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The positive effects of exogenous 5-aminolevulinic acid on the chlorophyll biosynthesis, photosystem and calvin cycle of Kentucky bluegrass seedlings in response to osmotic stress

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ABSTRACT

Frequently occurring drought stress is a worldwide environmental constraint that limits the growth and aesthetic quality of Kentucky bluegrass (*Poa pratensis* L.). Our previous study demonstrated that 5-aminolevulinic acid (5-ALA) could alleviate osmotic-stress-induced damage in Kentucky bluegrass seedlings. However, global gene expression profiling and the physiological mechanisms involved in the positive effects of exogenous 5-ALA on plant resistance to stress have not yet been well documented. In this study, RNA sequencing was used to explore the molecular roles of 5-ALA during the osmotic stress response. According to GO and KEGG analyses, photosynthesis was significantly enriched. The transcripts encoding the photosystem II oxygen-evolving enhancer protein, photosystem I subunit, light-harvesting chlorophyll protein complex I and II and ferredoxin were all up-regulated by the application of 5-ALA, accompanied by significant increases in Pn, Tr, ΦPSII, ETR and qP. Transcripts encoding several key enzymes involved in chlorophyll biosynthesis were also up-regulated, accompanied by significant increases in the contents of endogenous 5-ALA, magnesium-protoporphyrin IX, protochlorophyllide and chlorophyll *a*. In addition, carbon fixation was significantly enriched, accompanied by significant increases in the activities of Rubisco, FBA and TPI, which are key enzymes involved in the Calvin–Benson cycle. Notably, transcriptional and physiological analyses revealed that the underlying mechanisms of 5-ALA could involve a major reorientation of photosynthesis, carbon fixation and porphyrin and chlorophyll metabolism under osmotic stress conditions.

1. Introduction

As one of the most common abiotic stress factors, drought causes negative effects on the physiological and metabolic processes of both warm- and cool-season turfgrass species (Huang et al., 2014). The effects of drought stress on the molecular, physiological and metabolic mechanisms of drought tolerance in perennial grasses have been widely reported (Merewitz et al., 2011; Pirnajmedin et al., 2015; Zhou et al., 2013). As a prominent cool-season turfgrass and forage grass, Kentucky bluegrass (*Poa pratensis* L.) is widely used in arid and semi-arid lands. The growth, aesthetic quality and seed yield of Kentucky bluegrass can be influenced considerably by drought stress (Ebdon and Petrovic, 1998; Michaeld et al., 2008). However, with increasing water demand and contamination of potable water, the pressure to utilize more ecological strategies in the management of turfgrass has gradually increased in recent years (Huang et al., 2014). Therefore, studies on the strategies and potential mechanisms of plant growth regulators (PGRs) to ameliorate the adverse effects of drought stress on turfgrass have become increasingly important (Ma et al., 2016; Puyang et al., 2015; Zhang et al., 2016).

In plants, different forms of tetrapyrrole molecules have vital roles in electron carriers, signalling factors, and catalyst redox reactions. Tetrapyrrole biosynthesis starts at glutamate, and the subsequently formed 5-aminolevulinic acid (5-ALA) is metabolized to compound tetrapyrroles (e.g., chlorophyll, vitamin B12, billins and heme) through a variety of reactions (Beale and Weinstein, 1990; Phung et al., 2011). As an essential biosynthetic precursor of all heterocyclic tetrapyrrole molecules, 5-ALA is a potential PGR that is involved in regulating plant growth and yield under normal growth conditions and various abiotic stresses (Akram and Ashraf, 2013). In crops, many studies have demonstrated that 5-ALA enhances plant growth and yield by increasing the chlorophyll content, the chlorophyll *a/b* ratio and the activities of

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photosystems I and II (Awad, 2008; Xu et al., 2010). 5-ALA has a substantial role in antioxidant defence mechanisms in plants in response to various types of abiotic stress, including salinity (Nishihara et al., 2003; Yang et al., 2014), high or low temperatures (Korkmaz et al., 2010; Zhang et al., 2012), heavy metals (Ali et al., 2013a), and electromagnetic radiation (Aksakal et al., 2017). It is well known that the exogenous application of 5-ALA at low concentrations can stimulate a variety of physiobiochemical processes, such as chlorophyll biosynthesis, photosynthesis, the antioxidant system, essential mineral element uptake, and protein synthesis (Awad, 2008; Xu et al., 2009, 2010). These changes in plant physiological and metabolic processes are directly or indirectly involved in the plant mechanisms of stress tolerance to alleviate stress-induced damage and result in increased plant growth and yield (Akram et al., 2012; Ali et al., 2013a; Liu et al., 2011; Naeem et al., 2011).

Protoporphyrinogen oxidase (PPO) catalyses the biosynthesis of protoporphyrin IX (Proto IX), which is directed to the magnesium (Mg) and iron (Fe) branches for chlorophyll and heme biosynthesis, respectively (Sasaki et al., 2002; Nagata et al., 2005; Akram and Ashraf, 2013). Transgenic plants (expressing the PPO gene) had improved 5-ALA biosynthetic ability and protoporphyrin IX, Mg-protoporphyrin IX and protochlorophyllide contents, resulting in a higher shoot water potential and less oxidative damage than wild-type rice (*Oryza sativa*) when subjected to drought stress (Phung et al., 2011). As described above, the key genes regulated by exogenous 5-ALA could be an ideal target for the future genetic engineering of some plant species. However, the specific gene expression profile of 5-ALA-induced tolerance to abiotic stress is largely unknown.

