Proposal Category: _X__Research ___Outreach

Proposal Status: _X__ New Proposal _ Previously funded by SRSFC

Final Report of Project - 2018 R-09

Title: Response of Floral Buds to Frost and Recovery Treatments in Southern Highbush Blueberry CV. 'O'Neal'

Name, Mailing and Email Address of Principal Investigator(s):

Lauren E. Redpath (PI)
PhD Student
Department of Horticultural Science
North Carolina State University
2721 Founders Drive, 253 Kilgore Hall
Raleigh, NC 27695
Ph: (919) 805-8296
Email: leredpat@ncsu.edu

Hamid Ashrafi, PhD (Co-PI)*
Assistant Professor
Department of Horticultural Science
North Carolina State University
2721 Founders Drive, 234 Kilgore Hall
Raleigh, NC 27695
Ph: (919) 515-1216
Email: hamidashrafi@ncsu.edu

Rachel A. Itle, PhD (Co-PI)
Assistant Research Scientist
Department of Horticulture
University of Georgia
1109 Experiment Street
Griffin, GA 30223
Ph: (770) 233-6113
Email: ritle@uga.edu

* Hamid Ashrafi has faculty status at NCSU and will manage the account that will be associated with the fund.

Objectives

Our long-term goal is to identify blueberry genotypes that are cold hardy to early spring frost periods in North Carolina and in the southeastern U.S.

Our objective in this research is understand the mechanism(s) by which blueberry plants respond to sub-zero temperatures during early spring frost periods. Furthermore, we would like to determine how gene expression is affected by decreasing temperatures through a range of floral developmental stages: dormancy, bud break, tight cluster, and bloom. This knowledge would enable the development of tools in the breeding program that could be used to develop new blueberry cultivars that flower after detrimental spring freeze events. Cultivars that would flower after spring freezes would help the blueberry industry to avoid devastating crop losses that can result from these freeze events, and would help to increase grower profit.

Justification

Blueberry is the highest grossing fruit in terms of production value, if not in acreage, in many southern states ((Usda), 2016). Freeze events in which the floral buds are injured reduce the yield and annual production value. Low chill varieties, such as southern highbush blueberries (e.g. cultivars 'Emerald' and 'Star'), are grown extensively in the southern states and flower early, making them susceptible to freeze damage caused by late spring freeze events. North Carolina, and some parts of Georgia, are under the influence of two weather patterns: warm temperatures from the south and cold weather from the north, often times called Canadian cold blasts. As a result of the freeze events this past spring in 2017, blueberry growers in NC lost 50% of their crop (North Carolina Blueberry Council, Pers. Comm.).

Floral buds are the most susceptible part of the plant to freeze injury (Clark et al., 1996). As a plant develops towards bud break, it loses cold hardiness; later stages of bud development are more susceptible to cold injury at higher temperatures (Patten et al., 1991). Flower buds in different stages of development post deacclimation have not been studied for cold hardiness. As the plant develops into each progressive floral developmental stage, its flower buds are increasingly susceptible to cold injury. The combination of early spring bloom with subsequent early floral development and spring freeze events puts southern highbush blueberries at a greater risk for crop loss.

In order to protect their crop from early spring frost damage, blueberry growers usually spray water over their entire farm overnight and during the following day, or as long as the frost lasts, in a process commonly referred to as freeze protection. This process protects the flower buds from freezing by releasing a few degrees of heat around the flower buds when liquid water changes to solid water on the plant. If frost period lasts more than one or two nights, they may have to use their entire water reservoir (usually a nearby pond), which reduces their ability to protect against additional future freeze events that may occur. Further, fuel to pump the water over the plants is an additional expense, increasing the cost of protection. This is measure is very costly and requires growers to stay up the whole night to tend to the frost protection operation. It also reduces the profit margin of crop sale and it is not always 100% effective.

Certain southern high bush cultivars such as ‘O’Neal’ and ‘Reveille’ have a very distinct characteristic. They do not exhibit early break bud even under warm conditions seen in December or January, only breaking bud when the risk of frost is minimized. This characteristic sometimes is attributed to higher chilling hour requirement of these and similar cultivars. However, both cultivars have FL4B in their background, which is known to be a source of lower chill hour requirement for many southern high bush blueberries. Therefore, understanding the mechanism by which ‘O’Neal’ and similar cultivars do not initiate bud break until late March (in North Carolina), may help to develop markers that can be used in our marker assisted breeding program in future. These markers would be used as tools to help in selecting for new cultivars that would similarily avoid early bud break.

