Identification of a Gene in the Process of Being Lost from the Genus Agrostis\textsuperscript{1[w]}

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Lineage-specific gene loss is considered one of the processes contributing to speciation and genome diversity. Such gene loss has been inferred from interspecies comparisons of orthologous DNA segments. Examples of intraspecific gene loss are rare. Here we report identification of a gene, designated Crs-1, that appears to be in the process of being lost from heterozygous populations of the species creeping bentgrass (\textit{Agrostis stolonifera}). The Crs-1 gene encodes a protein with an N-terminal dirigent protein domain and a C-terminal lectin domain and is similar to the maize (\textit{Zea mays}) \(\beta\)-glucosidase aggregating factor. Most individual creeping bentgrass plants examined are lacking Crs-1. Some individuals are hemizygous for the Crs-1 locus, indicating major haplotype noncolinearity at that locus. Crs-1 was not detected in several other Agrostis species, indicating it is being lost from the genus. The Crs-1 locus in creeping bentgrass provides a rare example of the evolutionary process of gene loss occurring within a plant species.

Lineage-specific gene loss is considered an important phenomenon in eukaryotic evolution (Aravind et al., 2000; Krylov et al., 2003). Genome-scale analyses of gene loss have evaluated the extent of loss between species and how this contributes to the attributes of the species. Comparative analyses of the available eukaryotic protein sequences revealed that \textit{Saccharomyces cerevisiae} has lost about 300 genes since its divergence from its common ancestor with \textit{Schizosaccharomyces pombe} (Aravind et al., 2000). Analysis of HOX genes in seven nematode genera revealed an apparent loss of five genes in the model organism \textit{Caenorhabditis elegans} (Aboobaker and Blaxter, 2003). Numerous lineage-specific losses of Tyr kinases were found among eukaryotic species (Shiu and Li, 2004). Lineage-specific gene losses from plant mitochondrial and chloroplast genomes have also occurred (dePamphilis et al., 1997; Palmer et al., 2000). Gene loss is thus one of the mechanisms involved in the generation of genome diversity among eukaryotic species.

Within a species, much of the phenotypic and genotypic diversity can be attributed to allelic variation, both within coding sequences and in the regulatory regions (Buckler and Thornsberry, 2002; Lo et al., 2003; Guo et al., 2004). Loss of function alleles are often due to point mutations or indels that result in non-functional coding sequences or changes in regulation of expression, but the DNA region is often retained in the genome. Total gene loss through deletion of genomic segments is often the underlying basis for some human disease syndromes (Inoue and Lupski, 2002). Total gene loss (loss of the DNA region) within a species that does not result in obvious deleterious phenotypes is presumably also occurring at some frequency. Logically, prior to a gene being completely lost from a species, there must be some period of time when the species is composed of a mixture of individuals that have or do not have the gene. Presumably, such a situation must exist for some genes in extant species. A surprising example of apparent gene loss among individuals within a species comes from the sequence comparison of the \(bz\) genomic region in two maize (\textit{Zea mays}) inbred lines McC and B73. Ten genes are present in this region in McC, but only six in B73 (Fu and Dooner, 2002). The genes missing in B73 are obviously not essential, and paralogs present in other regions of the genome likely compensate for their loss (Fu and Dooner, 2002). A similar situation of differences in gene content in the zein 21C genomic region was observed in the maize inbreds B73 and BSS553 (Song and Messing, 2003). Recent information suggests that the apparent gene loss and haplotype noncolinearity observed between maize inbreds is actually a result of insertion of gene segments from elsewhere in the genome (Brunner et al., 2005; H. Dooner, personal communication).

There are few specific examples of widespread gene loss occurring within a species. As part of our investigations into the disease responses of creeping and colonial bentgrasses (\textit{Agrostis stolonifera} and \textit{Agrostis capillaris}, respectively), we unexpectedly identified a gene in creeping bentgrass that is apparently in the process of being lost from the species. Creeping and colonial bentgrasses are economically important turfgrass species that are used extensively on golf courses.

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in temperate regions (Warnke, 2003; Riemmele, 2003). Creeping bentgrass is highly susceptible to the fungal pathogen *Sclerotinia homoeocarpa* F.T. Bennett, the causal agent of dollar spot disease. The related species colonial bentgrass exhibits good resistance to the disease (Plumley et al., 2000) and may be a source of genes or alleles that could be used for genetic improvement of creeping bentgrass. Fertile interspecific hybrids between creeping bentgrass and colonial bentgrass could be recovered at low frequencies and some of the hybrids exhibited excellent dollar spot resistance (Belanger et al., 2003a, 2003b, 2004). To begin the investigation into the basis of the dollar spot resistance in the interspecific hybrids, suppression subtractive hybridization was used to generate subtracted cDNA libraries enriched for genes unique or overexpressed in either one of the resistant interspecific hybrids or its susceptible creeping bentgrass parent. Genes overexpressed in the dollar spot susceptible creeping bentgrass parent were of interest since they may be part of the response system of creeping bentgrass to the fungal pathogen. Surprisingly, one of the creeping bentgrass cDNAs isolated from this approach is from a gene that appears to be in the process of being lost from the creeping bentgrass genome. Presence of the gene was not detected in several related Agrostis species, suggesting it is being lost from the genus. A few similar genes have been reported from other grass species, but the functions of the encoded proteins have not been resolved.

