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# Chlorophyll loss associated with heat-induced senescence in bentgrass

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ABSTRACT

Heat stress-induced leaf senescence is characterized by the loss of chlorophyll from leaf tissues. The objectives of this study were to examine genetic variations in the level of heat-induced leaf senescence in hybrids of colonial (*Agrostis capillaris*) × creeping bentgrass (*Agrostis stolonifera*) contrasting in heat tolerance, and determine whether loss of leaf chlorophyll during heat-induced leaf senescence was due to suppressed chlorophyll synthesis and/or accelerated chlorophyll degradation in the cool-season perennial grass species. Plants of two hybrid backcross genotypes ('ColxCB169' and 'ColxCB190') were exposed to heat stress (38/33 °C, day/night) for 28 d in growth chambers. The analysis of turf quality, membrane stability, photochemical efficiency, and chlorophyll content demonstrated significant variations in the level of leaf senescence induced by heat stress between the two genotypes, with ColXCB169 exhibiting a lesser degree of decline in chlorophyll content, photochemical efficiency and membrane stability than ColXCB190. The assays of enzymatic activity or gene expression of several major chlorophyll-synthesizing (porphobilinogen deaminase, Mg-chelatase, protochlorophyllide-reductase) and chlorophyll-degrading enzymes (chlorophyllase, pheophytinase, and chlorophyll-degrading peroxidase) indicated heat-induced decline in leaf chlorophyll content was mainly due to accelerated chlorophyll degradation, as manifested by increased gene expression levels of chlorophyllase and pheophytinase, and the activity of pheophytinase (PPH), while chlorophyll-synthesizing genes and enzymatic activities were not differentially altered by heat stress in the two genotypes. The analysis of heat-induced leaf senescence of *pph* mutants of *Arabidopsis* further confirmed that PPH could be one enzymes that plays key roles in regulating heat-accelerated chlorophyll degradation. Further research on enzymes responsible in part for the loss of chlorophyll during heat-induced senescence could aid in the development of genotypes with stay-green traits either through marker assisted selection or transgenic approaches.



## 1. Introduction

Heat stress is a major abiotic factor which affects cool-seasons plants and frequently limits growth during summer months. Heat stress damage in plants is characterized by leaf senescence associated with the loss of macromolecules, including chlorophyll pigments [1]. Heat-induced chlorophyll loss has been noted in a number of species, including sorghum (*Sorghum bicolor*), wheat (*Triticum aestivum*), Kentucky bluegrass (*Poa pratensis*), and creeping bentgrass (*Agrostis stolonifera*) [2–5]. The metabolic factors underlying heat-induced loss of chlorophyll molecules in association with heat-accelerated leaf senescence are generally not fully understood.

Chlorophyll is the main pigment involved in the absorption of light energy for use in photosynthesis in the thylakoids [6]. Its structure consists of a porphyrin ring with a magnesium ion bound in the middle and a phytol chain [7]. Several important enzymes involved in chlorophyll synthesis include porphobilinogen deaminase (PBGD) which is responsible for combining four porphobilinogen subunits into a ring structure [8]; Mg-chelatase (MG-CHT) which is responsible for inserting the magnesium ion into the tertapyrrole ring [9]; and protochlorophyllide reductase

*Abbreviations:* 5-ALA, delta-aminolevulinic acid; CHL, chlorophyll content; CHLASE, chlorophyllase; CHL-PEROX, chlorophyll degrading peroxidase; EL, electrolyte leakage; Fv/Fm, chlorophyll fluorescence ratio; MG-CHT, Mg-chelatase; PBGD, porphobilinogen deaminase; PPH, pheophytinase; POR, protochlorophyllide reductase; qPCR, real-time polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction; TQ, turf quality; WT, wild type.

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**Table 1**
Primers used for qPCR to measure gene expression levels in bentgrass plants.

| Gene: | Accession: | Forward primer: | Reverse Primer: |
|---|---|---|---|
| AsSAG12 | GR281516 | CCCAGCAGTTTACTGGCTTT | AAGCAGGTGCCTTGAAACTT |
| Asl20 | DV853171 | GGGTAGACGGCAACGATACT | TACTTGGTTGAATCGTCGGA |
| Ash36 | GR280199 | TGGGAATGTGTTCAGGGTAA | TCACCTCGATGAGGTAGTCG |
| Porphobilinogen deaminase | DV861883 | TAGCGCTGCGGATTAGAACT | GAAGGATAACGAACCGCTGA |
| Mg-Chelatase (H subunit) | GR278806 | CATCAGGGCGGATAGAGAGA | TCTGCCACAATCAGCTTCAG |
| Protochlorophyllide-reductase | DV854057 | GCGTCTACTGGAGCTGGAAC | GTCACTTCATGCAGGTCACG |
| Chlorophyllase | DV859235 | GGTCGCATTCCTGAGGTCTA | ATCATATTCAACCGGGTCCA |
| Pheophytinase | JU113198 | GAATGTCATTGCCGTCTGAA | CAATGAAATGCTGGACCTGA |
| Peroxidase | FE527944 | CCCAACCTACAGGACATCGT | GGAAGCAGTCGTGGAAGAAG |
| Actin | DY543529 | CCTTTTCCAGCCATCTTTCA | GAGGTCCTTCCTGATATCCA |

