

Histological and molecular study of apical buds of cranberry (*Vaccinium macrocarpon* Ait.) and
study of vigor of plants from self- and cross-pollinations.

By

Jenny L. Bolivar-Medina

A dissertation submitted in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

(Plant Breeding Plant Genetics)

at the

UNIVERSITY OF WISCONSIN-MADISON

2016

Date of final oral examination: 12/17/2015

The dissertation is approved by the following members of the Final Oral Committee:

Juan E. Zalapa, Assistant Professor, Horticulture

Sara E. Patterson, Professor, Horticulture

Amaya Atucha, Assistant professor, Horticulture

Patrick Masson, Professor, Genetics

Shawn Steffan, Assistant Professor, Entomology

Table of Contents

Table of Contents	i
Acknowledgements.....	iii
Abstract.....	vi
Chapter 1 Literature review	1
1. 1 Cranberry	2
1.2. Flowering.....	5
1.3 Alternate bearing	12
1.4 Pollen source and vigor. (Studies of vigor of self vs cross pollinations)	17
1.5 References	21
Chapter 2 Histological Study of Apical Buds of Non-Alternate and Alternate Bearing Cultivars of Cranberry	26
2.1 Abstract	27
2.2 Introduction	28
2.3 Materials and methods	32
2.4 Results	36
2.5 Discussion.....	42
2.6 Acknowledgements.....	52
2.7 References	52
Chapter 3 Transcriptome analysis of apical buds from fruiting and vegetative uprights before, during and after floral initiation in an alternate bearing cranberry	66
3.1 Abstract	67
3.2 Introduction	68
3.3 Materials and methods	73
3.4 Results	83
3.4.1 Verification of Ben Lear cultivar in the bed	83
3.4.3 Phenology	84
3.4.11 Expression analysis of flowering genes in buds	94
3.5 Discussion.....	97

3.6 References	112
Chapter 4 A vigor study based on three self- and cross-pollinated cranberry cultivars.....	143
4.1 Abstract	144
4.2 Introduction	145
4.3 Materials and methods	147
4.3.1 Fruit collection from the field.	147
4.3.2 Outline of experiments using field collected fruits	148
4.3.3 Greenhouse experiments.	153
4.3.3.1 <i>Collection, storage, and sorting of uprights</i>	153
4.4 Results	156
4.4.1 Fruit collection from the field.	156
4.4.2 Greenhouse experiments	163
4.5 Discussion.....	165
4.6 References	173
Chapter 5 General conclusions and future perspectives	189

Acknowledgements

I want to recognize the support that I have received through my PhD studies here in UW-Madison. Even though sometimes we as graduate students could feel isolated and working alone, we are not. There are lots of persons surrounding us and giving all the support that we need to continue this path.

I am thankful with the members of my committee (Amaya Atucha, Patrick Masson, Shawn Steffan, Sara Patterson and Juan Zalapa) who, in one way or in another, guided me and offered all the support to successfully complete my studies. Also I want to recognize the help Sara has offered me as my first advisor here and then being part of my committee. In her lab, I learned molecular techniques and critical thought that have grown on me since then. To my current advisor, Juan, who gave me the opportunity to finish my graduate studies and allowed me the freedom to explore experimental ideas to carry out.

I would like also to recognize all the support that I have had from the former and present members of Sara Patterson Lab and Juan Zalapa Lab as well as the members from the Plant Breeding and Plant Genetics program and Horticulture Department. Around a good cup of coffee, I have created friendship bonds that helped in my growing as a person and to mature and develop my project ideas. I really hope these bonds will last forever. Special thanks also to Eric Zeldin, emeritus research, for help in the exploration of new ideas for study in cranberry, and Shelby Ellison, from Simon Lab, for her unconditional willing to guide me in all about RNA extractions. Her input continues to be greatly appreciated.

Another important component during my life and formation as a person and in the academy has been my family back there in Colombia and the one that surrounds me here in Madison. My Parents and siblings always have believed in my capabilities to carry out the goals that I have set for myself and they have kept me motivated to pursue them. A big source of inspiration, and work, is my family here. My husband, Humberto, who has been here for me at all times and shared all the good and bad moments in my life, as a good soulmate that he is. My children, Joshua and Karen; their arrival to this world have been a blessing and fulfilled my heart with tremendous amounts of joy and strength. They continue being my source of inspiration to move forward.

List of abbreviations

ABA: abscisic acid

AGL20: AGAMOUS-LIKE 20

AKS1: ABA-RESPONSIVE KINASE SUBSTRATE 1

AP: APETALA

ATGA2OX1, 2, and 8: GIBERELLIN 2-OXIDASE 1, 2, and 8

ATUBC1: UBIQUITIN PROTEIN CARRIER 1

ATX1: ARABIDOPSIS TRITHORAX 1

BLAST: Basic Local Alignment Search Tool

BRI1: BRASSINOID INSENSITIVE 1

CDF5: CYCLING DOF FACTOR 5

CO: CONSTANTS

COG: Clusters of Orthologous Group

COL5: CONSTANTS-LIKE 5

COP1: CONSTITUTIVE MORPHOGENIC 1

CRY2: CRYPTOCHROME 2

CUL4: CULLIN4

ELF8: EARLY FLOWERING 8

GA: Gibberelellin

GA3 : Gibberellic acid

GAI: GIBBERELIC ACID INSENSITIVE

GI: GIGANTEA

GO: Gene Ontology

HUA2: ENHANCER OF AGAMOUS-4 2

HUB2: HISTONE MONO-UBIQUITINATION BINDING 2

LHY: LATE ELONGATED HYPOCOTYL

NCBI: National Center for Biotechnology Information

NFYB1/HAP3A: NUCLEAR TRANSCRIPTION FACTOR Y SUBUNIT B-1/HEME ACTIVATED PROTEIN 3A

PHYA: PHYTOCHROME A

PTF1: PHYTOCHROME AND FLOWERING TIME REGULATOR

QTL: Quantitative trait loci

REF6: RELATIVE OF EARLY FLOWERING 6

SAM: shoot apex meristem

SMZ: SCHLAFMUTZE

SNZ: SCHNARCHZAPFEN

SVP: SHORT VEGETATIVE PHASE

TFL1: TERMINAL FLOWER 1

TOE: TARGET OG EARLY ACTVATION TAGGED

UBP26: UBIQUITIN-SPECIFIC PROTEASE 26

VRN2: REDUCED VERNALIZATION RESPONSE2

Abstract

The American cranberry (*Vaccinium macrocarpon* Ait.) is an edible fruit plant species, member of the Ericaceae family and one of few commercially important fruit crops native to North America. Cranberry has been cultivated for over two centuries and Wisconsin accounts for 67% of total production in the United States, but only recently it has become popular among consumers, mostly due to the health benefits inherent to the red berries

Cranberries are naturally adapted to acidic soils found in the swamps and sandy soils of temperate climates. Cranberry plants grow as stoloniferous vines that produce vertical branches called uprights. These uprights can be vegetative or reproductive in a given year, and they alternate between stages of growth yearly (alternative bearing). This behavior is most predominant in cultivars selected from wild populations, and less accentuated in bred cultivars released recently. Thus, similar to other woody fruit crops, the challenge in cultivated cranberries is to increase yield, quality, and nutrition of the crop while dealing with several complexities, including self-incompatibility, chilling requirement, singulated fruiting, preformed buds, and biennial bearing. However, little is known about genetic and physiological aspects of this species. Therefore, basic biological studies aiming to broaden our cranberry knowledge base are essential to continue the selection of cultivars with improved yield, quality, and nutritional traits. In addition, the simplicity of handling cranberry in greenhouses and *in vitro* culture allows for easy propagation and maintenance of genotypes of interest, thus making cranberry a good candidate as a model system to study traits of commercial importance as well as some of the intricacies of woody fruit crops.

This research thesis aims to study some of the important cranberry characteristics mentioned above. The introductory chapter (Chapter1) presents a literature review basic to understand the concepts studied. General concepts related to the characteristics and breeding of cranberry are covered, including the flowering process and implications for alternate bearing tendencies in woody species. Additionally, basic concepts related to transcriptome analysis are discussed, as well as its use to understand molecular aspects of particular process. The second chapter is focused on the study of apical buds in an alternate bearing cultivar (Ben Lear) and a non-alternate cultivar (HyRed) during the growing and fruiting season. This chapter shows histological evidence that floral initiation occurs distinctively in both cultivars, and that there is a positive relationship between bud size and the presence of floral initials. This chapter has been written following guidelines of the Botany Journal (formerly the Canadian Journal of Botany), and it is ready for submission. The third chapter analyzed the transcriptome of apical buds from vegetative and reproductive uprights of Ben Lear in three different floral initiation stages. Transcripts from candidate genes to be involved in flowering were detected. The fourth chapter studies the vigor of seedlings derived from self- and cross-pollinations in three different cultivars Ben Lear, Stevens, and HyRed. It is important to highlight that these three cultivars are genetically related, with HyRed being an F₁ offspring of Ben Lear and Stevens. The chapter examines the relationships between fruit size, seed and plant vigor, and genetic origin of the seedlings (self vs. cross) within and across cultivars. The final section of the thesis is dedicated to conclusions and suggested research topics to continue exploring morphological, phenotypical and molecular aspects of the important agronomical traits in cranberry.