**RESULTS**

**Isolation of *Crs-1* cDNA**

Suppression subtractive hybridization (SSH) was used to generate subtracted cDNA libraries that were enriched for sequences overexpressed in either a dollar spot susceptible creeping bentgrass plant (plant 5061) or one of its dollar spot resistant interspecific hybrids with colonial bentgrass (hybrid no. 15). Plant 5061 is a transgenic plant expressing the *bar* gene that confers resistance to the herbicide glufosinate. In a cross between creeping bentgrass plant 5061 and a colonial bentgrass plant, transmission of the herbicide resistant phenotype to progeny of the colonial bentgrass plant was used to identify the interspecific hybrids, one of which was interspecific hybrid number 15 (Belanger et al., 2003b). Several clones from the subtracted cDNA libraries were identified from reverse dot-blot as cDNAs that were overexpressed in the creeping bentgrass parent relative to the interspecific hybrid progeny plant. The suppression subtractive hybridization procedure includes a restriction enzyme digestion of the cDNAs produced, so none of the clones obtained from the resulting libraries were full length. RACE-PCR of one of the clones (designated *Crs-1* for creeping specific-1) identified from the creeping specific library was used to determine the full-length coding sequence.

The 1,283-bp sequence contained an untranslated 5′-upstream sequence of 60 bp, an open reading frame of 960 bp, and an untranslated 3′-sequence of 263 bp. A 319-amino acid protein with a molecular mass of 34,753 D is predicted from the cDNA sequence. The DNA and deduced amino acid sequences of *Crs-1* and the positions of the PCR primers used are shown in Supplemental Figure 1. Searches of the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) revealed some significant matches to similar amino acid sequences reported from other grass species. A comparison of the deduced amino acid sequence of the creeping bentgrass *Crs-1* clone with that of the similar wheat (*Triticum aestivum*) WCI-1 sequence is shown in Figure 1. The WCI-1 gene was induced in response to treatment with benzothiadiazole, an inducer of systemic acquired resistance (Gorlach et al., 1996). All of the sequences similar to *Crs-1* have an N-terminal dirigent protein domain (formerly designated a disease responsive domain) and a C-terminal jacalin-like lectin domain, as identified using the Conserved Domain Architecture Retrieval Tool (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rgs; Marchler-Bauer et al., 2003). These proteins likely originated from a fusion of a dirigent protein coding sequence with that of a lectin since numerous proteins have been reported from many plant species as either dirigent proteins or lectins. Dirigent proteins are involved in controlling stereoselective coupling of monolignols in the formation of lignans (Davin and Lewis, 2000). Jacalin-like lectins bind to Gal or Man residues (Raval et al., 2004). The functions of the proteins similar to creeping bentgrass *Crs-1* are unclear, but several were isolated through differential screens investigating the plant’s response to pathogen attack, as was *Crs-1* (Gorlach et al., 1996; Lee et al., 1996a; Williams et al., 2002).

Phylogenetic analysis comparing the nine similar amino acid sequences illustrates the relationship of the creeping bentgrass *Crs-1* sequence to the similar sequences reported from other species (Fig. 2). The nine other protein sequences included in the phylogenetic analysis in Figure 2 are the only ones reported to have both the dirigent protein and lectin domains fused in one coding sequence. Maximum parsimony analysis of the amino acid dataset was based upon 379 total characters, of which 92 were constant, 110 variable characters were parsimony uninformative, and 177 characters were parsimony informative. Gaps were treated as missing data. The tree was rooted with the maize sequence designated as the outgroup.

**Induced Expression of *Crs-1***

To confirm that *Crs-1* was overexpressed in creeping bentgrass plant 5061 relative to the interspecific hybrid number 15, the expression of *Crs-1* in the field-grown plants was evaluated by RNA blots (Fig. 3). Also included in the analysis was an unrelated colonial
bentgrass plant in the same field test. RNA was isolated from the plants before inoculation with the pathogen and at 56- and 82-d postinoculation. 