Therefore, the objectives of this study are to examine differential gene expression from RNA extracted from O’Neal blueberry buds collected in two different floral stages and treated to a range of freezing temperatures. We hypothesize that gene products involved in the floral bud break signaling pathway do not activate during short periods of higher temperatures and that longer high temperature periods are required to accumulate gene products to activate the floral bud break signal in ‘O’Neal’ or in similar cultivars. Further, it is expected that genes relating to cold hardiness will be expressed at higher rates in cultivars that break bud later. In regard to physiological recovery time, we hypothesize that with greater recovery time at 4 °C, the expression of genes relating to cold hardiness will decrease according to temperature and threat of freeze.

Materials and Methods

Plant material. One-year old shoots of ‘O’Neal’ containing floral buds were collected on 3 March 2017 from NC State Ideal Tract Farm in Castle Hayne Research Station. ‘O’Neal’ plants displayed a range of bud stages; both shoots with tight bud and tight cluster bud stages were collected (Fig. 1).

Freeze treatment. Samples were prepared by removing remaining leaves. Following, stems with intact attached buds were trimmed to 5 cm segments with the terminal bud removed (Fig. 1). Sixteen subsets containing stem segments with a minimum of three attached floral buds were prepared for both bud stages of ‘O’Neal’. Samples were placed on a tissue (Kimwipe®, Irving, TX), folded, and moistened with D.I. water. Sample sets were placed in individual freezer

‘O’Neal’ cultivar.

bags; two subsets of 'O'Neal' (per bud stage) were placed in each of the temperature-designated bags (Table 1). The bags were placed on a rack in the ESPEC EY-101 (Tabai Espec Corp., Osaka, Japan) freeze chamber with a freezing rate of 4 °C·h^{-1} (7.2 °F reduction every hour). Removal occurred every 3 °C, from -3 °C to -21 °C (approximately 45-minute intervals). A control bag of each bud stage was held at 4 °C (39.2 °F).

Recovery. The freeze treated and control floral buds were stored in a refrigerator at 4 °C, where they recovered for different time periods. Three buds per temperature treatment of each bud stage were removed from 4 °C one-day post freeze treatment and the three remaining buds were removed a week after storage at 4 °C. Bud weight was measured for 0.2 g, placed in 1.5 mL screw-cap tubes with a Biospeck 3.2 mm chrome steel bead, dipped in liquid nitrogen and stored at -80 °C. Buds stored for one week at 4 °C were prepared in the same manner as those removed one-day post freeze treatment (Table 1).

Table 1. Seven temperature treatments and two floral bud stage with recovery period of one day and one week were used for sampling. Only highlighted samples (24) will be used in this study.

Temperature Treatments		Floral Bud Stage		Recovery Period at 4 °C (39.2 °F)	
°C	°F	Tight Bud	Tight Cluster	1 day	1 week
4	39.2	6 buds	6 buds	3 tight buds 3 tight clusters	3 tight buds 3 tight clusters
-3	26.6	6 buds	6 buds	3 tight buds 3 tight clusters	3 tight buds 3 tight clusters
-6	21.2	6 buds	6 buds	3 tight bud 3 tight cluster	3 tight buds 3 tight clusters
-9	15.8	6 buds	6 buds	3 tight buds 3 tight clusters	3 tight buds 3 tight clusters
-12	10.4	6 buds	6 buds	3 tight buds 3 tight clusters	3 tight buds 3 tight clusters
-18	-0.4	6 buds	6 buds	3 tight buds 3 tight clusters	3 tight buds 3 tight clusters
-21	-5.8	6 buds	6 buds	3 tight buds 3 tight clusters	3 tight buds 3 tight clusters

RNA extraction. In order to keep the number of samples to manageable scale and in the scope of this project, three replicates of both bud stages of 'O'Neal' buds treated to 4 °C, as well as -12 °C with recovery times of 1-day and 1-week will be used for RNA extraction. These temperatures are chosen as a positive control at which the buds are expected to be viable (4 °C), and a predicted inflection point (-12 °C), where after the decreasing temperature is expected to be lethal to the buds. Samples will be pulverized with the 2010 Geno/Grinder® (SPEX® SamplePrep, Metuchen, NJ). RNA will be extracted from the ground tissue using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO). Extracted RNA samples will be quantified via Qubit™ 3.0 Fluorometer (Invitrogen, Carlsbad, CA) before using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) to evaluate RNA quality.