Chapter 1:

Literature review

1. 1 Cranberry

The American cranberry (*Vaccinium macrocarpon* Ait.) is an edible fruit plant species member of the Ericaceae family, *Vaccinium* genera, section *Oxycoccus* (S.P. Vander Kloet, 2009). Cranberry was the first species cultivated from the *Vaccinium* genus and one of few commercially important fruit crops native to North America (Vorsa and Johnson-Cicalese, 2012). The U.S. is the largest producer and exporter of cranberries, and Wisconsin is the top producing state, accounting for 67% of the total production in 2013 (USDA NASS, 2014). Even though cranberry has been cultivated for over two centuries, only recently it has become popular among consumers, mostly due to the higher availability of cranberries and to the health benefits inherent to the red berries (Lacombe *et al.*, 2010; Viskelis *et al.*, 2009).

Cranberry is a diploid ($2n = 2x = 24$), evergreen, small woody perennial species naturally adapted to acidic soils found in the swamps and sandy soils of temperate climates (S.P. Vander Kloet, 2009). In these environments, cranberry plants grow as stoloniferous vines (runners) that produce vertical branches called uprights (Vorsa and Johnson-Cicalese, 2012). Cranberry uprights can be vegetative or reproductive in a given year and alternate yearly between stages of growth. Therefore, this alternative bearing growth behavior is given by the capacity of uprights to set reproductive buds (or mixed bud) or vegetative buds. Additionally, reproductive buds contain one apical vegetative meristem and several lateral floral meristems, which after a period of dormancy will develop into reproductive upright with 2 to 7 flowers (McCown and Zeldin, 2005). In fact, cranberries require a minimum 800 chilling hours to produce flowers. Meanwhile, vegetative buds contain only vegetative meristems, which will become either vegetative uprights or runners after the dormancy period. Additionally, most wild cultivars selected from

the wild populations suffer from alternate bearing, which is less accentuated in cultivars produced through breeding efforts (Vorsa and Johnson-Cicalese, 2012). Thus, similar to other woody fruit crops, the challenge in cultivated cranberries is to increase the yield, quality, and nutrition of the crop while dealing with several complexities, including self-incompatibility, chilling requirement, singulated fruiting, preformed buds, and biennial bearing.

1.1.1 Cultivation and plant breeding approaches

The cultivation of cranberry began in early 1800s by the use of wild selections based mainly on fruit characteristics such as fruit size, color and early ripening (Vorsa and Johnson-Cicalese, 2012). In the mid 1900's, cranberry breeding programs in the U.S. (mainly USDA) began using native cranberry varieties in their breeding efforts against fungal diseases and insects by producing resistant varieties (Vorsa and Johnson-Cicalese, 2012). Since then, cranberry breeding has been conducted for only 2-3 generations, and still many of the most widely grown cultivars are the original wild selections (e.g., Ben Lear) or their initial hybrids released in the 1950's (e.g., Stevens).

Ben Lear is a Wisconsin native selection from 1901 that has good flower and fruit production and continues to be a popular cultivar among growers and breeders despite its susceptibility to fruit rot and fairy ring diseases (Caruso, 1998; USDA, 2012). Other native cultivars that remain popular among breeders and growers include Howes (MA, 1843), McFarlin (MA, 1874), Searles (WI, 1893), Potter (WI, 1895), and LeMunyon (NJ, 1960). On the other hand, breeding programs using wild selections have resulted in cultivars such as Stevens, which

originated from a cross between the native varieties McFarlin and Potter. Stevens is one of the six varieties tested and selected in the 1940s and released in the 1950s by the USDA, Wisconsin Agricultural Experimental Station, and Wisconsin Department of Agriculture, and WI Cranberry Sales Co. (Vorsa and Johnson-Cicalese, 2012). Stevens was selected for its increased fruit production, size and shape of fruit, and resistance to leafhopper feeding and fruit rot (Caruso, 1998; Vorsa and Johnson-Cicalese, 2012). Stevens is now the most widely grown cultivar with 40% of total U.S. acreage.

Currently, the main goals of cranberry breeding programs has been increasing yield and fruit quality, and nutrition improvement (Vorsa and Polashock, 2005). For example, HyRed is a high anthocyanin cultivar released by the University of Wisconsin-Madison in 2003 (McCown *et al.*, 2003). HyRed was developed from crosses between Ben Lear and Stevens and possesses early flowering and fruit ripening, large berry size, high plant vigor, and high yield combined with the capacity to produce reproductive buds in consecutive years (rebudding) (McCown *et al.*, 2003). Similarly, Crimson Queen® was recently released by Rutgers University (Vorsa and Johnson-Cicalese, 2012), and shares the same cultivar parents and many of the same fruit characteristics as HyRed.

Despite recent successes in the development of new varieties, cranberry breeding for superior yield, quality, and nutrition are still limited and there have been only a few new introductions (Vorsa and Johnson-Cicalese, 2012). Therefore, the phenotypic evaluation and analysis of populations derived from high performing cultivars will provide invaluable data about selectable traits that may influence the yield, quality, and nutrition in cranberries and

ultimately lead to the development of new cultivars. Another aspect that requires attention in cranberry is the study of the vigor of seedlings from self and cross pollinations as well as the influence of the pollination type in the fruit characteristics, which in the end could affect the quality of the berries.

1.2. Flowering

The transition from juvenile to adult phase is highly influenced by the ability of the plants to produce flowers once the environmental conditions are favorable to guarantee their reproduction. However, external and internal cues must occur so the flowering process can take place. Recent studies in *Arabidopsis* have indicated that at least two microRNAs (miRNAs) play an important role in regulating the changes of developmental stages of juvenility and maturity (Huijser and Schmid, 2011; Khan *et al.*, 2013).

The flowering process has been extensively studied and, in general, three different stages have been determined. During the *flower induction stage*, also called transition to flowering, a competent vegetative meristem transitions into a floral meristem due to the interaction of external and internal signals that induce flowering and the activation of flowering regulator genes (Hanke *et al.*, 2007). This stage is followed by the *flower initiation stage*, where morphological changes, not yet detectable, are occurring in the vegetative meristem to transform it into a floral meristem. Finally, the floral meristem goes through a *flower differentiation stage*, where morphological changes give rise to the floral organ specification process (Hanke *et al.*, 2007; Koutinas *et al.*, 2010). During floral organ specification, the floral meristem differentiates

primordia that will continue their development as floral organs (sepals, petals androecium, and gynoecium). The differentiation of the primordia follows a centripetal sequence, starting by the flanks of the floral apex with the differentiation of sepal primordia, followed by petal, androecium (anthers), and finally the gynoecium (carpel) primordia formation (Endress, 2006; Tan and Swain, 2006). The process is completed once all the primordia fully develop into each of the floral organs they were committed to (Alvarez-Buylla *et al.*, 2008). Although the flowering process differs in plants, in annual plants, flowering occurs as a continuum, while in perennial plants, flowering is temporally separated by a period of dormancy, commonly known as chilling period.

1.2.1 Flowering Pathways

Depending on the signal that induces the flowering process, 5 different signaling mechanisms that have been proposed and identified, and their names are based on these signals. Those pathways are: Photoperiodism, Vernalization, Gibberellic Acid, Autonomous and Phase Change. The first two pathways are induced by environmental cues, while the last three depend on internal signals from plants. In *Arabidopsis*, once the plant is capable of flowering, the signal is perceived by the plant, and a series of genes mainly coding for photoreceptors and transcription factors are activated throughout the flowering pathways (Tan and Swain, 2006). Most of the pathways start with the activation of flowering regulator genes: *CONSTANTS (CO)* and *FLOWERING LOCUS C (FLC)*. While *CO* is a positive regulator, since its expression promotes flowering, *FLC* is a negative regulator by repressing the process. The activation of these genes regulates, positively or negatively, the expression of downstream genes called floral pathway

integrators, which are responsible for the time when floral transition occurs. Among the floral pathway integrators are: *FLOWERING LOCUS T (FT)*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANTS 1 (SOC1)*, and *LEAFY (LFY)*. Subsequently, these genes regulate the expression of another set of genes responsible for the transition of the vegetative meristem to inflorescence meristem. Those genes are called floral meristem identity genes, and include genes such as *APETALA 1 (AP1)*, *FRUITFUL (FUL)*, *CAULIFLOWER (CAL)*, *LEAFY (LFY)*, and *SEPALLATA (SEP)*. The flowering pathway ends with the activation of floral organ identity genes (ABCDE genes), which are responsible for the differentiation of the floral organs (Khan *et al.*, 2013; Tan and Swain, 2006; Wigge *et al.*, 2005; Wilkie *et al.*, 2008)

1.2.1.1 Photoperiod pathway. Day length is the signal that activates the Photoperiod pathway in *Arabidopsis*, or more specifically, the circadian control exerted over *CO* (Amasino and Michaels, 2010; Khan *et al.*, 2013). *CO* is exclusively expressed in the vascular tissues of the leaf and encodes a CO protein, a crucial transcription factor for photoperiod perception that activates *FT* expression in leaves. The FT protein is key in the transition to flowering and needs to be transported to the shoot apical meristems (SAM) where it forms a complex with the FLOWERING LOCUS D (FD) protein, which is a meristem-expressed transcription factor. The FT/FD complex activates *AP1* and *SOC1*, floral meristem identity genes, and then the vegetative meristem becomes a floral meristem (Amasino and Michaels, 2010; Hanke *et al.*, 2012; Khan *et al.*, 2013).