**Crs-1** message was detected only in the 5061 sample and was at higher levels in the 56- and 82-d postinoculation samples, times when the plants were exhibiting disease symptoms (Belanger et al., 2004). Dollar spot symptoms generally begin to develop several weeks after inoculation. The environmental conditions in 2002 were not optimal for disease development, and consequently the symptoms were milder than usual and took longer to develop.

Some Creeping Bentgrass Plants Are Hemizygous for **Crs-1**

DNA-blot analysis was performed to determine if the observed differences in expression were due to absence of the gene in the interspecific colonial × creeping bentgrass hybrid and the colonial bentgrass plant or to differences in regulation of expression. DNA samples included in the analysis were the creeping bentgrass parent (5061) and two of its progeny interspecific hybrids (hybrid nos. 14 and 15), as well as DNA from individual plants of two creeping bentgrass cultivars (L93 and Crenshaw), a colonial bentgrass plant, and individual plants of other *Agrostis* spp. (*Agrostis gigantea* Roth), and dryland bentgrass (*Agrostis castellana* Boiss. and Reut.). The **Crs-1** gene was present only in the creeping bentgrass parent 5061 and one of its progeny, interspecific hybrid number 14 (Fig. 4). No signal was detected in any of the other samples.

Two major hybridizing bands, at approximately 4.5 kb and 2 kb, and a faint band at about 12 kb were seen in the **SpeI**-digested 5061 and hybrid number 14 DNA samples. Bands of these sizes could not be explained by a single copy of the **Crs-1** gene. Genomic PCR using primers at the 5′ and 3′ ends of the cDNA sequence yielded an amplified fragment of approximately 1,600 bp (data not shown). Since the cDNA sequence between the primers used was 1,150 bp, about 450 bp in the amplified fragment obtained from the DNA could be attributed to the presence of one or more introns. There is an **SpeI** site just 36 bp downstream of the region of the RACE-PCR clone used as the probe, so even if there were an **SpeI** site within an intron generating two fragments to which the 1.1 kb probe could hybridize, one of the fragments generated would be considerably smaller than the smaller hybridizing band of 2 kb. Therefore, the two major hybridizing bands must indicate the presence of at least two **Crs-1** genes. The lack of any signal in the interspecific hybrid number 15 and the presence of both bands in interspecific hybrid number 14 precluded the possibility that the two bands in the parent plant 5061 originated from two alleles of the **Crs-1** gene.

The absence of the gene in interspecific hybrid number 15 and its presence in interspecific hybrid number 14 was surprising and suggested that the two **Crs-1** genes were closely linked and were inherited together and that the parent plant 5061 was hemizygous for the **Crs-1** locus. Closely linked gene duplications are known to occur in plant genomes (Chen et al., 1998; Dubcovsky et al., 2001). Segregation of the **Crs-1** gene in other interspecific hybrid progeny from 5061, as assessed by PCR, supported this interpretation. In the analysis of eight interspecific hybrid progeny originating from 5061, five individuals had the **Crs-1** gene
and three did not, suggestive of a 1:1 segregation (data not shown). The sample size is small because in the original crosses between 5061 and a colonial bentgrass plant, only 12 interspecific hybrids were recovered (some later died; Belanger et al., 2003b).

Segregation analysis of Crs-1 in another population originating from a controlled cross revealed that another creeping bentgrass plant, not related to 5061, was also hemizygous for the Crs-1 locus. In 2002, the interspecific hybrid number 15 was crossed with a creeping bentgrass plant, 9188. From PCR, 9188 was determined to possess the Crs-1 gene. Individuals from the backcross population were examined for presence of the Crs-1 gene by PCR using primers designed from within the coding sequence (see Supplemental Fig. 1 for positions of the primers), and 18 out of 37 were positive for the Crs-1 gene (data not shown). DNA-blot analysis was carried out on nine PCR-positive and nine PCR-negative individuals of the backcross population. The creeping parent plant of the population, 9188, had a hybridization pattern that was identical to that of 5061, as did all the individuals scored as positive from the PCR analysis (Fig. 5). There was no hybridization signal from the individuals scored as negative from the PCR analysis. Since the other parent of the population, hybrid number 15, did not carry the Crs-1 gene (Fig. 4), the 1:1 segregation in the progeny indicated that 9188 was hemizygous for the Crs-1 locus, as was creeping bentgrass plant 5061.

The Crs-1 Locus Is Absent from Many Creeping Bentgrass Individuals

The lack of the Crs-1 gene in the two creeping bentgrass individuals from the cultivars L93 and Crenshaw (Fig. 4) was surprising since most genes would be expected to be present in all individuals within a species, although allelic variation among individuals would be expected. Since some creeping bentgrass individuals are apparently hemizygous for Crs-1, null genotypes could originate from crosses between two hemizygous individuals.

