Environmental and Experimental Botany 149 (2018) 43–50

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Characterization and transcriptional regulation of chlorophyll *b* reductase gene *NON-YELLOW COLORING 1* associated with leaf senescence in perennial ryegrass (*Lolium perenne* L.)

Bin Xu[a], Hui Li[a], Yu Li[a], Guohui Yu[a], Jing Zhang[a,*], Bingru Huang[b,*]

[a] College of Agro-grassland Science, Nanjing Agricultural University, Nanjing, 210095, PR China
[b] Department of Plant Biology and Pathology, Rutgers, The State University of New Jersey, New Brunswick, NJ, 08901, USA

ARTICLE INFO

Keywords:
Perennial ryegrass
NON-YELLOW COLORING 1
Chlorophyll degradation
ABA
Ethylene

ABSTRACT

Chlorophyll (Chl) degradation leads to leaf senescence and adversely affects biomass production of forage grasses and aesthetic appearance of turfgrasses. The objectives of this study were to characterize the function of a Chl catabolic gene, *NON-YELLOW COLORING 1* (*LpNYC1*), and to understand its transcriptional regulatory pattern involved in leaf senescence in perennial ryegrass (*Lolium perenne* L.). The *LpNYC1* was initially cloned using the RACE-PCR method. Its encoded protein was localized in chloroplasts and its expression pattern was correlated with the progression of leaf senescence. LpNYC1 shared the same biological function with *Arabidopsis* NYC1, as overexpression of its encoding gene accelerated chlorophyll degradation in *Nicotiana benthamiana* and rescued the stay-green phenotype of the *Arabidopsis nyc1* null mutant. A yeast one-hybrid cDNA library was prepared from senescent leaves of perennial ryegrass. Using the *LpNYC1* promoter as bait, five putative transcription factors upstream of *LpNYC1* were pooled out using this method, among which three (LpABI5, LpABF3, and LpEIN3) were orthologous to *Arabidopsis* transcription factors involved in the ABA and ethylene signaling pathways. The expression of *LpNYC1* was highly inducible by ABA and ethephon (ethylene releasing reagent) but was suppressed by treatment with AVG (ethylene biosynthesis inhibitor). Furthermore, LpABI5, LpABF3, LpEIN3 directly activated the expression of *LpNYC1* by binding to its promoter. The current result laid the groundwork for future in-depth analysis of the molecular regulation of *LpNYC1* and Chl metabolism during leaf senescence in perennial grass species.

1. Introduction

Stress-induced leaf senescence adversely affects the biomass production of crops grown for yield, such as forage grasses, as well as the aesthetic quality of ornamental plants and turfgrasses (Munné-Bosch and Alegre, 2004; Czyczyło-Mysza et al., 2013). The yellowing of leaves associated with chlorophyll (Chl) degradation is the hallmark of leaf senescence and severely impacts turf quality; therefore, the stay-green trait, which delays leaf senescence and allows plants to maintain greenness in senescent leaves, is a highly desirable trait for perennial grasses cultivated as turfgrass or for forage.

Delayed leaf senescence and slower Chl degradation rate are attributed to the stay-green phenotype. Knowledge of molecular factors controlling Chl degradation and its regulatory mechanisms are of utmost importance for genetic improvement and molecular breeding for the stay-green trait. Chl catabolism is initiated when Chl *b* is converted to 7-Hydroxymethyl-Chl *a*. This initial step is catalyzed by the two Chl *b* reductase compounds NON-YELLOW COLORING1 (NYC1) and NYC1-LIKE (NOL) (Kusaba et al., 2007; Horie et al., 2009; Sato et al., 2009; Jibran et al., 2015). Both *nyc1* and *nol* null mutants in rice (*Oryza sativa*) show a stay-green phenotype, and the stay-green phenotype of the *nyc1/nol* double mutant is stronger than that of each mutant alone (Sato et al., 2009). In *Arabidopsis*, only the *nyc1* mutant shows the stay-green phenotype (Horie et al., 2009), indicating the diversity of these Chl catabolism gene(s) across different plant species. In both rice and *Arabidopsis*, the NYC1 and NOL proteins physically interacted with each other and co-catalyzed Chl degradation (Sato et al., 2009; Sakuraba et al., 2012; Jibran et al., 2015). In addition, we reported the *in vivo* interaction between perennial ryegrass NYC1 and NOL proteins (named as LpNYC1 and LpNOL) using the perennial ryegrass transient expression system in mesophyll protoplasts established by our group (Yu et al., 2017). Despite findings of recent research, the functions and