1.2.1.2 Vernalization pathway: Another important environmental factor in flowering is temperature. Exposure to low temperatures (0°C to 10°C) for long periods of time can induce flowering. This process is mainly found in winter annual and biennial plants (Amasino and Michaels, 2010). The interaction of *FRIGIDA (FRI)* with *FLC* promotes the overexpression of *FLC*, and hence represses flowering. Cold temperatures perceived by the apical meristem, induce the expression of genes such as *VERNALIZATION 1 (VRN1)* and *VERNALIZATION INSENSITIVE 3 (VIN3)*, which encode plant specific proteins responsible for changes in DNA methylation resulting in repression of the expression of *FLC* (Amasino and Michaels, 2010; Hanke *et al.*, 2012).

1.2.1.3 Autonomous Pathway. Similar to the epigenetic control exerted over *FLC* in the vernalization pathway, in the autonomous pathway the expression of *FLC* is repressed by a group of genes called autonomous floral-promotion pathway genes (also known as AP genes; Veley and Michaels, 2008). Among the AP genes are *FCA*, *FPA*, *FLOWERING LOCUS K (FLK)*, and *FY*, which encode proteins with RNA binding domains and regulate *FLC* transcripts. These genes have also been suggested to regulate *FLC* via chromatin remodeling (Veley and Michaels, 2008). Other AP genes code for histone deacetylation factors and include *FVE*, *FLOWERING LOCUS D (FLD)* and *RELATION OF EARLY FLOWERING 6 (REF6)* (Amasino and Michaels, 2010; Hanke *et al.*, 2012; Khan *et al.*, 2013). It has also been shown by Veley and Michaels (2008) that AP genes are also involved in cellular organization and normal growth in *Arabidopsis*.

1.2.1.4 Gibberellic Acid (GA) pathway. GA is also known to induce flowering in *Arabidopsis*, particularly in short days when GA₄ is synthesized in leaves and transported to competent meristems. Within the meristem, GA₄ up-regulates *LFY* directly or through the activation of the floral integrator *SOC1* (Hanke *et al.*, 2012). Another gene involved in flowering through this pathway is *AGAMOUS LIKE 24 (AGL24)* which is up-regulated by *SOC1* (Khan *et al.*, 2013). Additionally, in *Arabidopsis*, GA also regulates the expression of *FT* (Khan *et al.*, 2013).

1.2.1.5 Phase transition Pathway More recently, micro RNAs (miRNAs), miR156 and miR172, have been found to play an active role in controlling the transition from juvenile to adult stages and the flowering process in plants (Khan *et al.*, 2013). Several studies reviewed by Huijser and Schmid (2011) and Khan *et al.* (2013) have demonstrated that miR156 negatively regulates the expression of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* genes, thus affecting the transition between juvenile to adult and adult to juvenile stages. Transition to flowering is particularly affected by the negative control exerted by *SPL3*, which activates floral meristem identity genes *LFY*, *FUL* and *AP1*; and therefore, delays the transition to flowering. In contrast, miR172 promotes flowering by targeting transcripts of flowering repressors such as *AP2*, *SCHLAFMUTZE (SMZ)*, *SCHNARCHZAPFEN (SNZ)*, *TARGET OF EAT 1 (TOE1)*, *TOE2* and *TOE3* (Khan *et al.*, 2013).

1.2.2 Flowering in perennial fruit crops

All plants annual, biennial, and perennial plants have to go through the transition from juvenile to adult stages to be capable of flowering. However, the flowering process becomes more

complex in temperate perennial plants, where flowering induction is influenced by dormancy as well as interactions among vegetative growth, flowering and fruit production of previous years. These interactions can result in biennial bearing. Additionally, in woody fruit crops, the flowering process is temporally separated between floral initiation and floral differentiation by a dormancy and chilling period. In general terms, floral initiation occurs in the growing season prior to anthesis (Hanke *et al.*, 2012).

Studies in perennial plants, reviewed by Andrés and Coupland (2012), in *Arabis alpina*, have revealed an *Arabidopsis TFL1* orthologous gene. In *A. alpina*, *TFL1* prevents flowering due to vernalization in young plants, while in *Arabidopsis* it delays flowering under short days. *TFL1* also has a role in shortening the time to maturity in poplar and apples (Andrés and Coupland, 2012). Orthologous to a negative regulator of flowering in *Arabidopsis*, an *FLC*-like gene, has also been identified in *A. alpina* and named as *PERPETUAL FLOWERING1 (PEP1)* controlling the flowering period by keeping it short. It also has a role in the transition to vegetative phase in this perennial plant, and represses flowering in vegetative meristems (Andrés and Coupland, 2012; Khan *et al.*, 2013). In addition, an ortholog to *TFL1* found in strawberries has been proposed to have a similar role as *PEP1* in restricting the length of the flowering period (Andrés and Coupland, 2012)

In perennial trees such as poplar, *FLC* orthologs have not been found yet, but *FT-like* genes, *FT1* and *FT2*, are thought to control flowering. While *FT1* is activated by winter and together with *CO* promotes flowering, *FT2* is induced by long days and promotes vegetative growth

(Andrés and Coupland, 2012). Homolog genes to *SOC1*, *LFY*, *AP1*, *TFL* have also been found in poplar, apples (Hanke *et al.*, 2012; Tan and Swain, 2006), citrus (Khan *et al.*, 2013; Tan and Swain, 2006); and several more in peonies (Zhou *et al.*, 2013), grapes and eucalyptus (Tan and Swain, 2006).

Studies about flowering in the family of the Ericaceae have just started to emerge (Borchert *et al.*, 2009) studied the morphology and molecular aspects of flowering in *Calluna vulgaris*, and identified homologs to *AP3*, *SEP1* and *SEP2* from *Arabidopsis*. During the study of expression of flowering genes in apical buds of *Rhododendron*, homologs to *FT*, *TFL1*, *SOC1*, and *LFY* from *Arabidopsis* were identified (Cheon *et al.*, 2012). For more closely related species to cranberry, the identification and functional analysis of an homolog to *FT* in blueberry (*VcFT*) is the first report published report (Song *et al.*, 2013). Through constitutive expression of *VcFT* in transformed tobacco plants, the authors suggest that this gene could act as a flowering activator and the overexpression of *VcFT* can induce early and continuous flowering (Song *et al.*, 2013).

Gibberellic Acid (GA) has been shown to inhibit, rather than promote, flowering in fruit trees, and studies by researchers including Muñoz-Frambuena *et al.* (1992) and (Khan *et al.*, 2013) have revealed that GA represses the expression of *FT* in the leaves, and inhibits flowering in citrus. Other studies performed in poplar have suggested that chilling treatments upregulate genes from GA3-oxidase and GA20-oxidase families; and as a consequence, the GA biosynthesis was affected (Khan *et al.*, 2013). Transcriptome studies in peony, an ornamental tree, revealed that genes involved in GA biosynthesis such as *PsGA20OX* and *PsGID* showed

low expression in shoot apices during floral induction (Zhou *et al.*, 2013). Indirect evidence by the application of GA treatments to apple have been shown to halt flower production (Wilkie *et al.*, 2008).

MicroRNAs, miR156 and miR172, which are involved in controlling the transition from vegetative to reproductive stages in *Arabidopsis*, have also been found controlling the transition of these stages as well as flowering genes in perennials such as *A. alpina* and trees such as poplar and citrus (Khan *et al.*, 2013).

1.3 Alternate bearing

Similar to many of the woody fruit crops, cranberries possess a natural tendency towards alternate bearing. This phenomenon is characterized by the alternation of a higher fruit production year (popularly called “on” year) and a low production year (“off” year), and it is highly correlated with the ability of plants to bloom. The mechanism itself is still poorly understood, although it has been found that factors such as the nutritional status (i.e., carbohydrate content) and plant growth regulators can affect the flower induction and initiation process (Rosecrance *et al.*, 1998; Smith and Samach, 2013; Yanik *et al.*, 2013). Based on this knowledge, theories such as the “nutritional theory” and hormonal control theory have been proposed to explain alternate bearing tendencies.

The nutritional theory states that there is a differential distribution of nutrients (carbohydrates) to sinks in the plant (buds and fruits). Under this theory, the allocation of nutrients to the growing fruit would be favored, and in turn, the nutrients' supply would be limited to the buds, impeding the development of floral meristems for the following growing season (Shalom *et al.*, 2012; Smith and Samach, 2013; Turktaş *et al.*, 2013).

In cranberry, there are studies that support this theory. For example, Vanden Heuvel and Demoranville (2009) observed that the area and weight of leaves in reproductive uprights of three different cultivars (Stevens, Howes and Early Black) declined during fruit growth. Hagidimitriou and Roper (1994) also found differences in total carbohydrate content between vegetative and reproductive uprights in two cranberry cultivars (Searles and Stevens). Similar results were obtained by Devetter (2013) when analyzing total carbohydrates and starch among alternate and non-alternate bearing cultivars. Interestingly, these studies found that although the carbohydrate content declined as the growing season progressed, uprights without fruits (vegetative uprights) had higher carbohydrate content compared to the uprights with fruits (reproductive uprights). Additionally, various studies involving labeling with ¹⁴Carbon or defoliation of reproductive uprights have also demonstrated that new growth above the fruits is the primary source of photosynthates for the growing fruits (Hagidimitriou and Roper, 1994; Roper *et al.*, 1991; Roper and Klueh, 1994) and floral buds (Roberts and Struckmeyer, 1943).