Transcriptome sequencing and data analysis. The 24 samples of 'O'Neal' floral buds will be sent to Novogene (Novogene, Sacramento, CA) for transcriptome library preparation and sequencing. Transcriptome data will be analyzed using CLC genomics workbench (https://www.qiagenbioinformatics.com/).

Results

Sequence Data. 185 Gbp of raw read data were obtained from Illumina sequencing. Using the CLC genomics Workbench (v11), reads were trimmed for polyadenylated tails and adapters and 150 Gbp nucleotides were retained. An 'O'Neal' transcriptome assembly was constructed from leaf, stem, flower, root (obtained from another study), and bud tissue samples (this study). The assembly was used as a reference perform differential gene expression analysis. A total of 180,487 contigs were assembled with an N_{50} of 1,100 base pairs and total length of 156,578,099.

Differentially Expressed Genes. Trinity (Grabherr et al., 2011)pipeline was used to analyze the data for differential gene expression. All possible pairwise comparisons were analyzed using DESeq2. DESeq2 identified 12,993 unique differentially expressed genes (DEG) with filters of log2 fold change of ±2 and a p-value of 0.05. The majority of DEGs were related to recovery (4,476), followed by temperature (3,383) and tissue type (1,701). Unigenes were not unique to these treatments, some unigenes were shared between two treatment parameters. Temperature and recovery treatments had the most shared contigs (1,939) (Fig. 1). Among these DE genes, 2,619, and 762 genes were up-regulated in freeze treated and non-freeze treated buds, respectively, and 3,123, and 1,321 genes were up-regulated in one-day and one-week recovery, respectively. Less DEGs were upregulated in sample buds prior to budswell (572) compared to buds post-budbreak (1121). Within the different bud stages, 2,581 DEGs were specific to freezing temperatures for buds prior to budswell, and 2,460 DEGs were specifically related to buds post budbreak treated to freezing temperatures. Data comparing freeze treated buds of either bud stage revealed 448 upregulated unigenes that were shared between the two bud stages. Within either bud stage at one-day recovery, freeze treated buds had greater upregulation (1,451) than non-freeze treated buds (436).

A heatmap produced from the trinity pipeline displayed relative consistency among the samples (Fig. 2). DEGs are largely upregulated on in similar regions for buds with one day recovery of either bud stage compared to those with recovery periods of one week which show more downregulation. Further, upregulation relating to non-freezing temperatures in buds prior to budswell at either recovery period is observed, compared to buds prior to bud swell treated to freezing temperatures. There is some slight patterning pertaining to the recovery periods, where buds with one-week recovery, regardless of other treatment parameters, have greater upregulation on the upper portion of the graph (Fig. 2).

One anomaly is noted looking at a bud prior to budswell treated to freezing temperatures and one-week recovery; the sample has notable upregulation compared to the two other biological replicate samples (Fig. 2, sample OBB_12C_1w_2). The upregulation of this sample is more consistent with buds prior to budswell and treated to freezing temperatures with one-day recovery (samples OBB_12C_1d_1-3). One of the hypotheses for this observation

is that the sample in question is still viable, with genetic regulation similar to buds soon after a freeze, whereas the other two replicates have turned necrotic due to the freeze treatment.

Cluster plot analysis of DEGs established 211 cluster plots. Multiple cluster plots complimented the heatmap patterns; samples displayed up and downregulated unigenes corresponding to the noted temperature and recovery patterns observed in the heatmap (Fig. 3 A&B).

Metabolic Pathways. In BLAST, 16.8 % of the unigenes mapped to the NCBI nonredundant protein database, a total of 11.8% were annotated. Regarding the annotated portion of unigenes were used to determine metabolic pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG). Comparing metabolic activity between bud stages, buds post budbreak had more DEGs and correspondingly had more enzymes involved in the metabolic pathways than the buds prior to budswell. KEGG determined 12,216 unigenes were manifested >40,000 times in 148 metabolic pathways. Purine metabolism, thiamine metabolism, and the biosynthesis of antibiotics were pathways for which the majority of unigenes in temperature regulation as well as recovery-related expression were involved; however, these subsets accounted for a relatively low frequency compared to the grand scale of all DEGs from all treatments that were involved in the pathway, accounting for <7%. This suggests that either these pathways are not unique to these treatments and occur across all treatments with similar levels of expression.

