Nitrogen Deficiency-induced Protein Changes in Immature and Mature Leaves of Creeping Bentgrass

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ADDITIONAL INDEX WORDS. *Agrostis stolonifera*, grass

ABSTRACT. Nitrogen (N) deficiency inhibits plant growth and induces leaf senescence through regulating various metabolic processes. The objectives of this study were to examine protein changes in response to N deficiency in immature and mature leaves of a perennial grass species and determine major metabolic processes affected by N deficiency through proteomic profiling. Creeping bentgrass (*Agrostis stolonifera* cv. Penncross) plants were originally fertilized with a diluted 36N–2.6P–5K fertilizer. After 14 days acclimation in a growth chamber, plants were grown in a nutrient solution containing 6 mM nitrate (control) or without N (N deficiency). Immature leaves (upper first and second not yet fully expanded leaves) and mature leaves (lower fully expanded leaves) were separated at 28 days of treatment for protein analysis. Two-dimensional electrophoresis and mass spectrometry analysis were used to identify protein changes in immature and mature leaves in response to N deficiency. The abundance of many proteins in both immature and mature leaves decreased with N deficiency, including those involved in photosynthesis, photorespiration, and amino acid metabolism (hydroxypyruvate reductase, serine hydroxymethyltransferase, alanine aminotransferase, glycine decarboxylase complex, glycolate oxidase), protein protection [heat shock protein (HSP)/HSP 70, chaperonin 60 and FtsH-like protein], and RNA stability (RNA binding protein). The reduction in protein abundance under N deficiency was greater in mature leaves than in immature leaves. The abundance of small HSP and metalloendopeptidase increased under N deficiency only in immature leaves. These results suggest that N deficiency accelerated protein degradation in immature and mature leaves of creeping bentgrass, particularly those proteins associated with energy and metabolism, but to a lesser extent in immature leaves. Immature leaves were also able to accumulate proteins with chaperone functions and for N reutilization, which could protect leaves from senescence under N deficiency.

Nitrogen is a major nutrient element that is required in large quantity by plants including turfgrasses to maintain active cell growth. However, reducing N fertility is often recommended in turfgrass management to prevent excessive shoot growth or clipping yield and potential nitrate leaching. Low N availability can lead to N deficiency and limit plant growth (Hull and Liu, 2005). Therefore, efficient use of N has been a major goal in turfgrass management and understanding the physiology of N metabolism by turfgrasses is critical for developing new approaches for turfgrass germplasm with improved effective use of N. Various studies have examined mechanisms of inorganic N uptake and recovery from the soil through the root system in turfgrass species (Bowman et al., 1989; Bushoven and Hull, 2001; Liu et al., 1993). Limited studies have investigated metabolic changes of leaves associated with deficient N uptake and availability in turfgrass species.

Nitrogen deficiency causes leaf senescence characterized by the chlorophyll content decline and protein degradation, limiting photosynthesis and various metabolic processes (Diaz et al.,

2008; Jiang et al., 2011; Smart, 1994; Thomas and de Villier, 1996). Several studies in perennial grass species demonstrated that N deficiency-induced leaf senescence has been associated with changes in enzymatic activities involved in organic N metabolism (Bushoven and Hull, 2001; Jiang et al., 2011; Lyons et al., 1990; Ourry et al., 1990). The metabolic processes and enzymes affected by N availability in immature leaves and mature leaves may vary. Nitrogen catabolism is predominant in mature or senescent leaves, in which proteins are degraded to produce NH_3 and amino acids, whereas in actively growing or immature leaves, N anabolism occurs and amino acids are used to synthesize proteins and chlorophyll (Liu et al., 2008). As a structural and functional component of the 20 protein amino acids, N is essential for protein synthesis, and therefore, N availability can have a significant impact on protein metabolism. Previous studies in cereal crops and *Arabidopsis thaliana* found that the abundance of many proteins decreases in response to N deficiency, including enolase, Rubisco activase, oxygen evolving enhancer protein (OEE), ATP synthase and methionine synthase (Bahrman et al., 2004), phosphoribulokinase, phosphoglycolate phosphatase, nitrite reductase, glutamine synthetase and glutamate synthetase (Feller et al., 2008a), and chaperone protein protease (Crafts-Brandner et al., 1998). Thomas and de Villier (1996) reported that the mRNA abundance of metallothionein-like protein and

Received for publication 19 Sept. 2011. Accepted for publication 14 Oct. 2011. We thank Emily Merewitz, David Jespersen, and Patrick Burgess for critical review of the manuscript. Thanks go to Rutgers Center for Turfgrass Science for funding support of this project.