Agrostis spp. are self-incompatible, wind-pollinated species. Cultivars are developed from a varying number of selected parent plants that are interpollinated. Cultivars are therefore composed of related, but genotypically unique, individuals. Because of the genotypic heterogeneity of bentgrass cultivars, the lack of the Crs-1 gene in a single individual from a cultivar does not mean the gene is completely lacking from that cultivar. Similarly, the lack of the gene in individuals of colonial bentgrass, velvet bentgrass, redtop bentgrass, and dryland bentgrass seen in Figure 4 does not mean the gene is necessarily absent from those Agrostis spp.
To further investigate the distribution of the Crs-1 gene within creeping bentgrass cultivars and in other Agrostis spp., we performed DNA blots on bulked DNA samples representing 24 individuals. In a screen of eight creeping bentgrass cultivars, presence of the Crs-1 gene could be clearly detected in 3, Cobra, L93, and ProCup (Fig. 6), all with the same bands seen in 5061. A weak signal was obtained with the cultivar Pennlinks. The lower intensity signal from these samples relative to the control 5061 plant indicated that not all individuals in the bulk sample had the Crs-1 gene. The transgenic line 5061 originated from the cultivar Cobra, so it was expected that Crs-1 would be present in Cobra. No signal was detected from the cultivars Penn A-1, Crenshaw, Penncross, Penn G-2, or in two Plant Introduction (PI) accessions. No signal was detected from any of the other Agrostis samples tested (Table I). A. stolonifera, A. capillaris, Agrostis castelana, A. gigantea, and Agrostis canina are all commercially used species (Brede and Sellmann, 2003; Ruemmele, 2003; Warnke, 2003). Agrostis mongolica, Agrostis nebulosa, Agrostis pallida, Agrostis transscaspica, and Agrostis vinalis are wild species.

PCR amplification of a Crs-1 fragment from individual seedlings was used to estimate the frequency of the presence of Crs-1 in the cultivars Cobra and L93. Amplified products were seen in seven out of 24 individuals from Cobra and in 11 out of 24 individuals from L93 (Fig. 7A). Based on the cDNA sequence, the primers were expected to amplify a fragment of 587 bp. A fragment around 850 bp was observed, indicating the presence of an intron within that region of the coding sequence. As a positive control for the quality of the DNA preparations, PCR amplification of a ubiquitin-conjugating protein sequence was also carried out on the same samples. Primers were designed from a clone that was obtained from random sequencing from a creeping bentgrass cDNA library. An amplified fragment of the expected size of about 200 bp was seen in all samples (Fig. 7B).

**DISCUSSION**

Through our investigations into the responses to dollar spot infection in creeping bentgrass, we have fortuitously identified a gene that appears to be in the process of being lost from the genus Agrostis. Our differential screen was designed to identify genes that may be important in the responses of susceptible and resistant genotypes to pathogen attack. The expression of the Crs-1 gene was indeed induced after inoculation with the pathogen in creeping bentgrass plant 5061 used for the SSH. However, since no similar sequence exists in the interspecific hybrid used for the subtraction (hybrid no. 15), the isolation of the Crs-1 fragment from the SSH subtraction library was actually based on it being unique to the creeping bentgrass plant used. Surprisingly, the Crs-1 gene was not found in many other creeping bentgrass individuals sampled. Based on DNA-blot hybridization, no similar sequences could be detected in any of the other Agrostis spp. examined.

One possible explanation for the lack of Crs-1 in many creeping bentgrass individuals, and the haplotype noncolinearity observed in some individuals, could be gene introgression from an as-yet unknown source. Creeping bentgrass can form fertile hybrids with other Agrostis spp. (Belanger et al., 2003b). Gain of the Crs-1 locus from another species is thus a formal
some species within these tribes (Avena sativa [7,624], Dactylis glomerata [27], Lolium multiflorum [5,818], Poa secunda [30], Schiedanora arundinacea [79,392]). No Crs-1 homologs were found among these ESTs.

The presence of Crs-1 homologs in grass lineages ancestral to creeping bentgrass supports the interpretation that the Crs-1 gene is in the process of being lost from creeping bentgrass, but is still present in some individuals. In fact, the Crs-1 gene was not detectable in most of the creeping bentgrass plants examined here. The lack of Crs-1 in other Agrostis spp. suggests it is being lost from the genus. There are 220 species in the genus Agrostis (Watson, 1990) and we have surveyed only nine other species for the presence of Crs-1. It is certainly possible that other Agrostis spp., or other accessions of the species examined here, may carry the Crs-1 gene. Even so, the lack of Crs-1 observed here in several species indicates this gene is being lost from the genus. As more sequence data from other species becomes available it will be interesting to see if Crs-1 homologs are present in any other species within the Aveneae or Poeae.