* Corresponding authors.
E-mail addresses: binxu@njau.edu.cn (B. Xu), 1049246453@qq.com (H. Li), 1272168448@qq.com (Y. Li), yuguohui86@126.com (G. Yu), nauzj@njau.edu.cn (J. Zhang), huang@aesop.rutgers.edu (B. Huang).

https://doi.org/10.1016/j.envexpbot.2018.01.017
Received 15 January 2018; Accepted 30 January 2018
Available online 03 February 2018
0098-8472/

upstream regulators of *LpNYC1* and *LpNOL* in perennial grass species are not well-understood.

It is well known that ABA and ethylene accelerate leaf senescence and Chl degradation through their signaling transcription factors on transactivation of senescence-associated genes (Grbic and Bleecker, 2003; Lim et al., 2007; Zhang et al., 2017). Several recent studies suggested a signaling linkage between these senescence-related hormones and the transcriptional regulation of Chl catabolic genes in *Arabidopsis* and rice (Delmas et al., 2013; Liang et al., 2014; Yang et al., 2014; Gao et al., 2016; Sakuraba, 2014; Qiu et al., 2015). The revelation of these signaling networks linked these senescence-causing hormones with the Chl degradation genes in model annual plant species, such as *Arabidopsis* and rice. Yet, how Chl degradation genes (CCGs) are transcriptionally regulated by those hormones in perennial grass species is still unclear.

A majority of current studies have been conducted on model annual plant species, and associated literature has suggested that knocking out the *Chl b reductase* gene could lead to a stay-green phenotype and that the expression of at least some CCGs are regulated by transcription factors involved in hormonal signaling pathways. The objectives of this study were to characterize the functions of *LpNYC1* in perennial ryegrass and to screen the upstream transcription factors directly regulating the expression of *LpNYC1* in perennial ryegrass during leaf senescence. Such knowledge would ultimately facilitate a strategy for molecular improvement of perennial ryegrass and related grass species in the future.

2. Materials and methods

2.1. Plant materials and growth conditions

Perennial ryegrass (cv. 'Pinnacle') was used for gene cloning and expression analysis. Wild tobacco (*N. benthamiana*) was used to test the role of *LpNYC1* in the regulation of leaf senescence by transient assay. *Arabidopsis* (*A. thaliana*) ecotype 'Col-0′ and the *nyc1* null mutant (T-DNA insertion line, SALK_017680C) were used to functional complement of *LpNYC1*. The *nyc1* mutant was obtained from the Arabidopsis Biological Resource Center (ABRC) (Alonso et al., 2003). The growth conditions of perennial ryegrass, wild tobacco, and *Arabidopsis* were the same as described previously by Zhang et al. (2016).

2.2. Hormone treatments

Perennial ryegrass leaf tissue (~5 cm in length) was excised from the center of the 3rd leaves from the top and incubated in 3 mM MES buffer (pH 5.8) supplemented with water (control), 200 μM ethephon, 25 μM AVG, and 50 μM ABA at 25 °C under dark. For the *Arabidopsis* senescence assay, leaves (numbers 4–6) of 28-day-old plants were excised and incubated on wet filter paper at 25 °C in total darkness for up to 6 d. Four biological replicates were used in each treatment.

2.3. Measurement of chlorophyll content

Chl content of perennial ryegrass and *Arabidopsis* leaves was measured by incubating approximately 0.1 g leaf tissue in dimethyl sulfoxide (DMSO) for 48 h in dark conditions until chlorophyll was completely extracted. Absorbance values of extracts were measured at wavelengths of 663 and 645 nm using a spectrophotometer (Spectronic in Instruments, Rochester, NY, USA) (Barnes et al., 1992). Chl content was calculated on a fresh weight basis using the equations described by Arnon (1949).