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catalase increased at the initiation of leaf senescence in *A. thaliana* and decreased in completely senescent leaves under N deprivation. The degradation of various proteins could be associated with leaf senescence, particularly in mature leaves in response to N deficiency, providing amino acids to immature leaves for reuse (Liu et al., 2008). However, few studies have reported changes in leaf protein profiles under N deficiency in perennial turfgrass species, particularly differential responses of immature leaves and mature leaves. Knowledge of leaf proteomic responses to N deficiency is critical for further understanding of metabolic processes affected by N availability to improve N use efficiency.

Therefore, the objectives of this study were to examine differential protein changes in response to N deficiency in immature and mature leaves of a C_3 grass, creeping bentgrass, and to identify major metabolic processes affected by N deficiency through proteomic profiling. Creeping bentgrass is a widely used grass species on putting greens in golf courses but requires frequent N fertility to maintain green turf on sand-based putting greens.

Materials and Methods

PLANT MATERIALS AND NITROGEN TREATMENTS. Creeping bentgrass (cv. Penncross) was obtained from the Rutgers University research field in New Brunswick, NJ, and planted in eight plastic pots (15 cm deep and 15 cm diameter with holes at the bottom for drainage) filled with fine sand. These pots of grass were grown in a greenhouse for 2 months (Feb. and Mar. 2010), watered daily, fertilized with a diluted 36N–2.6P–5K fertilizer, and clipped to a 1-cm height weekly. On the last clipping in the greenhouse, the pots of grass were then moved to a reach-in growth chamber and allowed to acclimate for 2 weeks. The grasses were not cut during the 14-d treatment period except sampling. The environment in the growth chamber was maintained at day/night temperatures of 20/15 °C, a photoperiod of 14 h with a photosynthetic photon flux density of 500 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and a relative humidity of 70%. Each pot of grass was watered daily with 200 mL half-strength Hoagland's nutrient solution, which contained 6.0 mM nitrate-N (Hoagland and Arnon, 1950). The complete composition was as follows: 0.92 mM KNO_3 , 2.56 mM $\text{Ca}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$, 0.6 mM KH_2PO_4 , 0.3 mM K_2SO_4 , 0.96 mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 47.28 μM $\text{Fe}(\text{EDTA})\text{Na}$, 11.22 μM $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$, 22.46 μM H_3BO_3 , 0.48 μM $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, 14.18 μM $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, and 0.08 μM $\text{Na}_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$. Any solution left in the saucer from the previous watering was emptied right before the new solution was applied.

At the end of the 14-d acclimation period (15 Apr. 2010), four pots of plants were randomly selected and continued to receive the half-strength complete nutrient solution with 6 mM nitrate-N (+N treatment) and another four randomly selected pots received the half-strength Hoagland's solution without N (–N treatment), in which KNO_3 was replaced with K_2SO_4 and $\text{Ca}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$ was replaced with CaCl_2 . Withholding N application (–N) allowed for plants to develop N deficiency, but not complete N starvation, because plants were previously supplied with nitrogen before the –N treatment initiation. These two treatments were made with 200 mL applied to each pot once every other day for 2 weeks and then daily for 2 weeks more because the +N plants had grown bigger and required more nutrient solution. All pots were randomly arranged in the growth chamber and each of the two N treatments had four replicate pots.

PROTEIN EXTRACTION. On the same tiller, the upper leaves are younger than the lower leaves because of the sequential leaf formation, maturation, and senescence. The upper two leaves, which were not fully expanded (immature), and the lower two leaves, which were fully expanded, (mature) were separately sampled for protein analysis at 28 d after initiation of the two N treatments. Immediately after each sampling, the samples were placed in liquid N and then stored at –80 °C until analysis. A previously described protein extraction protocol using acetone/trichloroacetic acid (TCA) precipitation was used for protein extraction (Xu et al., 2008). Approximately 0.5 g of leaf samples was homogenized and incubated with 10 mL of precipitation solution (10% TCA and 0.07% 2-mercaptoethanol in acetone) overnight at –20 °C. The precipitated proteins were pelleted and washed with ice-cold acetone containing 0.07% 2-mercaptoethanol until the supernatant was colorless. The pellet was vacuum-dried, resuspended in resolubilization solution containing 8 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), 1% pharmalyte, and then sonicated to extract proteins. Insoluble tissue was removed by centrifugation at 21,000 g_n for 15 min. Protein concentration was determined according to Bradford (1976) using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