(POR) which generates chlorophyllide in the presence of light [10]. Additionally a number of pathways for the initial steps of chlorophyll degradation have been proposed including chlorophyllase (CHLASE) which cleaves the phytol chain of chlorophyll molecules [11]; pheophytinase (PPH) which cleaves the phytol chain from pheophytin, a chlorophyll molecule with the magnesium ion removed [12]; and chlorophyll-degrading peroxidases (CHL-PRX) which oxidizes chlorophyll in the presence of $H_2O_2$ and phenolic compounds [13]. Recently several advances have allowed for a greater understanding into chlorophyll metabolism and how it differs from similarly structured heme pigments [14]. However, changes in chlorophyll metabolism and in particular how they relate to chlorophyll loss during stress-induced senescence are not well documented. In a number of species abiotic factors have been shown to alter chlorophyll synthesis. PBGD and MG-CHT in wheat (*Triticum aestivum*) and cucumber (*Cucumis sativus*) had decreased activity due to heat or chilling stress [15]. Aminolevulenic acid deaminase activity was found to be decreased in sunflower (*Helianthus annuus*) due to salt stress [16]. POR has also been found to be a regulatory step that is down-regulated in senescent leaves [10]. It has also been found that chlorophyll loss may be due to changes in chlorophyll degradation with several pathways being proposed as being responsible for losses in chlorophyll including CHLASE, PPH, or CHL-PEROX [17,18].

Bentgrasses (*Agrostis*) are a genus of cool-season perennial grasses which contain several species with fine textured leaves that are well adapted for use as turfgrasses on high value turf areas [19]. Bentgrasses have low to moderate levels of heat tolerance which results in damage to turf areas during summer months [20]. As with other cool-season plants, one of the major symptoms of heat stress in bentgrasses is a loss of chlorophyll associated with pre-mature leaf senescence [21]. Two important bentgrass species are colonial bentgrass (*A. capillaris*) and creeping bentgrass (*A. stolonifera*). A colonial (*Agrostis capillaris*) × creeping (*A. stolonifera*) bentgrass hybrid backcross population has previously been shown to have a range of phenotypic responses under abiotic stress conditions such as drought and heat [22,23]. Some hybrid backcross lines exhibited superior stay-green phenotypes or a lesser extent of heat-induced leaf senescence than others [23]. The question of whether heat-induced leaf senescence associated with loss of chlorophyll is related to heat-inhibition of chlorophyll synthesis and/or heat-accelerated chlorophyll degradation in cool-season grass species is unknown. Understanding factors regulating chlorophyll metabolism which play roles in the loss of chlorophyll during heat-induced senescence could allow for the development of genotypes with stay-green traits which are potentially more stress tolerant, either through marker assisted selection or transgenic approaches. It is hypothesized that the loss of chlorophyll in bentgrass is due to alteration in gene expression or metabolic activities of chlorophyll synthesis and chlorophyll degradation enzymes, contributing to the overall decline in turf quality during summer months. The objectives of this study were to examine genetic variations in the level of heat-induced leaf senescence in hybrids of colonial × creeping bentgrass contrasting in heat tolerance, and determine whether loss of leaf chlorophyll during heat-induce leaf senescence was due to suppression of gene expression and enzymatic activities involved in chlorophyll synthesis or stimulation of gene expression or enzymatic activities for chlorophyll degradation. Looking at both chlorophyll synthesis and degradation pathways will help elucidate key metabolic processes responsible for the loss in chlorophyll associated with heat induced senescence, which could facilitate the development of improved cultivars with stay-green characteristics.

## 2. Materials and methods

### 2.1. Growth and treatment conditions

Clonally propagated plants of two genotypes ('ColxCB169' and 'ColxCB190') from a colonial bentgrass (*A. capillaris)* × creeping bentgrass (*A. stolonifera)* hybrid backcross population generated at Rutgers University and previously shown to contrast in heat tolerance were examined in this study [23,24]. Plants were established in plastic pots (15 cm in diameter and 20 cm deep) filled with a mixture of 50% soil (fine-loamy, mixed mesic Typic Hapludult type soil) and 50% peat moss in a greenhouse for 6 weeks before being transferred to environmentally controlled growth chambers (Conviron, Winnipeg, Canada) controlled at 20/15 °C (day/night), 14-h photoperiod, and photosynthetically active radiation (PAR) at 600 μmol $m^{-2}$ $s^{-1}$ for a 7 d acclimation period prior to imposition of heat stress. Plants were maintained well-watered and fertilized weekly with half-strength Hoagland's nutrient solution during plant establishment and heat-stress periods [25].

Plants of both genotype were exposed to either heat stress conditions of 38/33 °C (day/night) or optimal temperature of 20/15 °C (day/night) for 28 d. Each temperature was repeated in four growth chambers and each genotype had four pots of plants which were randomly placed in four different growth chambers. The experiment design was a randomized split-plot design with temperature treatments as main plots and genotype as subplots, with each treatment and genotype having four replicates.

### 2.2. Physiological analysis of heat-induced leaf senescence

The extent of leaf senescence was evaluated using commonly-used parameters including chlorophyll content, chlorophyll fluorescence, and membrane stability. In addition, the content of a chlorophyll precursor, 5-aminolevulenic acid (5-ALA), was measured to assess changes in chlorophyll metabolic pathways as affected by heat stress. Whole-plant response to heat stress was evaluated by visually rating turf quality (TQ) on a 1–9 scale based on leaf color, density and uniformity to assess plant health and levels of leaf senescence with 1 representing dead plants and 9 representing completely healthy plants [19]. All