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Differential carbohydrate gene expression during preplanting temperature treatments controls meristem termination and bulbing in garlic

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ABSTRACT

Garlic is a sterile plant that reproduces vegetatively through cloves. We previously found that in 'Shani', a garlic cultivar adapted to Mediterranean climate, preplanting storage temperature alters plant development, architecture and yield during the growing period. To determine the possible mechanism underlying this phenomenon, the effects of two temperature treatments, 2 °C and 33 °C, were examined in the internal bud (IB) and storage leaf (SL) of garlic cloves using a transcriptomic approach. Higher representation of carbohydrate metabolism genes was found in cloves at 2 °C vs. 33 °C in both analyzed organs. Of the upregulated genes, 34% were common to IB and SL, whereas 15% and 51%, respectively, were organ-specific. Gene-ontology analysis confirmed that in both organs, carbohydrate metabolism-related genes are more highly expressed at 2 °C than at 33 °C. Chromatography analysis of fructan-degradation products sucrose and fructose in the IB and SL confirmed their higher availability at 2 °C vs. 33 °C. Expression analysis of garlic homologs of meristem-specific and flowering genes revealed differential expression patterns at 2 °C vs. 33 °C. RNA-Seq data were validated by real-time quantitative PCR of 10 candidate genes related to carbohydrate metabolism and flowering; results suggested that *FLOWERING LOCUS T2* (*FT2*) is affected by carbohydrate metabolism. Overexpression of garlic *FT2* in *Arabidopsis* resulted in an early-flowering phenotype and early termination of stem development. We suggest that preplanting environment temperature alters carbohydrate metabolism and consequently *FT2* expression, affecting the timing of meristem termination and bulbing after replanting the garlic clove.

1. Introduction

Garlic (*Allium sativum* L.) is the second most important *Allium* crop. Apart from being routinely used as a vegetable and condiment, it is known for its medicinal and nutraceutical properties (Bayan et al., 2014; Gebreyohannes and Gebreyohannes, 2013; Santhosha et al., 2013). Garlic cultivation ranges from the equator to about 50° latitude and it is produced in most countries with mild winters and some rainfall, followed by dry summers (Diriba-Shiferaw, 2016; Mhazo et al., 2014). The garlic cultivar Shani technically belongs to the hard-neck garlic category, which produces a flowering stem, but instead of an inflorescence, it develops a few topsets (Mathew et al., 2011; Rohkin Shalom et al., 2015). In hot climates, such as in Israel, 'Shani' is a short-day garlic cultivar that is cultivated in winter and harvested in spring.

Because all garlic cultivars are sterile, reproduction is generally vegetative with the cloves serving as propagation material (Kamenetsky, 2007). Garlic bulb maturation is characterized by the drying up of foliage leaves and prior to harvest, the bulbs enter a period of dormancy when they exhibit almost complete inactivity regardless of environmental conditions with a low rate of respiration (Diriba-Shiferaw, 2016; Mashayekhi et al., 2016). During storage, the internal meristems maintain a continuous process of slow but constant development (Rohkin Shalom et al., 2015). Several factors limit the rate of sprouting in garlic: cultivar, storage temperature, and maturity at harvest (Kamenetsky, 2007; Okubo, 2012). Plant growth in terms of leaf number and plant height increases until bulbing is initiated. Ideally, plants should achieve adequate growth before bulbing commences, so that the foliage is capable of producing large bulbs and high yields

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(Kamenetsky, 2007). Water and carbohydrates are the major constituents of garlic bulbs, accounting for about 65% of its fresh weight and 77% of its dry weight, respectively (Cardellina, 2013; Koch and Lawson, 1996). The major proportion of carbohydrates consists of water-soluble high-molecular-weight fructose polymers termed fructan (Santhosha et al., 2013). Garlic cloves also contain proteins, pectin, minerals, and polyamines (Suleria et al., 2015).

In garlic, the transition of the apical meristem from a vegetative to reproductive state occurs during the active growing phase (Kamenetsky and Rabinowitch, 2001). Its ontogeny is affected mainly by genetics and environmental factors, especially storage and growth temperatures, photoperiod, and the plant's phenological stage (Chen et al., 2013; Rohkin Shalom et al., 2015; Takagi, 1990). In general, plants have evolved mechanisms to control the timing of meristem transition by integrating plant development (maturity, carbohydrate status) and environmental factors (changes in photoperiod and cold temperatures) for their reproductive success (Amasino, 2010; Wahl et al., 2013). Recent studies in *Arabidopsis*, rice, wheat, *Medicago,* onion and several other plants have reported that environmental factors such as photoperiod, temperature and internal factors [plant age, gibberellic acid, and sugars such as trehalose-6-phosphate (Tre6P)] regulate flowering through expression of the Flowering Locus T (FT) protein, which acts as a systemic floral signal molecule (Putterill and Varkonyi-Gasic, 2016; Wahl et al., 2013). In *Allium*, different FT homologs have been attributed with control of flowering and bulbing (Lee et al., 2013; Rohkin Shalom et al., 2015).

