Environmental and Experimental Botany 106 (2014) 87–98

Contents lists available at ScienceDirect

Environmental and Experimental Botany

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

Elucidating cold acclimation pathway in blueberry by transcriptome profiling

Jose V. Die, Lisa J. Rowland *

U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD, USA

ARTICLE INFO

Article history:
Received 5 November 2013
Received in revised form
17 December 2013
Accepted 27 December 2013
Available online 6 January 2014

Keywords:
Annotation
GO terms
C3H
Non-coding RNA
qPCR
Transcription factors
Vaccinium

ABSTRACT

A fundamental goal of cold acclimation research is to understand the mechanisms responsible for the increase in freezing tolerance in response to environmental cues. Changes in gene expression underlie some of the biochemical and physiological changes that occur during cold acclimation. Detailed and comprehensive transcriptome annotation can be considered a prerequisite for effective analysis and a fast and cost-effective way to rapidly obtain information in the context of a given physiological condition. By computational predictions and manual curation, we have annotated 454 sequence assemblies from two blueberry cDNA libraries that represent flower buds in the first and second stages of cold acclimation. Gene ontology functional classification terms were retrieved for 4343 (80.0%) sequences. GO annotation files compatible with a commonly used annotation tool have been generated and are publicly available. By mining the dataset further, it was possible to associate presence of certain transcripts related to carbohydrate metabolism and lipid metabolism with different stages of cold acclimation. This was concomitant with differential presence of Zn finger functional domains and C3H-family transcription factors. The expression of a few selected genes was validated by quantitative real-time PCR assay. Results demonstrate that our transcriptome database is a rich resource for mining cold acclimation-responsive genes.

Published by Elsevier B.V.

1. Introduction

To survive the winter, plants of the temperate zone start preparing as early as the late summer or early fall by a process known as cold acclimation. This, in woody perennials, is generally considered a two-step process, first triggered by shortening day-length and then, declining temperatures (Powell, 1987; Weiser, 1970). The specific mechanisms plants use to survive freezing temperatures vary somewhat depending on the plant species and, to a certain extent, the particular tissue/organ exposed to freezing (Fujikawa et al., 2009; Levitt, 1972). Genetic and molecular evidence shows that cold acclimation is a complex phenomenon involving the alteration of metabolism with synthesis of specific metabolites, proteins, lipids and carbohydrates, and changes in membrane composition (Guy, 1990; Janská et al., 2010). As a whole, these processes have the specific outcome of protection against dehydration, oxidative damage and other stresses associated with freezing temperatures. With the advances in plant genomics research in recent years, fundamental knowledge of plant responses to cold is increasing at a rapid pace (Qin et al., 2011; Thomashow, 2010).

Blueberry (*Vaccinium* spp.) is an important fruit crop because of its high nutritional value, a rich source of antioxidants, and it is one of the major berry crops grown in the United States (USDA-NASS, 2013). Low temperatures are one of the critical environmental factors that limit its growth, survival and geographical distribution. Lack of cold hardiness, therefore, has been identified as one of the most important genetic limitations of current highbush blueberry (V. corymbosum) cultivars (Rowland et al., 2011). The use of *Arabidopsis* to understand the process of cold acclimation and general responses to cold temperature, while providing a wealth of information, has perhaps led to an over-extrapolation of the data, ignoring the context of the whole plant and its interaction with the environment (Gusta and Wisniewski, 2013). In addition, *Arabidopsis* and many herbaceous plants do not normally experience freezing temperatures and their cold acclimation capacity is generally less than that of woody species. Blueberry flower buds, on the other hand, can survive temperatures as low as ~−30 °C during the middle of winter yet suffer devastating damage from temperatures slightly below 0 °C during early spring when deacclimating (Ehlenfeldt et al., 2012; Rowland et al., 2013). Consequently, cold acclimation is probably more complex in woody perennials and, within this context, blueberry has and continues to serve as a model system for studying cold tolerance (Rowland et al., 2011).

Over the past decade, our laboratory has been working toward increasing our understanding of the genetic control and regulation

* Corresponding author.
E-mail address: Jeannine.Rowland@ars.usda.gov (L.J. Rowland).

0098-8472/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.envexpbot.2013.12.017

of cold hardiness in blueberry through a combination of genetic, molecular and physiological approaches. The ultimate goal is to integrate the knowledge obtained from these various approaches to develop more cold hardy cultivars for the industry. Particularly, genomics-based research has identified a number of potential genes associated with cold acclimation or contributing to cold hardiness in blueberry. The construction and analysis of cDNA libraries using RNA from cold-acclimated (CA) and non-acclimated (NA) floral buds have revealed a marked difference in the transcriptional regulation under the two conditions (Dhanaraj et al., 2007, 2004). Microarray technology has allowed the identification of genes that had not been previously reported as cold-induced in *Arabidopsis*. In addition, it has provided evidence for differences between field and cold room-based acclimation (Dhanaraj et al., 2007; Rowland et al., 2008), reflecting the importance of the environmental context to the physiology, growth habit and life cycle of the plant and how it plays a relevant role in the elucidation of cold hardiness (Gusta and Wisniewski, 2013). Additionally, the generation of subtracted libraries has resulted in valuable supplemental data leading to the identification of many potential regulatory genes such as transcription factors (Naik et al., 2007).