### Table 1. DNA-blot detection of Crs-1 in bulked Agrostis samples consisting of DNA from 24 individuals

<table>
<thead>
<tr>
<th>Sample</th>
<th>Presence (+) or Absence (−) of Crs-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creeping bentgrass (A. stolonifera)</td>
<td>−</td>
</tr>
<tr>
<td>Penn A1</td>
<td>−</td>
</tr>
<tr>
<td>Cobra</td>
<td>+</td>
</tr>
<tr>
<td>Crenshaw</td>
<td>−</td>
</tr>
<tr>
<td>L93</td>
<td>+</td>
</tr>
<tr>
<td>Penncross</td>
<td>−</td>
</tr>
<tr>
<td>Penn G2</td>
<td>+</td>
</tr>
<tr>
<td>Pennlinks</td>
<td>−</td>
</tr>
<tr>
<td>Procup</td>
<td>+</td>
</tr>
<tr>
<td>PI 235440 (Switzerland)</td>
<td>−</td>
</tr>
<tr>
<td>PI 318934 (Spain)</td>
<td>−</td>
</tr>
<tr>
<td>Colonial bentgrass (A. capillaris)</td>
<td>−</td>
</tr>
<tr>
<td>SR7100</td>
<td>−</td>
</tr>
<tr>
<td>PI 252045 (Italy)</td>
<td>−</td>
</tr>
<tr>
<td>Dryland bentgrass (A. castellana)</td>
<td>−</td>
</tr>
<tr>
<td>Highland</td>
<td>−</td>
</tr>
<tr>
<td>PI302830 (Spain)</td>
<td>−</td>
</tr>
<tr>
<td>Redtop bentgrass (A. gigantea)</td>
<td>−</td>
</tr>
<tr>
<td>Streaker</td>
<td>−</td>
</tr>
<tr>
<td>PI 406637 (Canada)</td>
<td>−</td>
</tr>
<tr>
<td>PI 499394 (China)</td>
<td>−</td>
</tr>
<tr>
<td>Velvet bentgrass (A. canina)</td>
<td>−</td>
</tr>
<tr>
<td>SR7200</td>
<td>−</td>
</tr>
<tr>
<td>A. mongolica (PI 362190, Mongolia)</td>
<td>−</td>
</tr>
<tr>
<td>A. nebulosa (PI 196319, Germany)</td>
<td>−</td>
</tr>
<tr>
<td>A. pallida (PI 238226, Spain)</td>
<td>−</td>
</tr>
<tr>
<td>A. transcapica (PI 283174, Former Soviet Union)</td>
<td>−</td>
</tr>
<tr>
<td>A. vinealis (PI 440110, Russian Federation)</td>
<td>−</td>
</tr>
</tbody>
</table>

By contrast, the nonshared genes were truncated versions of homologs present elsewhere in the genome (Brunner et al., 2005), suggesting insertion originated from genomic elements within the individual genome, not from introgression from another genome. The gene insertions for the maize inbred nonshared genes are thus not likely to be similar to the Crs-1 situation in creeping bentgrass, since the DNA-blot analysis indicated that no similar sequences were present in the genomes of individuals carrying the Crs-1 locus.

An alternate explanation to gene introgression of the Crs-1 locus is active gene loss in creeping bentgrass, with some individuals still retaining Crs-1. The evolutionary relationships of the grass species having Crs-1 homologs are illustrated in Figure 8. Rice (Oryza sativa) and maize are considered to have diverged from a common ancestor 50 million years ago and both species have Crs-1 homologs. Agrostis is classified in the tribe Aveneae and placed in a sister clade to that of Avena (Soreng and Davis, 1998), which diverged from rice 46 million years ago. Presence of Crs-1 homologs in grass lineages that diverged prior to the divergence of the bentgrass tribe Aveneae (Gaut, 2002) suggests that similar sequences would be expected to have been in the progenitors of the Aveneae. Crs-1 homologs are present in two members of the tribe Triticaceae (barley [Hordeum vulgare] and wheat), which diverged from the tribe Aveneae 25 million years ago. This further supports the hypothesis that the progenitors of the Aveneae had Crs-1 homologs. No Crs-1 homologous sequences have yet been reported from any other species within the Aveneae or the closely related tribe Poeae (Kellogg, 1998; Grass Phylogeny Working Group, 2001). In addition to characterized genes, numerous expressed sequence tag sequences are available for...
The hybridization pattern of Crs-1 in DNA blots and the segregation analysis in two populations originating from controlled crosses, suggested that creeping bentgrass plants 5061 and 9188 were both hemizygous for two closely linked genes of Crs-1. This was surprising and suggests major haplotype variation at the Crs-1 locus. Apparently, some genomic event occurred that resulted in a haplotype in which the Crs-1 locus was lost and this haplotype has come to predominate in the population. As discussed above, the haplotype noncolinearity observed between maize inbreds (Fu and Dooner, 2002; Song and Messing, 2003; Brunner et al., 2005) likely originated from gene insertion events from elsewhere in the genome, rather than deletion events, and so is different from the situation with Crs-1.