Other pathways occurred at a higher frequency of in select treatments compared to the total unigene contribution in the metabolic pathways. Specifically, lipopolysaccharide biosynthesis, glycosylphosphatidylinositol (GPI)-anchor biosynthesis, and steroid degradation were KEGG pathways which had a high frequency of unigenes related to freeze treated DEGs to total unigene relating to the KEGG pathways, accounting for frequencies of 60%, 50%, and 40%, in regard to total unigene contribution for the metabolic pathways, respectively. In recovery related DEGs, stilbenoid, diarylheptanoid and gingerol biosynthesis, lipoic acid metabolism, and lipopolysaccharide biosynthesis occurred at high frequency to total unigene upregulation of KEGG pathway, accounting for ≥20% for the total frequency the unigenes involved in each of the metabolic pathways.

Subclusters with large unigene representation (>400 in each of the selected subclusters) and expressing complimentary pattern to the heatmap were selected, unigenes of each subcluster were metabolically mapped with KEGG (Fig. 3 A &B). Upregulation of samples of either bud stage and treated to freezing temperatures with one-day recovery displayed similar KEGG frequencies to freeze-treated DEGs. Specifically, glycosylphosphatidylinositol (GPI)-anchor protein (AP) biosynthesis and stilbenoid, diarylheptanoid and gingerol biosynthesis had higher frequency of KEGG enzymatic expression. Under cold stress GPI APs increase on the plasma membrane, potentially aiding in cell wall restructuring and inhibiting continued extracellular ice propagation (Takahashi et al., 2009). Unigenes from the subcluster expressing upregulation and downregulation for one-day and one-week recovery periods also found similar KEGG frequencies to overall KEGG expression of recovery related unigenes; lipoic acid metabolism and stilbenoid, diarylheptanoid and gingerol biosynthesis. Both lipoicand stilbenoidsynthesis have functions in plant protection and defense (Chong et al., 2009; Navari-Izzo et al., 2002).

Conclusion

Based upon the results of the differential gene expressions analysis there were more DEGs relating to freezing temperature treatments, one-day recovery, and buds post budbreak in comparisons within each of these parameters. These results were anticipated; the upregulation of genes is often caused by environmental cues, e.g. moving a plant from a normal environment to a freezing environment. Note that the plants treated to non-freezing treatment had notably fewer upregulated DEGs with one-day recovery (wherein there was no temperature change from temperature treatment to recovery) than plants treated to freezing temperatures. Buds post budbreak are developing toward anthesis and are more metabolically active than buds prior to budswell with increased DEGs and enzymatic activity. Within subcluster analysis of expression patterns pertaining to freezing temperatures with one-day recovery as well as recovery period, corresponding KEGG pathway functions were associated with cold acclimation and plant defense, respectively.

Further analysis will examine candidate genes relating to plant defense stimulated by environmental cold stress.

Impact.

Subtropical climates, as commonly experienced in the southeastern United States, can signal premature budbreak in southern highbush blueberries. Post budbreak, buds become more susceptible to freeze events. The occurrence of freeze events can significantly reduce region-wide blueberry yield in all of the southeastern states, where blueberries are prominently produced. Understanding the regulation of these pathways in blueberry is a foundation for implementation for marker assisted breeding for cold tolerance in blueberry.

Presentations based upon this research:

1. Redpath, L. E. et al. *RNA-Seq Analysis of Floral Bud Response to Freeze Treatments in Southern Highbush Blueberry CV. 'O'Neal'.* National Association of Plant Breeders Conference, Guelph, Toronto. August 2018
2. Redpath, L. E. et al. *Differential Gene Expression of Southern Highbush Blueberry (Vaccinium corymbosum L.) Cv. 'O'Neal' Floral Buds in Response to Freeze Treatment and Recovery Periods.* American Society for Horticultural Science, Washington D.C. July 2018.
3. Redpath, L. E. et al. *Freeze Response Regulation in Vaccinium corymbosum (L.) Floral Buds.* To be presented at Plant and Animal Genomics XXVII, San Diego, CA. January 2019.