One of the major advances in the past decade of research on plant survival during winter has been the discovery of the C-repeat/binding factor/dehydration-responsive element binding factor (*CBF/DREB*) gene family. These transcriptional activators have been shown in *Arabidopsis* to regulate a number of downstream genes associated with low temperature response in plants (Shinozaki et al., 2003; Thomashow et al., 2001). *CBF* existence and importance has also been established in many other plants, including woody species through heterologous gene expression studies (Wisnieswski et al., 2013). Cloning of a *V. corymbosum CBF* followed by overexpression in *Arabidopsis* resulted in the induction of *COR* (cold-regulated) gene expression and constitutive freezing tolerance in transgenic plants (Polashock et al., 2010). Overexpression of the same *CBF* gene under the control of the *CaMV 35S* promoter in transgenic blueberry lines has also been shown to result in an increase in freezing tolerance in non-acclimated plants, although not to the level found in cold acclimated plants (Walworth et al., 2012). This suggests that manipulation of the *CBF* system may be potentially useful for the improvement of freezing tolerance in woody fruit crops, although it is probably not the only pathway involved (Wisnieswski et al., 2013). Therefore, there is a growing interest in cold-responsive genes that are potentially under the control of these *CBF* activators and other transcriptional activators and collectively, these results provide evidence for an emerging picture of blueberry response to cold temperatures. Determining the identity of cold-responsive genes and their regulators in blueberry will be useful in understanding their function and establishing their relative importance. Clearly such information may help in the development of strategies for the improvement of cold hardiness of woody perennials in general.

More and more large-scale, high throughput gene expression studies as well as the sequencing of entire transcriptomes and genomes, are being conducted today in actual crop species, because of the advent of affordable Next Generation Sequencing (NGS) technologies. Recently, the first blueberry transcriptome from 454 NGS has become publicly available making possible computational analyses of thousands of sequences within the blueberry and related species (Rowland et al., 2012). The availability of these kinds of resources with new bioinformatics tools provide the ability to address a more comprehensive analysis of complex and multigenic traits and will help to integrate the vast amount of data generated (Die and Rowland, 2013a).

In this study, we aimed to mine the blueberry 454 transcriptome database more in depth than previously done in order to study global changes in gene expression and identify relevant pathways or functional groups of genes associated with first and second stages of cold acclimation. We conducted new analyses such as identification of transcription factors and non-coding RNAs by computational predictions. The latest annotation files output are available and provided in a format compatible with publicly available tools. Our results show that this database is very useful for the ongoing cold acclimation research in blueberry to confirm previously reported changes, identify so far unreported genes, and establish which processes predominate during different stages of cold acclimation. Our dataset may be useful for comparative genomic studies among other woody perennial species as well.

2. Material and methods

2.1. Annotation, functional classification and bioinformatics tools

Blueberry cDNA libraries used in this study, from flower buds collected at 0 and 397 chill units (hours of exposure to temperatures from 0 to 7 °C) from northern highbush V. corymbosum cultivar 'Bluecrop', were constructed and described earlier (Rowland et al., 2012). The 0 and 397 chill unit time points corresponded to collection dates of 2006, September 7th and November 30th. Early September represents the first time point when it was possible to collect flower bud samples, as they were just being formed. Average temperatures and daylength for this period are shown in Suppl. Fig. 1.

In this work, the Gene Ontology Functional Annotation Tool Blast2GO version 2.6.6 (Conesa and Götz, 2008; Götz et al., 2008) was used to assign GO identities and enzyme commission numbers to contigs from assemblies of the 454 transcript sequences generated from these libraries. For the annotation, the following configuration settings were used: BLASTX against NCBI non-redundant (nr) protein database, *E*-value filter $\leq 10^{-6}$, HSP length cutoff of 33, maximum 20 BLAST hits per sequence and annotation cutoff of 50. Furthermore, to improve annotation ability, InterProScan was performed and results were merged to GO annotation.

The program Blast2GO was also used to assign biological functions, cellular components and cellular processes to the transcripts. Only sequences that were not successfully annotated were selected and re-annotated with more permissive parameters. Then, plant GOslim for all three independent GO categories were obtained from the AgBase database (http://www.agbase.msstate.edu/) to be further assigned to secondary categories and to be compared with those obtained in *Arabidopsis thaliana* (McCarthy et al., 2006). ATH GOslim *Arabidopsis* data were obtained from The *Arabidopsis* Information Resource (TAIR) database (http://www.arabidopsis.org/). The analysis of biological processes/pathways was carried out using the KEGG (Kyoto Encyclopedia of Genes and Genomes) map module supported by the Blast2GO bioinformatics tool. Blast2GO also enabled analysis related to over-representation of functional categories through the Gossip package (Blüthgen et al., 2005) for statistical assessment of annotation differences between two sets of sequences, using Fisher's exact test for each GO term. False discovery rate (FDR) controlled *P* values (FDR < 0.01) were used for the assessment of differentially significant metabolic pathways. To identify transcription factors, BLASTX searches against a comprehensive *A. thaliana* transcription factor collection maintained at Plant Transcription Factor Database (PlantTFDB v3.0; http://planttfdb.cbi.pku.edu.cn/) were performed (Jin et al., 2013). Finally, the Coding Potential Calculator website (http://cpc.cbi.pku.edu.cn) was used to identify non-coding RNAs (Kong et al., 2007). Parameters for the website were set to use only the forward strand. The output data was analyzed, and a list of transcript IDs described as "non-coding" and "weakly non-coding" was created.