TWO-DIMENSIONAL GEL ELECTROPHORESIS. An IPGPhor apparatus (GE Healthcare, Piscataway, NJ) was used for isoelectric focusing (IEF) with immobilized pH gradient (IPG) strips (pH 3.0–10.0, linear gradient, 13 cm). The IPG strips were rehydrated for 14 h at 20 °C with 250 μL rehydration buffer (8 M urea, 2 M thiourea, 2% w/v CHAPS, 1% v/v IPG buffer, 1% DTT, and 0.002% bromophenol blue) containing 300 μg proteins. The voltage settings for IEF were 500 V for 1 h, 1000 V for 1 h, and 8000 V to a total 56.50 kVh. After IEF, the protein in the strips was denatured with equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate, 0.002% bromophenol blue, 1% DTT) and then incubated with the same buffer containing 2.5% iodoacetamide instead of DTT for 20 min. The second dimension electrophoresis was performed on a 12.5% gel using an electrophoresis unit (Hoefer SE 600 Ruby; GE Healthcare).

Gels were stained with colloidal Coomassie brilliant blue G-250 to detect total proteins (Newsholme et al., 2000). Gel images were analyzed with SameSpots software (Nonlinear, Newcastle upon Tyne, U.K.). To correct the variability resulting from staining, the spot volumes were normalized as a percentage of the total volume of all spots on the gel. Data were subjected to analysis of variance to test for the effects of N deficiency. Means were separated by least significance difference test ($P \leq 0.05$). Hierarchical clustering was performed by the complete linkage clustering analysis using Cluster/Tree-View 2.11 software (Stanford University, Stanford, CA) based on the Pearson correlation coefficient (Eisen et al., 1998).

PROTEIN IDENTIFICATION. The gel spots were excised and washed with 30% acetonitrile in 50 mM ammonium bicarbonate before DTT reduction and iodoacetamide alkylation. Trypsin was used for digestion at 37 °C overnight. The resulting peptides were extracted with 30 μL of 1% trifluoroacetic acid followed by C_{18} ZipTip desalting. For the mass spectrometry (MS) analysis, the peptides were mixed with 7 $\text{mg}\cdot\text{mL}^{-1}$ α -cyano-4-hydroxy-cinnamic acid matrix in a 1:1 ratio and spotted onto a matrix-assisted laser desorption/ionization (MALDI) plate. The peptides were analyzed on a 4800 MALDI

time-of-flight (TOF)/TOF analyzer (Applied Biosystems, Framingham, MA). Mass spectra (m/z 880 to 3200) were acquired in positive ion reflector mode. Twenty-five most intense ions were selected for subsequent MS/MS sequencing analysis in 1 kV mode. Protein identification was performed by searching the combined MS and MS/MS spectra against the green plant database in National Center for Biotechnology information using a local Mascot search engine (Version 1.9; Matrix Science, Boston, MA) on a GPS Explorer™ server (Version 3.5; Applied Biosystems). Protein containing at least two peptides with confidence interval values no less than 95% was considered identified.

Results and Discussion

The level of N deficiency used in the present study was reported to accelerate leaf senescence as exhibited by the decline in chlorophyll, amino acid, and soluble protein content in both immature and mature leaves in our previous study (Jiang et al., 2011). To identify the proteins that are responsive to N deficiency, two-dimensional gel electrophoresis was performed and a representative gel image for immature leaves (Fig. 1A) and one for mature leaves (Fig. 1B) are presented. N deficiency caused changes (increase or decrease) in the abundance of 43 protein spots in immature and mature leaves. The expression patterns of N deficiency-responsive protein spots are shown in Figures 2 and 3. Analyses of hierarchical clustering indicated that most N deficiency-responsive protein spots exhibited a decline in protein abundance (downregulation) in either mature or immature leaves, and more spots were downregulated in mature leaves than in immature leaves (Fig. 3).