One possible mechanism for the loss of the Crs-1 locus could be due to deletion of a DNA segment containing most or all of the Crs-1 locus. Since grass genomes are largely composed of retrotransposons (Kumar and Bennetzen, 1999; Vicient et al., 2001), an alternate possibility to a DNA deletion is multiple insertions of retrotransposons into the Crs-1 locus disrupting the Crs-1 sequence such that the probe used would not be able to hybridize. Ultimately, understanding the underlying basis for the apparent gene loss of Crs-1 in many creeping bentgrass individuals will require sequence comparisons of large genomic regions of the plus and minus haplotypes.

Many plant genomes are either polyploids or ancient polyploids that have become diploidized (Masterson, 1994; Soltis and Soltis, 2000). Although creeping bentgrass is an allotetraploid (Jones, 1956), segregation analysis indicated that while there are at least two Crs-1 genes, they are closely linked and likely originated from a localized duplication event rather than from polyploidization. If Crs-1 was originally present in both genomes that generated the allotetraploid, it must have been previously lost from one genome and is now in the process of being lost from the other. Clearly, the Crs-1 protein is not essential for creeping bentgrass or the other Agrostis spp. tested. Genetic linkage maps for creeping bentgrass are currently being constructed (S.A. Bonos and G. Jung, personal communications). The Crs-1 gene has not yet been mapped. Noncolinearity of haplotypes within a germline cell would be expected to result in reduced meiotic recombination frequency in that genomic region. It will be interesting to see if any areas of reduced recombination are observed in the creeping bentgrass maps. Based on the information presented here for Crs-1, it is certainly possible that haplotype noncolinearity may be present in some creeping bentgrass individuals at other loci.

The physiological function of the creeping bentgrass Crs-1 gene product is unknown. The specific functions of the similar proteins from other species are also unknown, but the maize β-glucosidase aggregating factor has been the most studied (Blanchard et al., 2001). In maize, β-glucosidase cleaves hydroxamic acid glucosides, releasing the aglycones, which are toxic to some insects. The β-glucosidase aggregating factor binds to β-glucosidase but does not affect its enzymatic activity. The binding of β-glucosidase aggregating factor may function to protect β-glucosidase from proteolytic breakdown (Blanchard et al., 2001). cDNAs for some of the other similar sequences were isolated in differential screens looking for genes expressed in the plant's response to pathogen attack. The wheat WCI-1 gene was induced by treatment with the chemical benzothiadiazole, which induces systemic acquired resistance. The expression of WCI-1 was correlated with resistance to powdery mildew (Gorlach et al., 1996). The wheat Hfr-1 gene was induced by Hessian fly larvae in an incompatible interaction, suggesting the Hfr-1 gene product may play a role in plant defense (Williams et al., 2002). Two nearly identical genes in barley were induced by jasmonic acid, suggestive of a role in stress response (Lee et al., 1996a). In creeping bentgrass, the Crs-1 gene was also inducible and was expressed at higher levels in the field grown samples after inoculation with the dollar spot pathogen. Expression of Crs-1 in creeping bentgrass may be a component of the plant's response to the pathogen attack but is not enough to confer resistance to dollar
Gene Loss in Agrostis

spot since the plant expressing the Crs-1 gene was susceptible to the disease (Belanger et al., 2004). We have not yet identified any individuals carrying two plus Crs-1 haplotypes, so we do not know if there may be a dosage effect of Crs-1 regarding degree of disease susceptibility.

The Crs-1 locus in creeping bentgrass provides a rare example of the evolutionary process of gene loss occurring within a plant species. Gene loss is considered one of the processes contributing to speciation and genome diversity but previously could only be inferred from interspecies comparisons. Krylov et al. (2003) examined the factors important in orthologous gene loss observed among seven complete eukaryotic genomes. The propensity for gene loss was found most related to gene dispensability, but sequence evolution rate was also considered a moderately important factor. The Crs-1 gene is clearly dispensable. Its amino acid sequence also shows considerable variation relative to the similar sequences from other species, consistent with the conclusions of Krylov et al. (2003). The Crs-1 gene thus provides a specific example that could be used to further examine an ongoing process of gene loss in a population of heterozygous individuals.