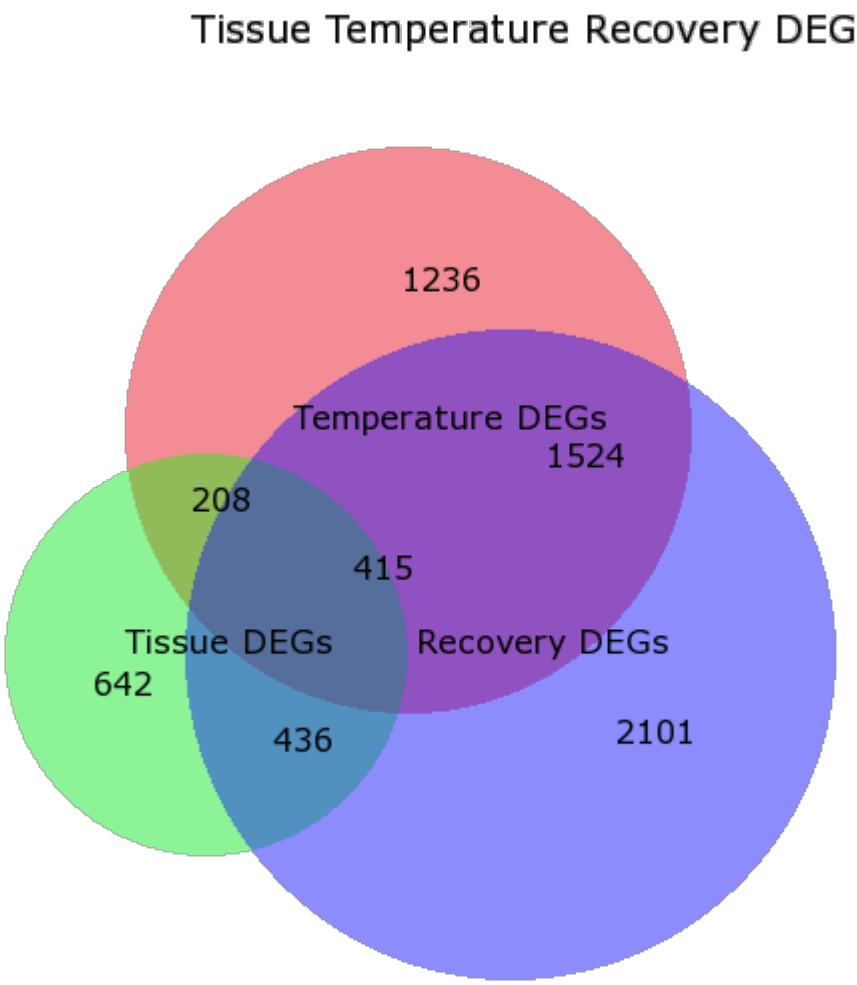

Figure 2. Venn Diagram of Differentially Expressed Genes and Genetic Interaction. Shown are the upregulated contigs unique to each treatment and contigs commonly upregulated among treatments.

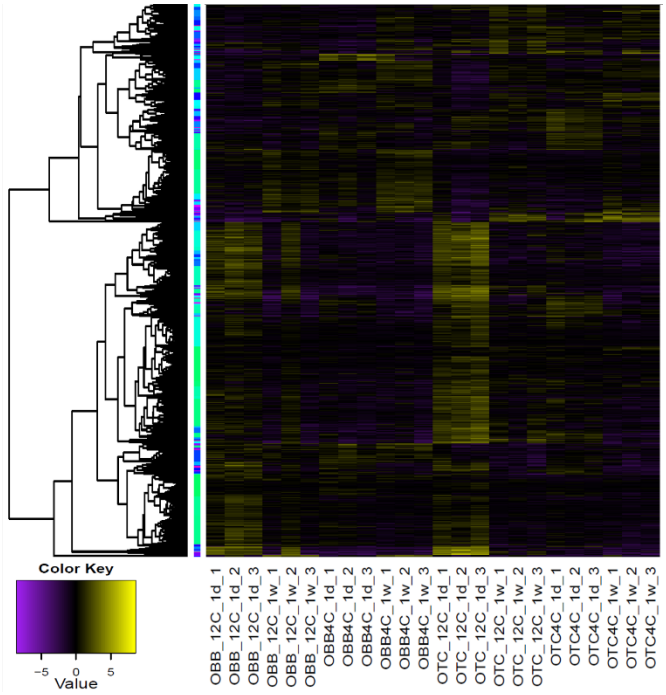

Figure 3. Heatmap of Differentially Expressed Genes per Treatment Group. Purple indicates downregulation while yellow indicates upregulation of genes. Buds at either stage treated to freezing temperatures and 1 day recovery had more genetic upregulation.