2.4. Cloning and sequence analysis of LpNYC1

The full length coding sequence (CDS) of *LpNYC1* was amplified from perennial ryegrass using the SMARTer® RACE 5′&3′ Kit (Clontech Laboratories, Mountain View, CA, USA). A pair of primers (*LpNYC1*-RACEF and *LpNYC1*-RACER, supplemental information 1) were designed according to the conserved sequence region of *NYC1* genes in rice, brachypodium (*Brachypodium distachyon*), and *Arabidopsis* and used to clone the first fragment of *LpNYC1*. Two gene-specific primers (*LpNYC1*-3′ RACE and *LpNYC1*-5′ RACE, supplemental information 1) were designed to amplify the two ends of the *LpNYC1* gene. DNAsp5 software (http://www.ub.edu/dnasp/) was used to calculate the ratio between non-synonymous and synonymous nucleotide substitutions (Ka/Ks) for selected pairs of homologous *NYC1* genes (Librado and Rozas, 2009). The *cis*-elements in orthologous *NYC1* promoters of perennial ryegrass and five model plants with sequenced genomes were predicted using the ExactSearch: a web-based plant motif search tool (http://sys.bio.mtu.edu/motif/index.php) for conserved *cis*-element sequences (Gunasekara et al., 2016).

2.5. Subcellular localization of LpNYC1

The CDS of *LpNYC1* was cloned into a modified Gateway-compatible p2GWF7.0 vector fused with a GFP tag at the C-terminal (Karimi et al., 2005) for subcellular localization analysis in perennial ryegrass protoplasts. Ryegrass protoplast transformation was carried out according to the protocol developed by Yu et al. (2017).

2.6. Transient overexpression of LpNYC1 in wild tobacco and Arabidopsis nyc1 null mutant complementation assay

The CDS of *LpNYC1* with BamHI and HindIII restriction sites was first cloned into the pENTR/D vector and then recombined into the destination vector pEarleyGate103 to produce a C-terminal GFP/His-tag fusion of LpNYC1 protein (Earley et al., 2006). The destination vector was transformed into *Agrobacterium tumefaciens* strain 'AGL1′. Transient transformation of *LpNYC1* in wild tobacco was carried out following the methods used in a previous work (Zhang et al., 2016). For the *Arabidopsis nyc1* null mutant complementation assay, AGL1/pEarleyGate103-*LpNYC1* was used to transform wild type and *nyc1* mutant using the floral dip method (Clough and Bent, 1998). Transgenic lines were selected through glufosinate ammonium resistance and PCR confirmation, and the T2 homozygous lines were used in the analysis.

2.7. Western blot

Western blot was carried out using an anti-GFP antibody (Life Technologies, Grand Island, NY, USA) to determine the expression of LpNYC1-GFP fusion protein in *Agrobacterium*-inoculated wild tobacco leaves. The procedure of Western blot was performed according to the methods described by Kogami et al. (1994) with some modifications. In brief, approximately 0.1 g transformed wild tobacco leaf tissue was ground into powder in liquid nitrogen and then homogenized in 2 ml cold protein extract solution [50 mM PBS (pH 7.8) containing 1% (w/v) polyvinylpyrrolidone and 0.2 mM EDTA]. Homogenates were centrifuged at 15,000 × *g* for 20 min at 4 °C and the resultant supernatant was transferred to a 1.5 ml microfuge tube and stored at −20 °C for further analysis. Crude protein content in the supernatant was quantified using the methods described by Bradford (1976). SDS-PAGE [7% (w/v) polyacrylamide gel and 0.1% sodium dodecyl sulphate (SDS)] was used to separate 1.0 mg protein from the supernatant. Following electrophoresis, the polypeptides were transferred from the SDS-PAGE gel to an Immobilon-P membrane (Millipore, Bedford, USA). The membrane was first treated with anti-GFP antibody solution and then with another containing horseradish peroxidase-conjugated secondary antibody. The blot was imaged using the Fusion Solo Imaging Chemiluminescence equipment (VILBER LOURMAT, Paris, France).