MATERIALS AND METHODS

Plant Materials

Creeping bentgrass (Agrostis stolonifera) plant 5061 is a transgenic plant expressing the bar gene, which confers resistance to the herbicide glufosinate (Lee et al., 1996b). This plant was used as the pollen parent in a cross with a colonial bentgrass (Agrostis capillaris) plant to produce the colonial bentgrass × creeping bentgrass interspecific hybrid number 15 (Belanger et al., 2003a). In 2001 and 2002, these plants were part of a field evaluation of interspecific hybrids inoculated with the fungal pathogen Sclerotinia homoeocarpa (Belanger et al., 2004) and were used as the source of plant material for SSH. Creeping bentgrass plant 9188 is an individual from the Rutgers breeding program. Bentgrasses can be vegetatively propagated, and all plants used in this study were also maintained in a greenhouse. Seedlings of several bentgrass cultivars and accessions were germinated and maintained in the greenhouse. Creeping bentgrass cultivars included Penn A-1, Penn G-2, Penncross, and Pennlinks (Tee-2-Green, Hubbard, OR), Cobra (CEBECO International Seeds, Halsey, OR), Crenshaw and L93 (Afbiotch, Somerset, NJ), and ProCup (Scotts, Marysville, OH). Creeping bentgrass accessions included P1235440 and P1518954. Colonial bentgrass samples included cv SR7100 (Seed Research, Corvallis, OR) and accession P152045. Dryland bentgrass (Agrostis castellana Boiss. and Reut.) included cv Highland (Great Western Seed, Albany, OR) and accession P1302830. Redtop bentgrass (Agrostis gigantea Roth.) included cv Steaker (Great Western Seed) and accessions P1406367 and P1409934. Velvet bentgrass (Agrostis canina) cv SR7200 was from Seed Research. Other Agrostis accessions included Agrostis mongolica Roshev. (P192190), Agrostis medina Boiss. and Reut. (P196319), Agrostis palida With. (P1328226), Agrostis transcaucasica Litv. (P1833174), and Agrostis vinalis Schreb. (P144010). The seeds of all the germplasm accessions were from the Western Region Plant Introduction Station, Pullman, WA. Leaves from valerian from this study are also listed in Table I.

Nucleic Acid Isolation

Total RNA was isolated from field-grown leaf samples using Tri-Reagent (Sigma-Aldrich, St. Louis). Field-grown samples were from the 2002 field test inoculated with the dollar spot fungus, and at several times after inoculation, and stored at ~0°C. For RNA isolation leaf samples were ground to a fine powder with liquid nitrogen and resuspended in Tri- Reagent (10 mL g-1).

Debris was removed by centrifugation and the supernatant was extracted twice with chloroform. The aqueous layer was precipitated with isopropanol and the RNA pellet was washed once with ethanol and dissolved in water.

Plant genomic DNA was isolated using commercial kits (DNeasy Plant Maxi and Mini kits, Qiagen USA, Valencia, CA). For the bulk DNA samples, 0.05 g each from 24 seedlings of a cultivar or accession were combined for DNA isolation.

SSH

SSH (Diatchenko et al., 1996) was used to generate a subtracted cDNA library enriched in sequences unique or overexpressed in the creeping bentgrass plant 5061 relative to its interspecific hybrid progeny plant hybrid number 15. Total RNA was isolated from the plants in the 2002 field test 56 d after inoculation with the fungal pathogen. SSH and construction of the subtracted cDNA library were carried out by BD Biosciences CLONTECH (Palo Alto, CA). Two SSH reactions were carried out. In one reaction, cDNA from the creeping plant 5061 was used as the tester and cDNA from hybrid number 15 was the driver resulting in a cDNA population enriched in 5061-specific sequences. In the other SSH reaction, cDNA from hybrid number 15 was used as the tester and cDNA from creeping plant 5061 was used as the driver, resulting in a cDNA population enriched in hybrid number 15-specific sequences. The 5061-specific cDNAs recovered following SSH were cloned into a pBlunt vector (BD Biosciences CLONTECH). Colonies from the subtracted cDNA library were screened for differential expression. Colonies were randomly picked and grown in 96-well microtiter plates with 100 µL Luria-Bertani plus ampicillin medium. The cDNA inserts from individual colonies were amplified using PCR with the nested primer 1, 5′-TCGAGCGGCCGCGCGCGGAGGT-3′ and nested primer 2R, 5′-ACCGTGTCGCGGCCGCGAGGT-3′. The 20-µL reactions contained 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl, pH 8.8, 2 mM MgSO4, 0.1% Triton X-100, 0.2 mM dNTPs, 0.2 µL Tag DNA polymerase (New England Biolabs, Beverly, MA), 18 ng nested primer 1, 18 ng nested primer 2R, and 1 µL bacterial culture. PCR was carried out in a GeneAmp 9600 thermocycler (Perkin-Elmer Life Sciences, Boston). The cycling parameters were 94°C for 30 s followed by 25 cycles of 10 s denaturation at 94°C and 3 min annealing/extension at 68°C. Amplification was confirmed by electrophoresis of a 5-µL aliquot of each reaction in a 1.5% agarose gel.