Among 43 N deficiency-responsive protein spots, 40 were identified by MS and their functional classification and identity information are presented in Table 1. The identified proteins in this study were classified according to the functional categories described by Bevan et al. (1998) (Fig. 4). N deficiency caused changes in proteins with diverse functions. In both immature and mature leaves, the largest group of proteins responsive to N deficiency was in the energy category (47.8% in immature leaves and 65.5% in mature leaves), which was followed by

Fig. 2. Venn diagram illustrating the expression patterns of spots responsive to nitrogen deficiency in mature and immature leaves of creeping bentgrass.

protein destination and storage (21.7% in immature leaves and 20.7% in mature leaves). In immature leaves, N deficiency-responsive proteins involved in stress defense accounted for 13.0% and the remaining proteins were in metabolism (8.7%), transcription (4.3%), and protein synthesis (4.3%). In mature leaves, proteins in metabolism, transcription, and stress defense accounted for 6.5%, 3.4%, and 3.4%, respectively. The N deficiency-responsive proteins that exhibited differential responses between immature and mature leaves and have known important biological functions in stress adaptation are discussed subsequently.

PROTEINS IN ENERGY PRODUCTION AND PRIMARY METABOLISM.

The functional group with the largest number of protein spots associated with N deficiency is the energy category (Table 1). In this category, the abundance of 20 protein spots decreased in mature leaves as a result of N deficiency (Fig. 3). The down-regulated proteins by N deficiency in mature leaves included light-harvesting complex I [LHC I (spots 4 and 5)], subunits of photosystem I [PS I (spots 6 and 7)], ferredoxin-NADP reductase (spot 16), Rubisco large and small subunit (spots 10–14), Rubisco activase (spot 15), hydroxypyruvate reductase (spot 25), glycine decarboxylase complex [GDC (spot 27)], glycolate oxidase (spot 28), transketolase (spot 17), cytosolic glyceraldehyde-3-phosphate dehydrogenase [GAPDH (spot 18)], ATP synthase (spot 29), and chloroplastic aldolase (spot 24) and GAPDH (spots 22 and 23). In immature leaves, only six protein spots were downregulated (spots 11 and 12: Rubisco large and small subunit; spot 16: ferredoxin-NADP reductase; spot 24: chloroplastic aldolase; spot 25: hydroxypyruvate reductase; spot 26: phosphoribulokinase) and five protein spots (spot 8: PS I subunit VII; spot 9: OEE; spots 19–21: cytosolic GAPDH) exhibited increases in abundance or were upregulated.

Most of those downregulated protein spots in energy category are

Fig. 1. Coomassie blue stained 2-DE gel of separated proteins from immature leaves (A) and mature leaves (B) of creeping bentgrass. Proteins were separated in the first dimension on an immobilized pH gradient strip (pH 3.0 to 10.0) and in the second dimension on a 12.5% gel. The numbered spots were responsive to nitrogen deficiency. MW = molecular weight.

Fig. 3. Hierarchical display of data from 40 spots responsive to nitrogen deficiency in the immature or mature leaves of creeping bentgrass. The values of abundance difference divided by the abundance of spot in the control were applied as input data. The dendrogram and colored image were produced as described by Eisen et al. (1998): green indicates downregulation; red indicates upregulation; dark indicates no change in response to stress.

involved in photosynthesis such as LHC I, subunits of PS I, ferredoxin-NADP reductase, Rubisco large and small subunit, Rubisco activase, phosphoribulokinase, and chloroplastic aldolase and GAPDH. Chloroplasts are known to be a major site of protein degradation during senescence (Khanna-Chopra, 2011). The degradation of Rubisco and other photosynthetic proteins is a common phenomenon during natural or stress-induced leaf senescence, which allows the N reutilization in other organs (Feller et al., 2008a, 2008b; Hörtensteiner and Feller, 2002). Tsai et al. (1991) suggested that the decreased abundance of Rubisco polypeptides in maize (*Zea mays*) exposed to low soil N was a result of enhanced proteolysis. In addition, more protein spots were downregulated in mature leaves than in immature leaves under N deficiency, and the extent of protein decline was smaller in immature leaves than in mature leaves (Figs. 2 and 3). These results suggested that immature leaves were better able to maintain proteins for photosynthesis under N deficiency, which could be associated with N remobilization from mature tissues to support the higher energy demand of immature leaves (Ourry et al., 1990; Smart, 1994). The more severe downregulation of proteins in mature leaves than in immature leaves under N deficiency also reflected that N deficiency-accelerated protein degradation and leaf senescence.

Three spots of cytosolic GAPDH (spots 19–21) were upregulated in immature leaves, whereas one spot (spot 18) was downregulated in mature leaves under N deficiency. The cytosolic GAPDH is involved in breaking down glucose and is an important protein in the pathway for energy and carbon supply. The downregulation of cytosolic GAPDH in mature leaves indicated that glycolytic activities may be inhibited, which limits carbon supply for respiratory energy production under N deficiency, whereas its upregulation in immature leaves suggests that immature leaves could be better able to maintain glycolytic activity for continuing energy production through respiration.