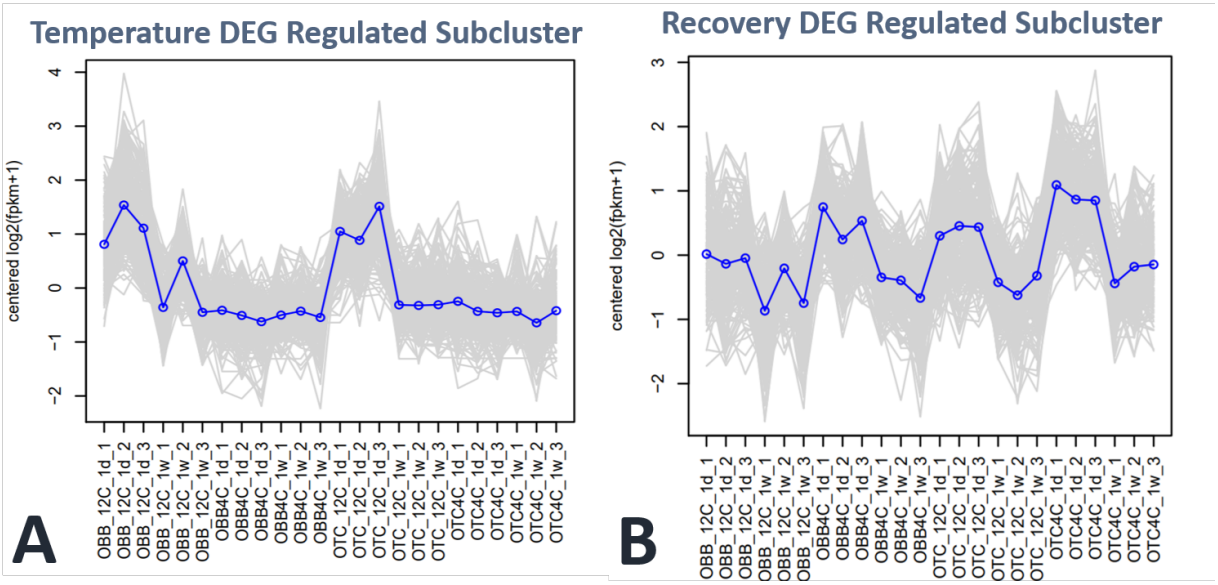

Figure 4. Cluster Plots of Differentially Expressed Genes. Cluster plots display upregulation of inflorescence freeze-treated buds with 1-day recovery within 508 traits (A). Plots show genetic upregulation with 1-day recovery compared to 1-week recovery (B).

Literature Cited

(USDA), U.S.D.o.A., 2016. Fruit and tree nuts yearbook, berries. In: (USDA), U. S. D. o. A. (ed.), 2016 Yearbook, Food Environment Atlas.

Chong, J., Poutaraud, A., and Hugueney, P., 2009. Metabolism and roles of stilbenes in plants. *Plant Science* 177:143-155.

Clark, J.R., Bourne, R., and Gbur, E., 1996. Flower bud and shoot hardiness of southern highbush blueberry cultivars. *Fruit Varieties Journal* 50:98-104.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., and Regev, A., 2011. Full-length transcriptome assembly from rna-seq data without a reference genome. *Nat. Biotechnol.* 29:644-652.

Navari-Izzo, F., Quartacci, M.F., and Sgherri, C., 2002. Lipoic acid: A unique antioxidant in the detoxification of activated oxygen species. *Plant Physiology and Biochemistry* 40:463-470.

Patten, K., Neuendorff, E., Nimr, G., Clark, J.R., and Fernandez, G., 1991. Cold injury of southern blueberries as a function of germplasm and season of flower bud development. *Hortscience* 26:18-20.

Takahashi, N., Goto, T., Taimatsu, A., Egawa, K., Katoh, S., and Kusudo, T., 2009. Bixin regulates mrna expression involved in adipogenesis and enhances insulin sensitivity in 3t3-l1 adipocytes through ppargamma activation. *Biochem Biophys Res Commun* 390.