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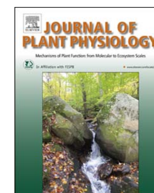

The contrasting microRNA content of a drought tolerant and a drought susceptible wheat cultivar

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ABSTRACT

Drought stress represents one of the most common stresses affecting the productivity of crop plants. A rather recently discovered component of the plant response to drought is the cellular population of microRNAs. Here, the microRNA content was revealed of two bread wheat cultivars contrasting strongly with respect to the ability to withstand drought stress. A total of 1813 miRNAs was identified, grouped into 106 families. Some 104 of these miRNAs were predicted to match 212 novel miRNA precursors. In the drought tolerant cultivar (SM), 105 (33 known and 72 novel) miRNAs were altered in abundance by the imposition of drought stress, while the equivalent number in the more sensitive cultivar (SW) was 51 (20 and 31). An *in silico* analysis predicted that these miRNAs target at least 1959 genes in SM and 1111 in SW, suggesting their broad contribution to the drought stress response. Among the target genes were several known stress-related genes, encoding, for example, superoxide dismutase, various MYB transcription factors, various ABA signaling proteins and various MADS-box transcription factors. In many cases, the more susceptible cultivar SW behaved in a contrasting manner. The suggestion is that miRNAs represent an important aspect of the drought stress response, post-transcriptionally regulating a range of stress-related genes.

1. Introduction

Among the various environmental stresses which negatively impact crop growth and productivity, drought is particularly important, not just on account of its damaging effects, but also due to its ubiquity. It is estimated to be responsible for about half of annual losses to crop production, exceeding the damage caused by all other stress agents combined (Hanson and Weltzin, 2000; Le Houérou, 1996; Parry et al., 2005). Plants have evolved various means of surviving a non-lethal episode of drought stress. These comprise a range of morphological, physiological, cellular and molecular changes which promote tolerance and/or escape from stress (Anjum et al., 2011). A very common morphologically-based response encountered among flowering plants is to accelerate flowering in order to complete the plant's life cycle before the severity of the stress proves to be fatal (Franks, 2011), however some reports mentioned delay in flowering and leaf senescence as symbols of tolerance response in plants (Rivero et al., 2007; Shan et al., 2012). At the cellular level, tolerance can be boosted by the accumulation of various osmolytes, some of which are sugars or sugar alcohols, some are amino acids, and some have the capacity to act as anti-oxidants and/or to neutralize reactive oxygen species (ROS) (Nakabayashi et al., 2014; Vinocur and Altman, 2005).

Gene activity is controlled by a number of regulatory processes; one of the most flexible of these occurs post-transcriptionally, mediated by short (19–24 nt) RNAs referred to as microRNAs (miRNAs); these sequences recognize their gene target via complementarity, and when bound, promote its cleavage. Each miRNA is believed to be transcribed by RNA polymerase II into a primary miRNA transcript, which is processed by DCL1 to form first an intermediate stem-loop precursor miRNA, and later a mature miRNA (Voinnet, 2009). In plants, miRNAs are intimately involved in the regulation of development, signaling and various metabolic processes (Huang et al., 2011), but the evidence now suggests their participation in the response to both biotic and abiotic stress as well (Khraiwesh et al., 2012). Their contribution to the

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drought stress response is likely related to their regulation of stress-related genes.

Both the regulatory role of miRNAs (Han et al., 2014; Meng et al., 2013; Sun et al., 2014; Yao et al., 2007) and their differential abundance in response to various stress agents in wheat (Khraiwesh et al., 2012; Kumar et al., 2015; Wang et al., 2013; Xin et al., 2010; Zhao et al., 2013) and also other plants (Bakhshi et al., 2016; Ehya et al., 2013) has been demonstrated. Although a small number of individual miRNAs have been shown to be critical to wheat's response to drought stress, there have been few attempts as yet to correlate differential abundance of specific miRNAs to a contrasting response to drought stress (Akdogan et al., 2015; Ma et al., 2015; Pandey et al., 2014). Here, high throughput sequencing methodology was employed to explore the miRNA content of the two wheat cultivars SM (SERI M 82) and SW (SW89.5193/kAu2), chosen because they contrast strongly with respect to their sensitivity to drought stress. The same pair of cultivars has recently been subjected to a comparative analysis at the physiological and proteomic level (Faghani et al., 2015) and same samples were also used in the current study to identify drought responsive miRNAs. Our results suggest that miRNAs potentially represent an important aspect of the drought stress response, post-transcriptionally regulating a range of stress-related genes.

2. Material and methods

2.1. Plant materials and sample preparation

Plant materials included in this work were same as those used in our previous study (Faghani et al., 2015). A detailed description of the raising of these plants has been given previously. Briefly, seeds of SM as a drought-tolerant genotype and SW as a drought-susceptible genotype were sown into soil-filled PVP pipes [measuring 7.5 cm (diameter) × 120 cm (height)], and the seedlings were raised under greenhouse conditions. The seedlings were watered three times a week until they had reached the two leaf stage. Drought stress was imposed by withholding water until the field capacity (FC) of the soil fell to 20%. The moisture content in control pipes, which were kept well-watered was around 80% of field capacity. To ensure plant materials were at the same developmental stage only fully developed leaves were sampled from each of the cultivars subjected to drought stress ($SM_{(S)}$ and $SW_{(S)}$, respectively) and those given adequate watering ($SM_{(N)}$ and $SW_{(N)}$, respectively). Each cultivar/treatment combination was represented by three biological replicates. The harvested leaf was snap-frozen in liquid nitrogen and stored at −80 °C.