The abundance of hydroxypyruvate reductase (spot 25), serine hydroxymethyltransferase [SHMT (spot 2)], and alanine aminotransferase (spot 1) decreased in immature leaves, whereas in mature leaves in addition to SHMT (spot 2) and hydroxypyruvate reductase (spot 25), the abundance of GDC (spot 27) and glycolate oxidase (spot 28) also decreased under N deficiency. These enzymes are involved in amino acid metabolism or photorespiration. Hydroxypyruvate reductase converts hydroxypyruvate to glycerate by an NADH-dependent reduction that occurs within peroxisomes. SHMT catalyzes the interconversion of serine and glycine and is a key enzyme in the biosynthesis of purines, lipids, hormones, and other compounds (Kopriva and Bauwe, 1995). Alanine aminotransferase is an enzyme involved in photorespiration catalyzing the interconversion of alanine and glycine. GDC catalyzes the degradation of glycine (Bourguignon et al., 1993). Glycolate oxidase is a peroxisomal, FMN-dependent oxidase that catalyzes the oxidation of α -hydroxy acids to the corresponding α -ketoacids. The downregulation of those proteins indicated that N deficiency could suppress amino acid metabolism and photorespiration. The extent of decreases in the abundance of proteins involved in metabolism under N deficiency was smaller in immature leaves than in mature leaves, suggesting that immature leaves were better able to maintain amino acid metabolism and photorespiration. Photorespiration acts as an energy sink and has antioxidant defense functions, preventing the overreduction of the photosynthetic electron transport chain, especially under stress conditions (Kozaki and Takeba, 1996; Wingler et al., 2000).

STRESS PROTECTION AND ANTIOXIDANT PROTEINS. The abundance of HSP70 (spot 35), chaperonin 60 (spot 36), and FtsH-like protein (spot 37) was reduced under N deficiency in both mature and immature leaves. The expression of HSPs is a fundamental stress response ubiquitous to plants, which is known to be an important adaptive strategy in plant tolerance to various stresses (Feder and Hoffman, 1999; Gazanchian et al., 2007; Lee et al., 2007; Zhang et al., 2010). HSP70 has essential functions in preventing aggregation and assisting refolding of non-native proteins under stress conditions, and it is also involved in protein import and translocation processes and in facilitating the proteolytic degradation of unstable proteins by targeting the proteins to lysosomes or proteasomes (Wang et al., 2004). Chaperonins play a crucial role by assisting a wide range of newly synthesized and newly translocated proteins to achieve their native forms (Wang et al., 2004). The reduction in the abundance of HSP70 and chaperonin 60 indicated that the chaperoning protection system could be weakened under N deficiency. FtsH proteins constitute a small family of membrane-bound zinc metalloproteases containing an ATPase domain. In *A. thaliana*, two chloroplast-targeted FtsH proteases have been shown to play a role in the repair of PSII after oxidative damage (Bailey et al., 2002; Sakamoto et al., 2003). Other

Table 1. Identification and functional classification of proteins responsive to nitrogen deficiency in mature or immature leaves of creeping bentgrass.