The hormonal control theory is based on an endogenous control where movement of hormones and signals are key factors in inducing or repressing the expression of genes promoting flower

initiation and fruit production during ‘off’ and ‘on’ years. QTL studies in populations produced by crosses of alternate and non-alternate bearing apples found that genes responsible for synthesizing gibberellins (GAs) were located in a QTL region associated with alternate fruit production, which indicates that this hormone has a negative effect in the induction of floral initials (Guitton *et al.*, 2012). Similar, results from Goldberg-Moeller *et al.* (2013) have shown that consecutive applications of GA in apple trees for three years interfered negatively in the development of floral buds in mandarin.

It has also been shown that spraying GA₃ to branches of ‘Hass’ Avocado trees disrupted the development of early stage floral initials and stimulated the production of vegetative shoots (Salazar-Garcia and Lovatt, 2000). Additionally, genes related to flowering and the synthesis of hormones such as abscissic acid (ABA) and auxins were differentially expressed among citrus buds from ‘ON’, ‘OFF’, and de-fruited trees (trees in which the fruits were removed). Flowering genes and ABA genes were upregulated in ‘OFF’ and de-fruited trees, while auxin genes were upregulated in ‘ON’ trees. These results, as well as the higher number of floral buds in ‘OFF’, and de-fruited trees in ‘ON’ trees, suggest that the hormones previously mentioned can play a signal network that affects floral initiation (Shalom *et al.*, 2014). Whether or not these networks of endogenous hormones, signals and gene expression could induce flower initiation in cranberry buds, still remains to be studied.

Based on field studies, it has been proposed that gibberellins could be a limiting factor for the development of cranberry floral initials in apical buds, as it was shown by Devlin and

Demoranville (1967) when GA₃ was applied to evaluate its effects in fruit set and yield in a commercial plantation. One of the mechanisms hypothesized is that the production of GAs from the seeds of developing fruits could send an inhibitory signal to the floral initiation process in the buds (Devetter *et al.*, 2013). However, whether or not endogenous GAs play a role in limiting bud set is still to be verified.

Several techniques have been tested to reduce the alternate bearing tendency in different crops by controlling the physiological mechanisms involved. For example, in apples and mandarin pruning, girdling or the application of growth regulators has been used to reduce alternate bearing (Samach and Smith, 2013; Wilkie *et al.*, 2008). Unfortunately, there are no methods that can guarantee a yearly and stable fruit production in most woody fruit crops, including cranberries (Smith and Samach, 2013). Until now, the only option to overcome alternate bearing in cranberry has been the cultivation of “rebudding” varieties. These varieties set an apical bud with vegetative and floral meristems called reproductive buds (also known as mixed buds) every year. These reproductive buds will grow as fruiting uprights the next season, guaranteeing stable fruit production throughout the years. In spite of the importance of rebudding, little is still known about the mechanisms that govern flower induction and initiation in cranberry.

1.3.1 Molecular techniques to understand alternate bearing and flowering in woody crops

New technologies for genetic analysis such as next generation sequencing (NGS), permit to efficiently and economically generate and analyze massive amounts of sequences from DNA or RNA. Data obtained from NGS can be used to discover genes that are expressed within specific

plant developmental stages or to identify polymorphisms. These sequences can then be used as markers in breeding programs. The application of these technologies in cranberry has allowed the development of microsatellites (Zhu *et al.*, 2012) that are useful in establishing fingerprinting techniques to discriminate cranberry cultivars (Fajardo *et al.*, 2012) and to determine inter and intra-cultivar variation (Zalapa *et al.*, 2012). The application of NGS technologies such as the analysis of the cranberry transcriptome through RNA sequencing (RNA-seq) will provide additional insights and more comprehensive information on gene expression during the flower induction and initiation process.

As new technologies are evolving and becoming more available for research, studies of the transcriptome in woody fruit crops to study alternate bearing and flowering have increased in number. The findings in these studies are contributing to our general knowledge on flowering in woody plants. For example, Yanik *et al.* (2013) used RNA-seq and found higher levels of expression in specific micro RNAs (miRNAs), in the samples of olive during “off” years rather than in “on” years. In the same study, high miRNA expression was only associated within fruiting samples (“on” years). Obviously, further studies need to be performed to clarify the role of specific genes in the alternate bearing process in olive. By combining transcriptome data and real time RT-PCR analysis in apical buds during floral induction in reblooming and non-reblooming cultivars of peony tree, Zhou *et al.* (2013) identified a subset of genes orthologous to known *Arabidopsis* genes such as *FT*, *VERNALIZATION INSENSITIVE 3 (VIN3)*, *CO*, *GIBBERELIN 20 OXIDASE (GA20OX)*, *PsVIN3*, *PsCO* and *PsGA20OX*, suggesting involvement in the regulation of the reblooming process. Similar approaches have been used to study dormant flowering buds of Japanese pear (*Pyrus pyrifolia* Nakai). However, no relationship between the

expression of the *FT* gene and endodormancy was observed in pear. In contrast, previous studies with poplar showed a strong correlation between *FT* and flowering (Bai *et al.*, 2013). Regarding the Ericales, the study by Rowland *et al.* (2012) in blueberry using RNA-seq generated a collection of transcriptome sequences from four different stages of fruit development, buds and leaves of commercial highbush blueberry (*Vaccinium corymbosum* L.) that could be used as a base for obtaining molecular markers in blueberries and related species.

1.4 Pollen source and vigor. (Studies of vigor of self vs cross pollinations)

In order to guarantee reproductive success, plants have developed different mating systems such as self and cross fertilization, and apomixis. Some plant species have developed only one of these mechanisms and others have the capability of being self or cross-pollinated depending on the environment and the availability or lack of pollinators. In cultivated plant species, particularly fruit crops, factors such as pollen source (same or different genotype), the number of successful pollinations, and fruit set are important factors in fruit production (Tuan and Yen, 2013).

Studies in fruit production of wax apple and mandarins testing self and cross pollinations, have found that fruit set and fruit characteristics, such as size and number of seeds are improved in cross-pollinations. Moreover, the cultivar of the pollen donor influenced variation of fruit traits (Tuan and Yen, 2013; Wallace and Lee, 1999). Positive effect of cross-pollinations was also observed in fruit set of *Macadamia* (Trueman and Turnbull, 1994), and seed number in capsules

of Lousewort (*Pedicularis sp.* Yang *et al.*, 2005). In contrast, Snow and Spira (1993) did not find differences in seed number and size when flowers of hibiscus were self-pollinated or cross-pollinated, but the seed germination and biomass of seedlings were significantly reduced compared to the seeds from cross-pollinations.

The effect of the type of pollination (self vs. cross) and the origin of the pollen have been studied in blueberry. In the study of Dogterom *et al.*, (2000) self and cross pollinations in two cultivars of highbush blueberry (*Vaccinium corymbosum*), it was found that the pollen load size highly influenced the time of fruit set and fruit size and mass. Interestingly, in both cultivars tested, cross pollinations with higher loads of pollen tetrads produced higher number of seeds compared to self-pollinations. Vander Kloet and Tosh (1984) studied the effect of the number of pollen donors in seed production and weight in highbush blueberry. Their study showed that when flowers were pollinated with pollen of three donors, the fruit set, seed number and weight were higher than when pollen of a single donor was used. Even though the study was performed with plants collected from different places and states in the USA, it is not clear if the plants used were from the same cultivar or all different.

In cranberry, studies with two early selections (Early Black and Howes) concluded that fruits from cross-pollinations had higher number of fully developed seeds than fruits from self-pollinations (MacKenzie, 1994). Later, Sarracino and Vorsa (1991), analyzed the effects of self vs. cross pollinations on fruit traits and number of seeds per berry through a diallel experiment with 8 cultivars. Three cultivars were early selections (from mid-1800s) and the other five, came

from the first breeding efforts of Vorsa and Johnson-Cicalese (2012) and Fajardo *et al.* (2012). Differences in seed production were observed depending on the cultivar used as the maternal parent. This study also showed different degrees of self-sterility among the cultivars; and a lower number of developed seeds were observed in the self-pollinations than in cross-pollinations (Sarracino and Vorsa, 1991). From an observational standpoint, the authors noticed that seedlings from cross pollinations were more vigorous than seedlings from self-pollinations. However, no data was shown to support this statement. In addition, none of the studies mentioned above found differences in fruit set, berry size and total seed number between self and cross pollinations. In spite of this information, no studies of self-pollination have been done using recently released cultivars, which have shown increased plant vigor and fruit characteristics: and no information about the vigor of seedlings and their relationship to pollen origin (self vs cross-pollinations) has been reported.