2.2. Construction of small RNA libraries and their sequencing

Total RNA was extracted from a 100 mg sample of powdered leaf, using a High Pure miRNA Isolation Kit (Roche, Germany), following the manufacturer's protocol. The quality and quantity of the RNA preparations were checked using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA). The RNAs obtained from each of the cultivar/treatment replicates was pooled on an equimolar basis, and submitted to BGI (http://www.genomics.cn) for sequencing using an HiSeq2000 sequencing platform (Illumina, USA).

2.3. Data analysis

Low quality reads, those including 5′ primer sequence, those lacking the 3′ primer, those without an insert tag, those including a poly-A tail and those below 18 nt in length were all removed. The remaining set of small RNAs were mapped onto the wheat genome sequence (ftp://ftp.ensemblgenomes.org; *Triticum aestivum*; IWGSP1 release-23) and only those which found an exact match were retained. Non-coding RNAs (rRNAs, tRNAs, snRNAs and snoRNAs) were identified by a Blast-based search of both GenBank (www.ncbi.nlm.nih.gov/genbank/) and Rfam (www.sanger.ac.uk/software/Rfam), and removed from the set of small RNAs. The remaining sequences were compared with the multi-species database of miRNAs housed in mirbase.org/release 21. This version of miRBase contained 19207 mature miRNAs from plants (Viridiplantae), 2200 mature miRNAs from cereals, and 119 matures miRNAs belonging to wheat. Finally, the miRNA prediction software Mireap (sourceforge.net/project/mireap/) was run under default setting to identify novel miRNAs, the sequences of which were then used as query terms to identify known cereal miRNAs represented in miRBase, allowing a maximum of two mismatches.

A normalized miRNA abundance (the number of reads × 10^6/total number of filtered reads in the library) was calculated for each of $SM_{(N)}$, $SW_{(N)}$, $SM_{(S)}$ and $SW_{(S)}$. Those associated with a count of below ten per ten million (TPTM) in all four libraries were excluded from further analysis. A miRNA was considered as 'drought-responsive' if it both showed a fold change of at least two ($|\log_2\text{Ratio}| > 1$) and the abundance difference was statistically significant according to Audic and Claverie's p-value (Audic and Claverie, 1997). Potential target genes were predicted using psRNATarget (plantgrn.noble.org/psRNATarget/) tool: the miRNA query sequences were searched against the wheat unigene v12 DFCI gene Index (TAGI), applying a prediction score cutoff value of 3.0, a length for complementarity score of 20 and a target accessibility of 25. In addition, the set of predicted targets were searched in recently published miRNAs data of wheat in which the correlation of miRNAs with their targets have been demonstrated experimentally (Kaur et al., 2016, Ma et al., 2015; Pandey et al., 2014; Tang et al., 2012). Gene ontology analysis was applied to putative targets to provide further information with respect to function (Pandey et al., 2014).

2.4. Validation of the miRNA abundance using stem-loop RT-PCR

Primers for stem-loop RT-PCR were designed in accordance with Chen et al. (2005a): the resulting primer sequences are listed in Table S1. Quantitative real-time PCR (qRT-PCR) was conducted using an iCycler iQ Real-Time PCR System (Bio-Rad, USA), based on iQ SYBR Green Supermix Kit (Bio-Rad) in 25 µL reactions which each contained 10 pM of each primer and 50 ng cDNA. The amplification regime comprised a 94 °C/2 min denaturation, followed by 35 cycles of 94 °C/30 s, primer-pair dependent annealing temperature/60 s and 72 °C/90 s. Each cultivar/treatment sample was represented by three biological replicates and each replicate by three technical replicates. The wheat 18S rRNA gene was used as the reference sequence. Fold changes in miRNA abundance were calculated using the comparative ΔC_t method, following the $2^{-\Delta\Delta ct}$ formula. Statistical significance was assigned to differences in abundance using the Students' *t*-test ($p < 0.05$).

3. Results

3.1. Analysis of sequence data and the identification of miRNAs

The number of clean reads recovered from each of the four libraries averaged 26–29 million. Quality trimmed reads were then subjected to a database search (genebank and rfam) for the removal of reads originating from rRNA, tRNA, snRNA, snoRNA, chloroplast and mitochondrial RNA sequences (Fig. 1). To discard mRNA degradation products, the sRNA sequences were aligned against the transcripts of wheat protein encoding genes. The remaining sequences resolved into 1.2–1.5 million unique sRNA reads with the $SM_{(N)}$ showing the lowest and the $SW_{(N)}$ showing the highest number of unique sequences. An analysis of their length distribution revealed a prevalence 18–24 nt reads (Fig. 2). In the set of non-clustered reads, those of length 20–21 nt were most prevalent, however, in clustered reads, 24 nt sRNA reads were the most strongly represented. This data suggests that 21 nt size sRNA reads that are highly redundant than 24 nt ones are representa-

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