Spot no.	Protein name (species)	Accession no.	MOWSE score	Peptides matched (no.)	Peptide sequences
Metabolism category					
1	Alanine aminotransferase (<i>Arabidopsis thaliana</i>)	gi 16604499	150	2	GYWGECGQR/KEV/AFIQR
2	Glycine hydroxymethyltransferase (<i>Oryza sativa</i> ssp. <i>japonica</i>)	gi 31126793	183	2	GFVEEDFAK/GYELVSGGTNDNHLVLVNLK
27	Glycine decarboxylase complex (<i>O. sativa</i> ssp. <i>japonica</i>)	gi 2565305	381	3	APPMQFTGK/FCDALISIR/EYAAFPAAWLR
Energy category					
4	Light-harvesting complex I; (LHC I) (<i>Hordeum vulgare</i>)	gi 544700	154	2	YPGGAFDPLGFSK/KYPGGAFDPLGFSK
5	LHC I (<i>H. vulgare</i>)	gi 544700	201	3	YPGGAFDPLGFSK/FKSEIYHCR/KYPGGAFDPLGFSK
6	Photosystem I (PSI) subunit III (<i>Triticum aestivum</i>)	gi 548604	232	2	SYLIAVSGEK/FGLLCGSDGLPHLIVSGDQR
7	PSI subunit IV (<i>O. sativa</i> ssp. <i>japonica</i>)	gi 131176	246	2	YPVVVR/VNYAGVSTNNYALDEIK
8	PSI subunit VII (<i>O. sativa</i> ssp. <i>japonica</i>)	gi 11466848	273	3	VYLGPEPTR/CESACPTDFLSVR/IYDTCIGCTQCVR
9	Oxygen evolving enhancer (<i>O. sativa</i> ssp. <i>japonica</i>)	gi 131394	143	2	EPFGQVLR/QYYISITVLTR
10	Rubisco large subunit (<i>Psathyrostachys fragilis</i> ssp. <i>fragilis</i>)	gi 14017580	332	4	DTDILAAFR/LTYYTPEYETK/TFQGPPIGQVER/LTYYTPEYETKDTDILAAFR
11	Rubisco large subunit (<i>T. aestivum</i>)	gi 61378666	1,020	12	ASVFGQAGVK/ACYECLR/DTDILAAFR/DNGLLLHHHR/FVFCAEIYK/EMTLGFVDLLR/FEFEPVDIDK/LTYYTPEYETK/TFQGPPIGQVER/YGRPLLGCITKPK/WSPELAAACEVWK/GGLDFTKDDENVNSQPFMR/VGFIFR/IIGFDNIR/EHGSTPGYYDGR
12	Rubisco small subunit (<i>Avena maroccana</i>)	gi 6409343	308	3	VGFIFR/YWTMWK/IIGFDNIR/EYPDAYVR/WVPCLEFSK/EHGSTPGYYDGR
13	Rubisco small subunit (<i>T. aestivum</i>)	gi 6573202	672	6	YWTMWK/IIGFDNIR/EYPDAYVR/KEYPDAYVR/WVPCLEFSK/FETLSYLPPLSTEALLK/KFETLSYLPPLSTEALLK
14	Rubisco small subunit (<i>Bromus catharticus</i>)	gi 4038725	484	7	FYWAPTR/MCCLFINDLDAGAGR/LVDTFPGQSIDFFGALR/VPIVTGNDFSTLYAPLIR
15	Rubisco activase (<i>T. aestivum</i>)	gi 115334975	336	4	KSEQWNVVY/LYSIASSALGDFGDSK/DPNATIMLATGTGIAPFR
16	Ferredoxin–NADP reductase (<i>Zea mays</i>)	gi 162458489	293	3	NPYWFNR/VTTTIGFGSPNK/VSLEAGSTLGWQK/FEALGWHTTWVK
17	Transketolase (<i>O. sativa</i> ssp. <i>japonica</i>)	gi 28190676	340	4	YDVTVHGHWK/AASFNIIPSSSTGAAG/VPTVDVSVDLTVR/TLFGEKPVTVFGVR/LVSWYDNEWGYSNR/GIMGYVEEDLVSTDFVGDGR/VINDNFGIIEGLMTTVHAITATQK
18	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cytosolic (<i>T. aestivum</i>)	gi 120680	572	7	VPTVDVSVDLTVR/GILGYVDEDLVSTDFQGDGR
19	GAPDH, cytosolic (<i>T. aestivum</i>)	gi 120668	112	2	

continued next page

Table 1. Continued.