RNA-Seq technology is a high-throughput platform for transcriptomic analysis to efficiently and economically investigate various types of gene expression, even for numerous non-model species (Wilhelm and Landry, 2009; Ma et al., 2017). Our previous study indicated that the application of 5-ALA could improve turfgrass quality and reduce oxidative damage in Kentucky bluegrass under osmotic stress (Niu et al., 2017a). Nevertheless, little information has been available to elucidate the effects of 5-ALA pretreatment on the chlorophyll biosynthesis, photosystem activity, and transcriptomics in plants under osmotic stress. In this study, we reveal the genes associated with osmotic stress and the associated signalling pathways regulated by 5-ALA using RNA-Seq analysis. In addition, physiological analyses of chlorophyll biosynthesis and photosystem activity were performed to demonstrate the key pathways of transcriptional regulation. The objectives of this study were to (1) elucidate the transcriptomic response to 5-ALA and osmotic stress, (2) uncover the genes and pathways associated with 5-ALA-induced osmotic tolerance, and (3) identify the interaction effects of 5-ALA and osmotic stress on leaf chlorophyll biosynthesis, photosystem activity and carbon fixation in Kentucky bluegrass. All of these findings will provide important insight into the mechanisms that turfgrass species utilize to respond to osmotic stress and PGR regulation.

2. Materials and methods

2.1. Plant culture and stress treatment

The plant material, culture and growth conditions, as well as the concentration of 5-ALA applied, were all the same as those used in our previous study (Niu et al., 2017a). Briefly, at 50 days after planting (5–6 leaf stage), seedlings of uniform size were selected and divided into two groups (six pots for each group) for pretreatment. One group was sprayed with a 10 mg L^{-1} 5-ALA solution (50 mL per pot), and the other group was sprayed with the same volume of double-distilled water. Three days later, the seedlings in each pretreatment group were divided into two groups (3 pots for each group). In one group, the seedlings continued to grow in half-strength Hoagland's nutrient solution without stress, and the other group was grown in half-strength Hoagland's nutrient solution with 10% PEG 6000 (osmotic stress). The solution was refreshed every three days. There were four treatment groups: (1) WC: well-watered control plants without 5-ALA pretreatment; (2) WA: well-watered plants with 5-ALA pretreatment; (3) DC: osmotic control plants without 5-ALA pretreatment, and (4) DA: osmotic-stressed plants with 5-ALA pretreatment. A completely randomized block design with four treatments was used in this study. Each treatment had three replicates of three pots with multiple plants in each pot. Leaf samples were collected at 1, 3, 6 and 9 days after PEG treatment, frozen in liquid nitrogen and stored at −80 °C until use.

2.2. RNA extraction, library construction and RNA sequencing

The second leaves of four treatment plants were collected at 1 d after the PEG treatment, and leaf samples were immediately frozen in liquid nitrogen for RNA extraction. The total RNA from 12 samples was extracted using a mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. All the RNA samples isolated were divided into two aliquots for RNA sequencing and real-time PCR analysis. For RNA sequencing, the samples were adjusted to the same concentration (400 ng/μL), and an equal volume of RNA from three replicates was pooled to prepare the cDNA library. The libraries were constructed using a TruSeq Stranded mRNA LTSample Prep Kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. Four libraries (WC, WA, DC and DA) were sequenced on an Illumina sequencing platform (HiSeqTM 2500 or Illumina HiSeq X Ten) at the OE Biotech Company (Shanghai, China), and 125 bp/150 bp paired-end reads were generated. Raw Illumina sequence data were deposited in the National Center for Biotechnology Information (NCBI) and can be accessed in the sequence read archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra) under accession numbers SRR6413558-SRR6413561 for WC, WA, DC and DA, respectively.

2.3. Sequence assembly and annotation

Each library generated more than 6 gigabytes of raw data. To ensure the reliability of the libraries, clean reads were obtained by filtering the raw reads, including the removal of reads containing adapter or ploy-N and low quality and empty reads. De novo assembly was applied to assemble these clean reads because of the lack of reference genomic sequences. Trinity (version: trinityrnaseq_r20131110) was used for the transcriptome assembly, with min_kmer_cov set to 2 by default and all other parameters set to default (Grabherr et al., 2011; Shi et al., 2017). Taxonomic and functional annotation of the transcripts was performed as described by Shi et al. (2017).

2.4. Differential expression analysis

To identify differentially expressed genes (DEGs) in Kentucky bluegrass treated with 5-ALA and PEG, differential gene expression analysis between any two samples among the four libraries was performed using the DEGseq (2010) R package. DEGs were defined as genes having a p-value < 0.05 and $|\log_2(\text{foldchange})| > 1$. To further characterize the function of the DEGs, they were subjected to Gene Ontology (GO) enrichment analysis using the GOseq R package (Young et al., 2010). The statistical enrichment of the DEGs in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was performed using KOBAS software (Mao et al., 2005).

2.5. qRT-PCR confirmation

A total of 21 transcripts was selected to verify the RNA-Seq analysis. The transcripts and gene-specific primer design can be found in the supplementary document (Table S1). Actin (ACT) and S-adenosylmethionine decarboxylase (SAM) were used as reference genes to normalize the data. The primers for reference genes, qRT-PCR, and the