In summary, cranberry is an important woody crop species with important health properties in the U.S. and Wisconsin. The species occupies a unique, native natural growing habitat and shares many of the traits and challenges of other major woody fruit crops such as alternate bearing and chilling requirements. Additionally, the relatively short juvenile cycle and ease of propagation through vegetative or tissue culture make cranberry an excellent candidate for a model species for woody plants. However, the physiological, histological, and genetic factors that govern the growth and development of cranberry are still understudied. Such knowledge will bring new perspectives to continue and accelerate the breeding efforts of this species and in general of *Vaccinium* genera. Considering all the above issues, the goals of the thesis were the following:

1. Perform a comparative histological study of apical buds during the fruit production season from vegetative and reproductive uprights of an alternate bearing cultivar and a non- alternate bearing cultivar; and determine the differences in development of floral initials across upright types and cultivars.
2. Analyze the transcriptome of apical buds in three different stages of floral induction (before, during, and after floral induction).
3. Study the fruit characteristics and vigor of seedlings from self and cross pollinations in three commercial cultivars: Ben Lear, Stevens, and HyRed.

1.5 References

- Alvarez-Buylla, E. R., Benítez, M., Corvera-Poiré, A., Chaos Cador, L., de Folter, S., Gamboa de Buen, A., ... Sánchez-Corrales Yara, E. (2008). Flower Development. *The Arabidopsis Book*, (1), 1–57. doi:doi:10.1199/tab.0127
- Amasino, R. M., & Michaels, S. D. (2010). The Timing of Flowering. *Plant Physiology*, 154 (2), 516–520. doi:10.1104/pp.110.161653
- Andrés, F., & Coupland, G. (2012). The genetic basis of flowering responses to seasonal cues. *Nature Reviews Genetics*, 13 (9), 627–639. doi:10.1038/nrg3291
- Bai, S., Saito, T., Sakamoto, D., Ito, A., Fujii, H., & Moriguchi, T. (2013). Transcriptome Analysis of Japanese Pear (*Pyrus pyrifolia* Nakai) Flower Buds Transitioning Through Endodormancy. *Plant and Cell Physiology*, 54 (7), 1132–1151. doi:10.1093/pcp/pct067
- Borchert, T., Eckardt, K., Fuchs, J., Krüger, K., & Hohe, A. (2009). “Who’s who’ in two different flower types of *Calluna vulgaris* (Ericaceae): morphological and molecular analyses of flower organ identity. *BMC Plant Biology*, 9 (1), 148. doi:10.1186/1471-2229-9-148
- Caruso, F. L. (1998). Cranberry cultivars. In H. A. Sandler (Ed.), *Cranberry production: A guide for Massachusetts* (pp. 40–50). East Wareham, MA: University of Massachusetts Extension. Retrieved from [http://atlanticcranberry.ca/Presentations/CARUSO Cultivars.pdf](http://atlanticcranberry.ca/Presentations/CARUSO_Cultivars.pdf)
- Cheon, K. S., Nakatsuka, A., Tasaki, K., & Kobayashi, N. (2012). Seasonal changes in the expression pattern of flowering-related genes in evergreen azalea “Oomurasaki” (*Rhododendron×pulchrum*). *Scientia Horticulturae*, 134 (FEBRUARY), 176–184. doi:10.1016/j.scienta.2011.11.006
- Devetter, L. (2013). Understanding Yield of Cranberry : Bud Development , Carbohydrate Allocation , and Yield Component Analysis. By Lisa Wasko DeVetter A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Horticulture). University of Wisconsin.
- Devetter, L. W., Harbut, R., & Colquhoun, J. (2013). Bud Development , Return Bloom , and External Bud Appearance Differ among Cranberry Cultivars. *J. Amer. Soc. Hort. Sci*, 138(5), 338–343.
- Devlin, R., & Demoranville, I. (1967). Influence of Gibberellic Acid and Gibrel on Fruit Set and Yield in *Vaccinium macrocarpon* cv. Early Black. *Physiologia Plantarum*, 20, 587–592.
- Dogterom, M. H., Winston, M. L., & Mukai, A. (2000). Effect of pollen load size and source (self, outcross) on seed and fruit production in highbush blueberry cv. “Bluecrop” (*Vaccinium corymbosum*; Ericaceae). *American Journal of Botany*, 87 (11), 1584–1591. Retrieved from <http://www.amjbot.org/content/87/11/1584.abstract>
- Endress, P. (2006). Angiosperm floral evolution: Morphological developmental framework. *Advances in Botanical Research. Incorporating Advances in Plant Pathology*, 44, 2–46. doi:10.1016/S0065-2296(06)44001-5

- Fajardo, D., Morales, J., Zhu, H., Steffan, S., Harbut, R., Bassil, N., ... Zalapa, J. (2012). Discrimination of American Cranberry Cultivars and Assessment of Clonal Heterogeneity Using Microsatellite Markers. *Plant Molecular Biology Reporter*, 31 (2), 264–271. doi:10.1007/s11105-012-0497-4
- Goldberg-Moeller, R., Shalom, L., Shlizerman, L., Samuels, S., Zur, N., Ophir, R., ... Sadka, A. (2013). Effects of gibberellin treatment during flowering induction period on global gene expression and the transcription of flowering-control genes in Citrus buds. *Plant Science*, 198 (0), 46–57. doi:http://dx.doi.org/10.1016/j.plantsci.2012.09.012
- Guillon, B., Kelner, J. J., Velasco, R., Gardiner, S. E., Chagné, D., & Costes, E. (2012). Genetic control of biennial bearing in apple. *Journal of Experimental Botany*, 63 (1), 131–149. doi:10.1093/jxb/err261
- Hagidimitriou, M., & Roper, T. R. (1994). Seasonal Changes in Nonstructural Carbohydrates in Cranberry, 119 (5), 1029–1033.
- Hanke, M.-V., Flachowsky, H., Hoenicka, H., & Fladung, M. (2012). Functional genomics of flowering time. In R. J. Schnell & P. M. Priyadarshan (Eds.), *Genomics of tree crops* (p. 369). New York: Springer New York Heidelberg. doi:10.1007/978-1-4614-0920-5
- Hanke, M.-V., Flachowsky, H., Peil, A., & Hättasch, C. (2007). No Flower no Fruit – Genetic Potentials to Trigger Flowering in Fruit Trees. *Genes, Genomes and Genomics*, 1(1), 1–20.
- Heuvel, J. E. Vanden, & Demoranville, C. J. (2009). Competition Between Vegetative and Reproductive Growth in Cranberry, 44 (2), 322–327.
- Huijser, P., & Schmid, M. (2011). The control of developmental phase transitions in plants. *Development (Cambridge, England)*, 138 (19), 4117–29. doi:10.1242/dev.063511
- Khan, M. R. G., Ai, X.-Y., & Zhang, J.-Z. (2013). Genetic regulation of flowering time in annual and perennial plants. *Wiley Interdisciplinary Reviews: RNA*, n/a–n/a. doi:10.1002/wrna.1215
- Koutinas, N., Pepelyankov, G., & Lichev, V. (2010). Flower induction and flower bud development in apple and sweet cherry. *Biotechnology and Biotechnological Equipment*, 24 (1), 1549–1558. doi:10.2478/V10133-010-0003-9
- Lacombe, A., Wu, V. C. H., Tyler, S., & Edwards, K. (2010). Antimicrobial action of the American cranberry constituents; phenolics, anthocyanins, and organic acids, against *Escherichia coli* O157:H7. *International Journal of Food Microbiology*, 139 (1–2), 102–107. doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2010.01.035
- MacKenzie, K. E. (1994). Pollination requirements in cranberry. *Journal of Small Fruit and Viticulture*, 2 (3), 33–44. doi:10.1300/J492v06n02_04
- McCown, B. H., & Zeldin, E. L. (2005). *Vaccinium* spp. Cranberry. In R. E. Litz (Ed.), *Biotechnology of Fruit and Nut crops* (pp. 247–261). Cambridge, MA: CABI publishing.
- McCown, B. H., Zeldin, E. L., & Normington, P. (2003). HyRed. UNITED STATES: United States Plant Patent. Retrieved from <http://assignments.uspto.gov/assignments/q?db=pat&pat=PP14225>