Spot no.	Protein name (species)	Accession no.	MOWSE score	Peptides matched (no.)	Peptide sequences
20	GAPDH, cytosolic (<i>T. aestivum</i>)	gi 120668	546	6	EVAVFGR/YDTVHGQWK/AGIALNDNFVK/ VPTVDVSVVDLTVR/LVSWYDNEWGYSTR/ GILGYVDEDLVSTDFQGDSR
21	GAPDH, cytosolic (<i>T. aestivum</i>)	gi 120680	432	5	YDTVHGHWK/VPTVDVSVVDLTVR/ TLLFGEKPVTVFVGR/ LVSWYDNEWGYSNR/VINDNFHIEGLMTTVHAITATQK
22	GAPDH, chloroplast (<i>O. sativa</i> ssp. <i>japonica</i>)	gi 120661	285	3	GTMTTTHSYTGDQR/VIAWYDNEWGYSQR/ GILDVCDPLVSVDFR
23	GAPDH, chloroplast (<i>O. sativa</i> ssp. <i>japonica</i>)	gi 166702	289	3	AVALVLPNLK/VIAWYDNEWGYSQR/GILDVCDPLVSVDFR
24	Chloroplastic aldolase (<i>O. sativa</i>)	gi 218157	194	3	VAPEVIAEYTVR/KVAPEVIAEYTVR/FASINVENVEDNRR
25	Hydroxypyruvate reductase (<i>Solenostemon scutellarioides</i>)	gi 118723307	325	4	GQTVGVIGAGR/EADVLSLHPVLDK/GPVIDEVALVEHLK/ MNLVYDLYQSTR
26	Phosphoribulokinase (<i>O. sativa</i> ssp. <i>japonica</i>)	gi 125578	394	4	LTSVFGGAAEPPR/FYGEVTQQMLK/KPDFDAYIDPQK/ ANDFDLMEYQVK
28	Glycolate oxidase (<i>Solanum lycopersicum</i>)	gi 1063400	374	4	AIALTVDTPTPR/FFQLVYVK/QLDYVPATISALEEVVK/ MDQANDSGLASYVAGQIDR
29	ATP synthase subunit (<i>T. aestivum</i>)	gi 285014508	528	5	VAVVLTGER/FLELAGIPTVK/GLCGAFNNNVLK/ ALQESLASELAAR/SDPIIQITLLPMSPK
Transcription category					
31	RNA binding protein (<i>A. thaliana</i>)	gi 3850621	270	4	FIGLFLSR/YVTFDGLAK/EGHQVTLFTR/DQHFFASVEK
Protein synthesis category					
32	Ribosomal protein L12 (<i>O. sativa</i> ssp. <i>japonica</i>)	gi 2331135	330	2	TEFDVVEEVPSAR/VLELGDIAIAGLTLEEAR
Protein destination/storage category					
30	Metalloendopeptidase (<i>A. thaliana</i>)	gi 22331173	139	2	HISNTWLWDR/GSLHTFLNAFTYPDR
33	Heat shock protein (HSP) 17.4 (<i>A. thaliana</i>)	gi 4456758	299	3	FRLPENAK/VEVEDGNVLQISGER/ EEVKVEVEDGNVLQISGER
34	HSP 20 (<i>T. aestivum</i>)	gi 86439765	97	2	DGVLYLNIPK/GRTPWEIK
35	HSP 70 (<i>Cucumis sativus</i>)	gi 6911551	659	6	HLNITLTR/QYSPSQIGAFVLTK/AVITVPAYFENDAQR/ IINEPTAAALSYGTNNK/SQVFSTAADNQTVGIR/ QAVTNPQNTFFGTK
36	60 kDa chaperonin (<i>A. thaliana</i>)	gi 10697184	333	2	AIELPNAMENAGAALIR/EIAFDQK
37	FtsH-like protein (<i>O. sativa</i> ssp. <i>japonica</i>)	gi 18423214	542	4	TAIAEGLAQR/AIDLIDEAGSR/GELQCIGATTLDYRK/ LIGSPGYVGYTEGGQLTEAVR
38	Cyclophilin A-2 (<i>T. aestivum</i>)	gi 13925734	97	2	VIPDFMCQGGDFTK/HVVVFGEVVEGMDVVK
Disease/defense category					
39	Peroxisome ascorbate peroxidase (APX) (<i>H. vulgare</i> ssp. <i>vulgare</i>)	gi 15080682	207	2	ALLDDPEFR/YEEYYTHGSNAGLK
40	APX, cytosolic (<i>A. thaliana</i>)	gi 31980500	132	2	FIAVGLGPR/DKFIADVGLGPR
3	Xylanase inhibitor (<i>T. aestivum</i>)	gi 153918942	161	2	TPVQLTATPR/TGQVAVFWGR

MOWSE = molecular weight search.

Fig. 4. Assignment of the protein spots in the immature or mature leaves of creeping bentgrass responsive to nitrogen deficiency according to functional categories described by Bevan et al. (1998).

studies have suggested that the FtsH protease complex may also degrade an unassembled Rieske Fe-S thylakoid protein and D1 proteins of PSII (Adam, 2000; Sinvany-Villalobo et al., 2004). The decreased abundance of FtsH protease may indicate reduced ability to repair damage to the D1 polypeptide under N deficiency.