Screening of clones for differential expression was done with dot blots. Five microclones of each PCR product were denatured with 5 µL of freshly made 0.6 N NaOH in a 96-well plate. Two microclones of each denatured sample were spotted onto two pieces of nylon membranes (Magnagraph, Osmonics, Minnetonka, MN) to generate duplicate blots. The membranes were neutralized by soaking in 0.5 x Tris-HCl, pH 7.5, for 4 min, followed by water for an additional 4 min. The DNA was then fixed to the membrane with a UV crosslinker (Stratagene, La Jolla, CA).

One blot was probed with the 5061-subtracted cDNA and the other blot was probed with the hybrid number 15-subtracted cDNA. Probe labeling and hybridization conditions were the same as for the RNA and DNA blots. Samples exhibiting higher intensity hybridization with the 5061-subtracted cDNA probe than with the hybrid number 15-subtracted cDNA probe were classified as being overexpressed in the creeping bentgrass 5061 plant. Plasmids from colonies containing the differentially expressed cDNA clones were isolated using a commercial kit (QIAprep Spin Miniprep kit, Qiagen) and sequenced (Davis Sequencing, Davis, CA).

RACE-PCR

The full-length coding sequence for Crs-1 was obtained through RACE PCR by using a commercial kit (SMART RACE cDNA Amplification Kit, BD Biosciences CLONTECH). One microgram of total RNA was used to generate RACE-ready first-strand cDNA. For 5′-RACE PCR, the SMART II A oligo (5′-AACGACTGCTATCAACCGAGAATCCGGG-3′) and a gene-specific primer (5′-CGCCGACACGCCCGTAGAACCCCACAATG-3′) were used. Amplification was done using the Advantage 2 PCR kit (BD Biosciences CLONTECH) according to the manufacturer’s instructions. Touchdown PCR (Don et al., 1991) cycling parameters were used. Initial denaturation was conducted at 94°C for 2 min. Cycle temperatures were 94°C for 5 s, annealing at 70°C for 10 s, and extension at 72°C for 2 min. Every five subsequent cycles, the annealing temperature was decreased by 2°C until 64°C was reached. An additional 30 cycles at an annealing temperature of 64°C were performed.
PCR products were resolved on a 1% (w/v) agarose gel. A DNA fragment of the expected size was excised and purified using a commercial kit (QIAquick Gel Extraction Kit, Qiagen). The purified fragment was ligated into the pGEM-T Easy vector (Promega, Madison, WI), and transformed into DH5α Escherichia coli competent cells. Plasmids from E. coli transformants were purified (Plasmid Miniprep kit, Qiagen) and sequenced (Davis Sequencing).

Phylogenetic Analysis

The ClustalX (Thompson et al., 1997) program was used to align amino acid sequences with the Gomori scoring matrices. Phylogenetic analysis was performed with the PAUP program (version 4.0b10 for Macintosh; Swofford, 2002). A rooted phylogenetic tree was generated using the maximum parsimony method employing a heuristic search strategy. Gaps were treated as missing data. For rooting the tree, the maize (Zea mays) sequence was designated as the outgroup. To determine relative level of support for the tree topology, bootstrap values were generated from 1,000 replicates.

Gel-Blot Analyses

For RNA gel-blot analyses, 25 μg of total RNA was subjected to electrophoresis in formaldehyde agarose gels and transferred to nylon membranes (Magnagraph) as described by Selden (1987). RNA was fixed to the membrane with a UV crosslinker (Stratagene).

For DNA gel-blot analysis, genomic DNA was digested with SpeI and subjected to electrophoresis through a 1% (w/v) agarose gel. DNA in the gel was depurinated by washing in 0.25 N HCl for 10 min. The gel then was washed in water and the DNA was transferred to a nylon membrane (Zetabind, Probe, Bio-Rad, Hercules, CA) overnight with 0.4M NaOH (Reed and Mann, 1985). The membrane was washed in 2× SSC (20× SSC is 3 mM NaCl, 0.3 M sodium citrate) and fixed by drying completely.

A 1.1-kb EcoRI restriction fragment from the RACE-PCR clone was labeled with [32P]dCTP using a commercial kit (Prime-It II Random Primer Labeling kit, Stratagene) for use as a probe for all RNA and DNA gel blots. For both DNA and RNA gel blots, filters were prehybridized at 42°C overnight.

Hybridized membranes were washed with 2× SSPE, 0.5% (w/v) SDS for 15 min at 65°C. The membrane was washed in 2× SSC (20× SSC is 3M NaCl, 0.3 M sodium citrate) and fixed by drying completely.

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LITERATURE CITED


Gene Loss in Agrostis


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