- Roberts, R. H., & Struckmeyer, B. E. (1943). Blossom Induction of the Cranberry. *Plant Physiology*, 18 (3), 534–536.
- Roper, T. R., & Klueh, J. S. (1994). Removing New Growth Reduces Fruiting in Cranberry, 29(3), 199–201.
- Roper, T. R., Stang, E. J., & Hawker, G. M. (1991). Early Season Leaf Removal Reduces Fruit Set and Size in Cranberry (*Vaccinium macrocarpon* Ait.), 1986.
- Rosecrance, R. C., Weinbaum, S. A., & Brown, P. H. (1998). Alternate Bearing Affects Nitrogen, Phosphorus, Potassium and Starch Storage Pools in Mature Pistachio Trees. *Annals of Botany*, 82, 463–470.
- Rowland, L. J., Alkharouf, N., Darwish, O., Ogden, E. L., Polashock, J. J., Bassil, N. V., & Main, D. (2012). Generation and analysis of blueberry transcriptome sequences from leaves, developing fruit, and flower buds from cold acclimation through deacclimation. *BMC Plant Biology*, 12(1), 46. doi:10.1186/1471-2229-12-46
- Salazar-Garcia, S., & Lovatt, C. J. (2000). Use of GA(3) to manipulate flowering and yield of “Hass” avocado. *Journal of the American Society for Horticultural Science*, 125 (1), 25–30.
- Samach, A., & Smith, H. M. (2013). Constraints to obtaining consistent annual yields in perennials. II: Environment and fruit load affect induction of flowering. *Plant Science : An International Journal of Experimental Plant Biology*, 207, 168–76. doi:10.1016/j.plantsci.2013.02.006
- Sarracino, J. M., & Vorsa, N. (1991). Self and cross fertility in cranberry. *Euphytica*, 58(2), 129–136. doi:10.1007/BF00022813
- Shalom, L., Samuels, S., Zur, N., Shlizerman, L., Doron-Faigenboim, A., Blumwald, E., & Sadka, A. (2014). Fruit load induces changes in global gene expression and in abscisic acid (ABA) and indole acetic acid (IAA) homeostasis in citrus buds. *Journal of Experimental Botany*, 65(12), 3029–3044. doi:10.1093/jxb/eru148
- Shalom, L., Samuels, S., Zur, N., Shlizerman, L., Zemach, H., Weissberg, M., ... Sadka, A. (2012). Alternate Bearing in Citrus: Changes in the Expression of Flowering Control Genes and in Global Gene Expression in ON- versus OFF-Crop Trees. *PLoS ONE*, 7(10), e46930. Retrieved from <http://dx.doi.org/10.1371/journal.pone.0046930>
- Smith, H. M., & Samach, A. (2013). Constraints to obtaining consistent annual yields in perennial tree crops. I: Heavy fruit load dominates over vegetative growth. *Plant Science : An International Journal of Experimental Plant Biology*, 207, 158–67. doi:10.1016/j.plantsci.2013.02.014
- Snow, A. A., & Spira, T. P. (1993). Individual Variation in the Vigor of Self Pollen and Self-Fed Progeny in *Hibiscus moscheutos* (Malvaceae). *American Journal of Botany*, 80 (2), 160–164. Retrieved from <Go to ISI>://A1993KM63500007
- Song, G., Walworth, A., Zhao, D., Jiang, N., & Hancock, J. (2013). The *Vaccinium corymbosum* FLOWERING LOCUS T-like gene (*VcFT*): a flowering activator reverses photoperiodic and chilling requirements in blueberry. *Plant Cell Reports*, 32 (11), 1759–1769. doi:10.1007/s00299-013-1489-z

- Tan, F.-C., & Swain, S. M. (2006). Genetics of flower initiation and development in annual and perennial plants. *Physiologia Plantarum*, 128 (1), 8–17. doi:10.1111/j.1399-3054.2006.00724.x
- Trueman, S. J., & Turnbull, C. G. (1994). Effects of cross pollination and flower removal on fruit set in madamia. *Annals of Botany*, 73, 23–32.
- Tuan, N. M., & Yen, C.-R. (2013). Tuan and Chung-Rey, 2013 Effect Pollen-Sources-to-Ability-Fruit-Set-in wax apple-(1). *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*, 7 (2), 74–77.
- Turktas, M., Inal, B., Okay, S., Erkilic, E. G., Dundar, E., Hernandez, P., ... Unver, T. (2013). Nutrition metabolism plays an important role in the alternate bearing of the olive tree (*Olea europaea* L.). *PloS One*, 8 (3), e59876. doi:10.1371/journal.pone.0059876
- USDA. (2012). Cranberry Cultivars and Selections. <http://www.ars.usda.gov/SP2UserFiles/Place/53581500/catalogs/vaccran.html>
- USDA NASS. (2014). Statistics of fruits, tree nuts, and horticultural specialties. In R. Holcomb, C. Foster, P. Hilliard, T. Ray, & S. Pendarvis (Eds.), *Agricultural Statistics* (pp. v1–v61).
- Vander Kloet, S. P. (2009). *Vaccinium* Linnaeus. In Flora of North America Editorial Committee (Ed.), *Flora of North America, North of Mexico* (Vol. 8, p. 515). New York: Oxford University Press. Retrieved from www.efloras.org
- Vander Kloet, S. P., & Tosh, D. (1984). Effects of Pollen Donors on Seed Production, Seed Weight, Germination and Seedling Vigor in *Vaccinium corymbosum* L. *American Midland Naturalist*, 112 (2), 392–396. doi:10.2307/2425444
- Veley, K. M., & Michaels, S. D. (2008). Functional Redundancy and New Roles for Genes of the Autonomous Floral-Promotion Pathway. *Plant Physiology*, 147 (2), 682–695. doi:10.1104/pp.108.118927
- Viskelis, P., Rubinskiene, M., Jasutiene, I., Sarkinas, a, Daubaras, R., & Cesoniene, L. (2009). Anthocyanins, antioxidative, and antimicrobial properties of American cranberry (*Vaccinium macrocarpon* Ait.) and their press cakes. *Journal of Food Science*, 74 (2), C157–61. doi:10.1111/j.1750-3841.2009.01066.x
- Vorsa, N., & Johnson-cicalese, J. (2012). American Cranberry. In M. L. Badenes & D. H. Byrne (Eds.), *Fruit Breeding* (pp. 191–223). Boston, MA: Springer US. doi:10.1007/978-1-4419-0763-9
- Vorsa, N., & Polashock, J. J. (2005). Alteration of Anthocyanin Glycosylation in Cranberry Through Interspecific Hybridization, 130 (5), 711–715.
- Wallace, H. M., & Lee, L. S. (1999). Pollen source, fruit set and xenia in mandarins. *The Journal of Horticultural Science and Biotechnology*, 74 (1), 82–86.
- Wigge, P. A., Kim, M. C., Jaeger, K. E., Busch, W., Schmid, M., Lohmann, J. U., & Weigel, D. (2005). Integration of Spatial and Temporal Information During Floral Induction in *Arabidopsis*. *Science*, 309(5737), 1056–1059. doi:10.1126/science.1114358

- Wilkie, J. D., Sedgley, M., & Olesen, T. (2008). Regulation of floral initiation in horticultural trees. *Journal of Experimental Botany*, 59 (12), 3215–28. doi:10.1093/jxb/ern188
- Yang, C. F., Sun, S. G., & Guo, Y. H. (2005). Resource limitation and pollen source (self and outcross) affecting seed production in two louseworts, *Pedicularis siphonantha* and *P. longiflora* (Orobanchaceae). *Botanical Journal of the Linnean Society*, 147 (1), 83–89. doi:10.1111/j.1095-8339.2005.00363.x
- Yanik, H., Turktas, M., Dundar, E., Hernandez, P., Dorado, G., & Unver, T. (2013). Genome-wide identification of alternate bearing-associated microRNAs (miRNAs) in olive (*Olea europaea* L.). *BMC Plant Biology*, 13 (1), 10. doi:10.1186/1471-2229-13-10
- Zalapa, J. E., Cuevas, H., Zhu, H., Steffan, S., Senalik, D., Zeldin, E., ... Simon, P. (2012). Using next-generation sequencing approaches to isolate simple sequence repeat (SSR) loci in the plant sciences. *American Journal of Botany*, 99 (2), 193–208. doi:10.3732/ajb.1100394
- Zhou, H., Cheng, F.-Y., Wang, R., Zhong, Y., & He, C. (2013a). Transcriptome comparison reveals key candidate genes responsible for the unusual reblooming trait in tree peonies. *PloS One*, 8(11), e79996. doi:10.1371/journal.pone.0079996
- Zhou, H., Cheng, F.-Y., Wang, R., Zhong, Y., & He, C. (2013b). Transcriptome comparison reveals key candidate genes responsible for the unusual reblooming trait in tree peonies. *PloS One*, 8 (11), e79996. doi:10.1371/journal.pone.0079996
- Zhu, H., Senalik, D., McCown, B. H., Zeldin, E. L., Speers, J., Hyman, J., ... Zalapa, J. E. (2012). Mining and validation of pyrosequenced simple sequence repeats (SSRs) from American cranberry (*Vaccinium macrocarpon* Ait.). *TAG. Theoretical and Applied Genetics*. 124 (1), 87–96. doi:10.1007/s00122-011-1689-2

Chapter 2

Histological Study of Apical Buds

of Non-Alternate and Alternate Bearing Cultivars of Cranberry

2.1 Abstract

Similar to other woody crops, cranberry exhibits alternate bearing; however, this trait is not well understood. It is believed that the potential of uprights to initiate floral initials or not for the following year is one of the factors that regulates the alternate bearing tendency. Unfortunately, little is known about the floral initiation process, especially in new cultivars. This study evaluates the presence of floral initials and bud widths through the characterization of longitudinal sections of apical buds from vegetative and fruiting uprights of cultivars Ben Lear and HyRed. Samples were collected weekly during the growing season. Based on histological analysis, we observed that floral initials were formed in vegetative uprights of both cultivars and in fruiting uprights of HyRed. Bud widths increased when floral initials were present. Ben Lear uprights exhibited a significant difference in the growth rate between vegetative and reproductive terminal buds. In addition, our results show a strong tendency for alternate bearing in Ben Lear as compared to HyRed. In contrast, HyRed displayed the capacity to form flower initials in terminal buds of uprights irrespective of the previous years fruiting condition. The results of our study show significant differences in the timing of flower induction between cultivars prone to alternate bearing and those that do not present this trait. In addition, our results show a strong relationship between the width of the terminal bud and the presence of flower primordia.

