies, which have less pseudogenes when compared to the mean of identified RGL, may be at the evolving stages. In contrast, RGL subfamilies, which have a number of pseudogenes may be at the degenerative stages.

Au: specify them in " of them had no ORF"; ORF = open reading frame?

The 5'-RACE analyses were performed to investigate the 5' upstream, untranslated region (5' UTRs) of the identified *R* gene-like sequences in *Buchlo'* species. This analysis helped locate translation start codon position more precisely. Twelve distinct 5'-RACE products were isolated. Sequence analysis of the identified RACE in this species showed a high level of similarity, ranging from 75 to 98%. The 5'-RACE products also indicated that an average size of 80 bp for the 5'-UTR of all transcripts identified from this species. Analysis of 5' upstream region indicated that all regulatory elements such as TATA box were conserved among the sequences.

The RGL sequences cloned from buffalograss were highly divergent at the nucleotide and amino acid levels. This indicates that buffalograsses contain a large and diverse family of genes for proteins within this domain. Our results indicate that conserved domains of *R* genes cloned from a wide range of plant taxa can be used to isolate RGL sequences or *R* gene candidates in buffalograss germplasm. The PCR-based strategy performed in this study was successful. However, there is likelihood that we sequenced some pseudogenes that have structural similarities to the *R* genes. Several researchers have demonstrated that sequencing of paralogs of *Xa21*, *Cf9*, and *Dm3* indicated the presence of pseudogenes ***(38–40)***. Only one functional *R* gene identified in 800 *R* gene analogs from 20 plant species was reported ***(41)***.

Cloning and sequencing of a set of *R* gene candidates in buffalograss were reported by PCR using degenerate primers in this study. The RGL sequences isolated from buffalograss appear to be part of a multigene family. These results indicate that the PCR-based strategy is also useful for systematically searching for RGL sequences in buffalograss and our previous work indicates that it can be applied to other turfgrass species as well ***(7)***. Markers designed by using conserved domains can be used in linkage maps to trace *R* genes. Initial screening of potential diploid buffalograss parents at a convenient ploidy level revealed considerable polymorphism for RGL markers designed in this study. Hence, the RGL markers designed (**Table 1**) have potential in buffalograss breeding programs through marker-assisted selection. Tracing *R* genes using these markers is much faster and less expensive when compared to other DNA-based methods such as restriction fragment length polymorphism (RFLP). For instance, integrated rust *R* genes in wheat were detected using *R* gene analog-derived PCR methods ***(42)***. Although we have no evidence from genetic linkage mapping that these sequences are linked to any diseases or abiotic stress *R* genes in buffalograsses, map positions of cloned *R* genes appear to be well conserved ***(22)***. Conserved linkages were reported in several plant species ***(24,43–45)***. Furthermore, the markers designed in this study could be used in buffalograss breeding programs for indirect selection or map-based cloning of related gene families, which might govern quantitative traits for disease and insect resistance.

Au: RGA = R gene analog?

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Au: ref 34: location of conference?

Au: ref 42: initial for Sivasithamparam?