Rohkin Shalom et al. (2015) found that preplanting temperature treatments (2 °C or 33 °C) can inhibit internal bud growth. Bulb formation started 30 and 60 d after planting cloves incubated at 2 °C or 33 °C, respectively, and was found to be associated with transition of the shoot apical meristem to reproductive state, through regulation of an *FT2* homolog (Rohkin Shalom et al., 2015). Plants from cloves treated by warmer temperature developed multiple leaves and a normal floral stem, whereas cold-treated cloves developed fewer leaves and the floral stem was very small or undetectable (Rohkin Shalom et al., 2015). However, the mechanism for early meristem termination and bulbing in plants, originate from propagation material stored at 2 °C prior to planting, is still not clear. We therefore investigated the effect of temperature treatment (2 °C or 33 °C) on garlic transcriptome, to gain further insight into the pathways, signaling molecules and genes involved in causing this phenotype in garlic plants.

2. Materials and methods

2.1. Plant material

The bulbs of garlic cultivar Shani were produced in a commercial field in the southern Arava in Israel, using standard commercial growth practices. Garlic bulbs were harvested and dried in the field, then incubated in the dark at two temperature regimes: constant 2 °C or 33 °C, with relative humidity of 85 and 45%, respectively, for 90 d. By the end of the incubation period, internal buds (IB) and storage leaves (SL) were sampled in three biological replicates, 10 cloves of different bulbs per replicate, pooled together and immediately dipped in liquid nitrogen, and then stored at −80 °C until RNA extraction.

2.2. RNA isolation and sequencing procedures

Total RNA of each sample was extracted according to the CTAB protocol (Chang et al., 1993). Sample purity and integrity were verified by RNA 6000 Nano Assay with an Agilent 2100 Bio Analyzer (Agilent Technologies, Waldbronn, Germany) with a minimum RNA integrated number value of 7, and then samples were treated with DNase (Epicenter, Madison, WI, USA) according to the supplier's instructions. Total RNA samples were shipped to the Roy J. Carver Biotechnology Center, W.M. Keck Center for Comparative and Functional Genomics, Urbana, IL, USA, for library preparation and sequencing. The constructed libraries were used for transcriptome sequencing using Illumina HiSeq 2000 and TrueSeq protocols. Twelve libraries of 100-nucleotide-long single-ended RNA sequences (three libraries per treatment) were generated for the IB and SL of cloves at 2 °C or 33 °C treatment.

2.3. Transcriptome analysis

Raw reads were subjected to a cleaning procedure using the FASTX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html, version 0.0.13.2) which included: (i) trimming read-end nucleotides with quality scores < 30 using fastq_quality_trimmer; (ii) removing read pairs if either one had less than 70% base pairs with quality score ≤30 using fastq_quality_filter. An organ-specific *A. sativum* transcriptome catalog from six vegetative and reproductive organs was employed as a reference (Kamenetsky et al., 2015). The cleaned reads from each library were aligned separately to the transcriptome catalog using Bowtie aligner (Langmead et al., 2009), and the abundance estimation was calculated using the Expectation-Maximization method (RSEM), by estimating maximum likelihood expression levels (Li and Dewey, 2011). Differential expression analysis of the sequence count data for each pair of samples (four comparisons overall) was performed by Bioconductor DESeq package in the R environment (Anders and Huber, 2010). Differential expression was defined as an over fourfold difference in transcription expression with a false discovery-corrected statistical significance of at most 0.001 (i.e., FDR < 0.001) (Benjamini and Hochberg, 1995). The transcripts that were differentially expressed in at least one pairwise sample comparison were examined using cluster analysis. Following the Trinity protocol (Haas et al., 2013), expression normalization was calculated by trimmed mean of M values (TMM) normalization and fragments per kilobase of transcript per million mapped reads (FPKM) calculation. Based on the average values in the three biological repeats, hierarchical clustering of transcripts and samples was performed and clusters were extracted using R scripts. Gene Ontology (GO) enrichment analysis was carried out using Blast2GO software (Conesa et al., 2005) based on GO terms obtained from the garlic transcriptome catalog (Kamenetsky et al., 2015). Analysis was carried out using Fisher's Exact Test (Upton, 1992) with multiple testing correction of False Discovery Rate, FDR (Benjamini and Hochberg, 1995). Threshold was set as FDR with corrected P-value of less than 0.05. GO analysis was done by comparing the GO terms in the test sample to the GO terms in a background reference. The sugar metabolism- and flowering-related genes were sorted manually based on the GO terms and corresponding sequences therein (test comp) to a particular GO term. The RNA-Seq data were deposited in the NCBI sequence read archive (SRA) as bioproject PRJNA384121 and biosample SAMN06828997.

2.4. Real-time quantitative (q) PCR for transcript validation

Total RNA was isolated from the garlic cloves incubated at 2 °C or 33 °C and used for c-DNA synthesis. Total RNA of each sample was extracted according to the CTAB protocol (Chang et al., 1993). The cDNA was prepared using 500 ng of total RNA with the Verso c-DNA Reverse Transcription Kit (Applied Biosystems, Forster City, CA, USA). The expression patterns of 10 genes were analyzed using gene-specific primer pairs (Supplementary Table 1), and actin was used as an internal control for normalization. Real-time qPCR was performed in the Step One Plus real-time PCR System using Fast Syber-Green Master Mix (Applied Biosystems). Fold expression was calculated by a previously described method (Livak and Schmittgen, 2001), and specificity of the real-time qPCR was monitored by melting curve analysis.