Keywords: cranberry, alternate bearing, biennial bearing, longitudinal sections, light microscopy, floral initials, bud width, Ben Lear, HyRed

2.2 Introduction

American cranberry (*Vaccinium macrocarpon* L.) is an important fruit crop in the U.S. with the leading producing states being Wisconsin, Massachusetts, New Jersey, Oregon, and Washington. Cranberry plants grow longitudinal stems, called runners from which lateral buds develop stems that grow vertically, called uprights. In each growing season cranberry plants develop two types of uprights, which can be vegetative or fruiting. Vegetative uprights elongate and produce leaves while fruiting uprights grow flowers that subsequently develop into fruits. The annual production of cranberries is highly dependent on the ability of cranberry plants to produce fruiting uprights, and thus a greater understanding of flower induction and flower bud development is critical.

Because cranberry is a slow growing woody perennial, breeding efforts have been relatively slow, and the generation of crosses and the release of cultivars has been complicated due to climatic conditions, field space constraints, expensive and intensive management techniques, and limited long term field phenotypic evaluations (Schlautman et al., 2015). Today, only a handful of cranberry cultivars grow commercially; and many are wild selections from the 1800s and 1900s, (Ben Lear, Howes, and McFarlin). Selectively bred cultivars have been developed using wild germplasm during initial cranberry breeding efforts in the 1940s and 1950s (e.g., Stevens). More recently, a second cranberry breeding cycle used both wild and bred cultivars, and resulted in new improved cultivars such as HyRed and Crimson Queen. Both new introductions possess high yield and improved anthocyanin content (McCown and Zeldin, 2003; Fajardo et al., 2012; Vorsa and Johnson-cicalese, 2012). An important challenge in cranberry production is that the

majority of wild and first generation hybrid cultivars display strong alternate bearing tendencies. However, new cranberry cultivars have been selected to promote the continual production of uprights that induce and sustain the development of floral initials and healthy floral meristems. These meristems subsequently determine fruit production the following season; however, little is known about how the floral induction process occurs and whether this process differs between old and new cultivars.

Alternate or biennial bearing, the tendency of certain plants to display seasons of high and low fruit production ('ON' and 'OFF' years, respectively; Smith and Samach 2013), is a cyclical fruiting pattern shared among woody perennial crops. Although this natural adaptation guarantees fruit production over the long run under different climatic conditions, biennial bearing is generally considered an undesirable trait under most horticultural production systems (Goldschmidt, 2013). Alternate bearing has been observed in several species such as pistachio, apple, and avocado, which exhibit floral meristem inhibition during the 'ON' production years and differentiation only resumes after one or several 'OFF' years (Shalom et al. 2014; Smith and Samach 2013; Rosecrance et al. 1998; Turktas et al. 2013b; Guitton et al. 2012).

Several mechanisms have been proposed to explain alternate bearing. One theory states that alternate bearing is due to the differential allocation of nutrients (i.e., carbohydrates or photoassimilates). In other words, plants need to go through a vegetative stage ('OFF' year) to accumulate enough nutrients to sustain the formation and development of floral meristems for the following season. Meanwhile, during the reproductive stage, most of the carbohydrates are

allocated to sustain fruit set and growth thus allowing an ‘ON’ year (Rosecrance et al. 1998; Turktas et al. 2013a).

Another mechanism proposed to explain alternate bearing is that hormonal control is transported from other organs (i.e., sinks such as fruits and seeds) into the buds resulting in inhibition or promotion of floral meristems. In support of this idea, studies in apple have revealed quantitative trait loci (QTL) associated with gibberellin (GA) biosynthesis genes in trees undergoing ‘ON’ fruiting years (Guitton et al., 2012). More recently, alternate bearing has been attributed to a complex network of interacting hormones, including GA, auxin, and abscissic acid (ABA), involved in signaling the buds to initiate or repress floral induction (Smith and Samach 2013; Shalom et al. 2014). In addition, sugar and calcium transport may also be involved in regulating alternate bearing in woody crops Shalom et al. 2014).

In cranberry, alternate bearing is thought to be due the partitioning of the nutrients based on evidence of source-sink translocation of ¹⁴C-assimilates (Roper and Klueh 1996), carbohydrate contents in fruiting and vegetative uprights (Hagidimitriou and Roper 1994; Roper and Klueh 1994; Roper et al.1991), and dry weight of leaves in both types of uprights across growing seasons (Vanden Heuvel and Demoranville, 2009). However, most of these studies only provide information about wild selections (e.g., Early Black, Searles, and Howes) and from early breeding cycle cultivars (e.g., Stevens). The only study comparing old and new cultivars was conducted by Devetter (2013) who studied carbohydrate content in fruiting and vegetative

uprights in Stevens, HyRed, and GH1. However, this study was inconclusive as to the role of nutrient depletion in alternate bearing cranberry.

Based on the need for a better understanding of alternate fruit bearing in cranberry, we studied the presence of floral initials and bud width from longitudinal sections of apical buds collected weekly during the cranberry growing season. While some information about the morphology of cranberry buds has been published, these studies have been performed using unknown cultivars (Goff, 1901) or using cultivars that are no longer grown commercially (Lacroix, 1926). Moreover, little is known about the histology of cranberry reproductive buds and the time when floral initials are formed.

The goal of the present study has been to determine the timing of floral bud initiation in both alternate and non-alternate bearing cultivars. In addition, we observed the relationship between bud size and the presence of floral meristems. To carry out these objectives, histological analysis was conducted using the apical buds from vegetative and fruiting uprights of two cranberry commercial cultivars: an alternate bearing cultivar (Ben Lear) and a non-alternate bearing cultivar (HyRed). We compared apical buds from these alternate and non-alternate bearing cultivars throughout the growing season; thus providing the first comprehensive study of bud development in relationship to the phenological stages of cranberry uprights. These observations provide new insights on alternate bearing in cranberry and potentially other crops.

2.3 Materials and methods

2.3.1 Plant material. Apical buds of cultivars Ben Lear and HyRed were collected from two cranberry plantings (beds) grown on a commercial farm in Necedah, WI. Both beds were managed using similar horticultural practices (Hansen 2014, Personal communication). Because cranberry cultivars are clonal, genetic contamination in commercial beds can be a problem, particularly for older cultivars and beds (Fajardo et al. 2012) and thus we confirmed the genetic integrity of each bed. Uprights within each bed were fingerprinted using 12 microsatellite markers (Fajardo et al., 2012) and confirmed that all samples were true to type. Additionally, we evaluated six stages of development in 300 fruiting uprights of Ben Lear in order to associate the progression of bud development in terms of phenological stages. The phenological stages evaluated were: bud break, floral bud (prehook), hook, open flower, pinhead, and berry (Figure 2.1). Bud break compiles the early stages of bud development (Workmaster et al. 1997). Fruiting uprights hold flowers for the current growing season and can have between two to seven flowers. The flowers in a single fruiting upright can be in different phenological stages at a given time. Therefore, to ensure that buds were sampled and compared at the same developmental stage, fruiting uprights were classified into a specific stage by evaluating only the development of the floral organs in the first two positions in an acropetally gradient (Figure 2.1). Subsequently, such phenological stage information was used to plan histological apical bud comparisons among Ben Lear and HyRed using similar phenological stages.

Starting in June 26, 2014, when blooming in Ben Lear was most predominant, 10 fruiting and 10 non-fruiting uprights (also called vegetative uprights) of Ben Lear and HyRed were collected

weekly until the first week of August (7th, 2014). A final upright collection was performed 7 weeks later corresponding to the fruit-harvesting season (September 25th). Uprights were collected and immediately stored in sealed plastic bags with a moist paper towel at 4°C to avoid dehydration, until the apical buds were excised and fixed.

2.3.2 Light microscopy. *Fixation:* 10 apical buds (one centimeter in length from the tip of the bud) of Ben Lear and 5 of HyRed from both vegetative and reproductive uprights were excised and immediately fixed by immersion in 0.4% (v/V) glutaraldehyde 0.05M KPO₄ solution at 4°C overnight.

Dehydration: Buds from each week and from each cultivar and upright type were kept in the same vial, properly labeled. Subsequently, the buds were rinsed 3 to 4 times with 0.1M KPO₄ and dehydrated by immersing them in a graded ethanol series of 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% for approximately 20 minutes at each concentration. Subsequently samples were rinsed three times in 100% (v/v) for approximately 2 hours or overnight.

Embedding: Immediately after the last transfer to 100% ethanol, buds were slowly infiltrated by slow swirling on a nutator for a minimum of 4 hours each solution with 25%, 50%, 75%, 87.5 % LR White Resin medium grade (Fisher Scientific cat # 50980420) at room temperature. After 3 additional changes to 100% LR White, samples were transferred to 24mm flat bottom aluminum tins (Fisher scientific cat # 08732105) and the buds were distributed throughout the entire surface of the tin. Another tin was carefully placed over the resin and samples were placed in an incubator at 60°C for 36 hours to permit resin polymerization in the absence of oxygen. Tins

were subsequently removed from the oven and stored at room temperature until samples were mounted.