Nitrogen deficiency, however, caused upregulation of the small HSP (spot 34) and metalloendopeptidase (spot 30) in immature leaves and downregulation of small HSP (spots 33 and 34) in mature leaves. Small HSPs have a high capacity to bind nonnative proteins and to stabilize and prevent protein aggregation, thereby facilitating their subsequent refolding by ATP-dependent chaperones (Lee and Vierling, 2000; Mogk et al., 2003). The degradation and aggregation of proteins such as Rubisco can be formed within chloroplasts as a result of stresses, including N deficiency (Feller et al., 2008a). Our results indicate that under N deficiency, immature leaves could be better able to provide protection from protein degradation or aggregation through increasing the abundance of small HSPs. Metalloendopeptidase is a proteolytic peptidase that breaks peptide bonds of nonterminal amino acids. It has been reported that metalloendopeptidase plays an important role in breaking down degraded Rubisco fragments for the N reutilization (Feller et al., 2008b). The upregulation of this protein in immature leaves could facilitate the reutilization or recycling of N derived from protein degradation under N-deficient conditions.

Under N deficiency, the abundance of cytosolic ascorbate peroxidases [APX (spot 40)] increased in both mature and immature leaves similarly, whereas the abundance of the APX isoform in the peroxisome (spot 39) decreased only in mature leaves. Cytosolic APX detoxifies peroxides such as hydrogen peroxide using ascorbate as a substrate. Its enhanced abundance suggested that N deficiency might cause oxidative stress and both mature and immature leaves were able to activate the scavenging ability to detoxify reactive oxygen species through the ascorbate reduction pathway. The reduced peroxisomal APX might result from the inhibition of photorespiration in mature leaves, because several other proteins in the photorespiration processes were also downregulated in mature leaves under N deficiency as discussed previously.

PROTEINS FOR RNA BINDING AND CELL WALL DEGRADATION. RNA binding proteins (RBPs) are involved in the synthesis, processing, transport, translation, and degradation of RNA. RBPs may bind and stabilize specific transcripts and play an important role in RNA post-transcriptional control such as splicing, polyadenylation, mRNA stability, mRNA localiza-

tion, and translation (Curtis et al., 1995; Johnstone and Lasko, 2001; Wickens et al., 2000). In the present study, RBP (spot 31) was downregulated in both mature and immature leaves under N deficiency, but the extent of protein decline was lower in immature leaves than in mature leaves. The differences in RBP responses to N deficiency indicated that N deficiency could cause damage to RNA and the immature leaves were better able to repair or maintain RNA stability than the mature leaves.

The abundance of xylanase inhibitor (spot 3) was downregulated under N deficiency in immature leaves. Two distinct classes of proteinaceous xylanase inhibitors, xylanase inhibitor protein (XIP)-type and *Triticum aestivum* xylanase-inhibitor-type inhibitors, have been reported in various cereals such as durum wheat (*Triticum sativum durum*), rye (*Lolium perenne*), barley (*Hordeum vulgare*), and maize (Elliott et al., 2003; Goesaert et al., 2003, 2004). However, the physiological functions and their relation to stress tolerance are not yet clear. A study in rice (*Oryza sativa*) through the analysis of the genes encoding XIP suggested that XIP-type xylanase inhibitors may be involved in general plant defense by preventing the cell wall from degradation by xylanase (Tokunaga and Esaka, 2007). The downregulation of xylanase inhibitor in immature leaves could be associated with cell wall degradation under N deficiency.

In summary, N deficiency caused significant changes in proteins involved in various metabolic functions in both immature and mature leaves of creeping bentgrass. The abundance of many proteins involved in photosynthesis, photorespiration and amino acid metabolism, and stress protection declined with N deficiency in both immature and mature leaves, but to a lesser extent in immature leaves than in mature leaves. The suppression of proteins involved in photosynthesis, photorespiration and amino acid metabolism, and stress protection could result in slower shoot growth and lower leaf clipping yield as often observed under low N fertility conditions. The small HSP as chaperones and metalloendopeptidase involved in N reutilization was upregulated only in immature leaves. In addition, the abundance of the xylanase inhibitor associated with cell wall degradation was downregulated under N deficiency only in immature leaves. The differential proteomic responses in immature and mature leaves to N deficiency could be associated with the differences in physiological responses such as leaf senescence to N deficiency between immature and mature leaves as reported in our previous study (Jiang et al., 2011). However, the specific expression patterns and functions of N deficiency-responsive proteins deserve further confirmation through molecular techniques such as Western blotting analysis. Confirmed N deficiency-responsive proteins could be potentially used as molecular markers to select for retaining-green grass germplasm under low N fertility conditions.

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