Mounting and sectioning: Individual bud samples within the resin discs were excised and mounted on plexiglass rods or on mounting cylinders (Ted Pella Inc cat # 10580). One to two micron sections were made on a Sorvall MT2-B ultra-microtome (Sorvall, CT), and samples were mounted on slides (ProbeOn™ Plus slides, Fisher Scientific) to favor the flattening of each cut section. Mounted sections were stained with Toluidine Blue O 0.5% (v/v) for 10 seconds and rinsed with distilled water. The slide was allowed to air-dry and mounted with Cytoseal™ 60. At least 5 independent buds from each upright type (vegetative and fruiting) and cultivar (Ben Lear and HyRed) per week of collection (8 weeks) were observed under an Olympus BX60 microscope (Olympus Optical Company, Tokyo, Japan) using bright field microscopy. Images were captured with an Olympus digital camera DP70. Presence/absence of floral initials was recorded and the width of each bud was measured through image J software (Schneider et al. 2012).

2.3.3 Data analysis. All statistical analyses were performed using R environment (R Core Team (2013). At least 5 apical buds per upright type per cultivar per week were analyzed; and a total of 112 buds from Ben Lear, and 80 from HyRed were evaluated. Presence of floral initials data was analyzed using a regression tree approach in order to determine the effect of cultivar, upright type, and date of collection (phenological stage) in the presence or absence of floral initials (Hothorn et al. 2006). Briefly, regression trees algorithm test and based on a p-value ,we selected the strongest variable (the root) that influenced the variable responses. Floral initials were

identified as the strongest variable, and subsequently data was split (recursive binary splitting) and all variables tested again to determine nodes. The splitting of data was controlled by p-values from the chi-square distribution. Additional nodes were only split when p-values were less than 0.05 (Hothorn et al. 2006; James et al. 2013). At the end of each branch (produced by the splitting process), the proportion of the presence or absence of floral initials was displayed, as well as the number of individuals in each group.

Bud width data was analyzed following a linear regression model. Specifically, the effects of cultivar, upright type, date of collection (phenological stage), and presence of floral initials on bud width were analyzed. Based on preliminary data analysis, increases in bud width did not display a similar pattern between the two cultivars and that the presence of floral initials and upright type were collinear (highly correlated and competing against each other). A linear regression model was used for each cultivar and upright type variable was excluded from the analysis. Thus, the model for each cultivar is as follows:

$$\text{Bud width}_{(\text{BenLear or HyRed})} \sim \beta_0 + \beta_1 (\text{Collection date}) + \beta_2 (\text{floral initials}) + \beta_{12} (\text{Collection date} \times \text{floral initials}) + \epsilon$$

Where, β_0 represents the intercept, β_1 slope for number of days, β_2 increment over intercept for presence of floral initials, β_{12} increment over slope for presence of floral initials, and ϵ as the experimental error.

2.4 Results

2.4.1 Phenological evaluation. By the second week of May 2014, after ice melting, the apical buds of Ben Lear started to swell and burst by the end of the month. Approximately 32% of the elongated uprights had floral buds by the first week of June. Subsequently, fruiting uprights went through the phenological stages as depicted in Figure 2.1. Excepting for the Stage VI, corresponding to growing berries, fruiting uprights progressed through each stage of development weekly (Figure 2.1 and 2.2). Particularly, the phenological stage VI, spanned for three weeks, from July 17th to July 31st.

From June 12th to July 10th, it was noticeable that the majority of the uprights within each time period fell into one of the two predominant phenological stages (Figure 2.2). By June 12th about 35% of the fruiting uprights had floral buds (Stage I) and 40% were in hook stage (Stages II. Figures 2.1 and 2.2). During the following week (June 24th), 42% were in hook (Stage II) and 43% in open flower (Stage III). Subsequently, in the week of July 2nd, 35% and 48% of the uprights were in open flower (Stage III) and pinhead stages (Stage IV), respectively, and 33% of the uprights were in pinhead (Stage IV), and 46% in berry stages (Stage V) occurred during the week through July 10th (Figure 2.2).

By July 17th, and through July 31st, more than 50% of the uprights had developed berries, which continued to grow (Stage VI) and mature until the harvesting period (Stage VII and VIII, reached by August 7th and September 25th, respectively). This tendency of berry production increased

throughout the weeks. However, in the last two weeks of the study (August 7th and September 25th), some of the fruits (15-25%) did not progress through the development and maturation process; and in turn, they became necrotic, stony and died; but remained attached to the pedicel (arrested berries; Figure 2.2).

Based on the most predominant phenological stage observed in Ben Lear, apical buds from fruiting uprights (fruiting uprights) of both Ben Lear and HyRed were collected weekly until July 10th. This collection strategy allowed us to histologically compare Ben Lear and HyRed apical buds from uprights in similar phenological stages while considering that HyRed fruiting uprights go through the developmental stages earlier than Ben Lear. Thus, uprights in Stage IV (Figure 2.1) were collected in July 2nd; Stage V in July 10th; Stage VI in July 17th, 24th, and 31st; Stage VII in August 7th, and in Stage VIII in September 25th.

2.4.2 Histological analysis.

2.4.2.1 General morphology of apical buds in cranberry. Differences in the morphology of the shoot apex were observed as the growing season advanced. By July 2nd, when pinhead was the most predominant phenological stage (Stage IV. Figure 2.1), the shoot apex of Ben Lear uprights were in constant growth with well-defined leaf primordia growing vertically and the leaf tip growing parallel to the meristematic dome (Figure 2.3, A and E). By July 17th, when fruiting uprights were growing berries (Stage VI), the leaf primordia growing close to the meristematic area gave the first indications of bud scales. Instead of growing vertically, these structures grew into the area associated with the shoot apex meristem (SAM). By the following week, with

uprights still in Stage VI, the bud was clearly organized and more scales (4-5 lateral pairs observed from the bud sections) were surrounding and protecting the meristematic dome, leaf primordia, and floral initials, if any (Figure 2.3 B and F). In HyRed uprights, bud scales were observed in most of the sectioned apices of the uprights in Stage IV (collected July 2nd). When uprights were in Stage VII (August 7th) and VIII (September 25th), 5 to 6 lateral pairs of scales were observed in all the buds sectioned from both types of uprights and cultivars (Figure 2.3 C, D, G and H).

2.4.2.2 Floral initials. In this study, the first signs of floral initials in apical buds of cranberry was determined by the observation of globular structures at the axillar zone in the first and second bud scale (counting from the leaf primordia delimiting the meristematic dome) as observed in Figure 2.3H. Later in their development, these globular areas became floral meristems with noticeable structures differentiating into floral primordia. In general, when floral initials were present in HyRed, no floral initials in apical buds from fruiting tissues of Ben Lear were observed.

In the case of Ben Lear, none of the apical buds collected from fruiting uprights showed evidence of floral initials throughout the growing season. In contrast, floral initials were observed only in apical buds collected from Ben Lear vegetative uprights (Figure 2.4 J). When the floral initials were observed for the first time in vegetative uprights (Jul 24th) of Ben Lear more than 90% of its fruiting uprights were holding berries (Stage VI) with an average size of 12mm of diameter and 8mm of length. By the time fruiting uprights were in stage VII (August 7th), most of the basal floral initials observed in vegetative uprights became floral meristems (Figure 2.4 N); and after a month, (Stage VIII of fruiting uprights), all floral meristems in apical

buds sectioned from vegetative uprights had well defined sepal and petal primordia (Figure 2.4 R).

In contrast, apical buds collected from both, fruiting and vegetative uprights of HyRed developed flower initials. Fruiting uprights of HyRed in Stage VI showed floral initials; and, this was similar to when uprights from Ben Lear developed visible floral initials (Figure 2.4 K). These floral initials grew in a similar fashion to those observed in Ben Lear (Figure 2.4 O and S). Floral initials were observed in vegetative uprights of HyRed 2 weeks earlier than those in fruiting uprights of HyRed and the vegetative uprights of Ben Lear (Figure 2.4 H). This was also approximately the same time when fruiting uprights were starting to grow berries (stage V). Due to the earliness of developing of floral initials in vegetative uprights, by the time of harvesting, it was evident that the floral meristems were more developed in vegetative than in fruiting uprights (Figure 2.4 L, P, and T).

Based on the regression tree analysis, collection date was the variable with the highest influence (root) in differences between buds with and without floral initials (p-value <0.001; Figure 2.5). Previous to the time when uprights reached Stage VI (July 17th), the presence of floral initials was explained by cultivar (node 2. p-value <0.001) showing that none of the uprights of Ben Lear had formed visible floral initials. For HyRed, previous to July 17th (stage VI), the presence of floral initials was determined to be dependent on collection date/development (Node 4, p = 0.005). Before the uprights reached the stage IV (before July 2nd), 5% of the uprights of HyRed had visible floral initials. When fruiting uprights reached Stage IV and V (between July 2nd and

17th), the proportion of uprights with floral initials increased up to 60%. Even though the statistical analysis could not discriminate between the type of upright in which the buds developed (fruiting or vegetative uprights), our observations showed that only vegetative uprights in HyRed possessed floral initials by stage VI in fruiting uprights.

After the uprights reached stage IV (later to July 17th, right side of the regression tree; Figure 2.5), the presence of floral initials was explained by upright type (p-value < 0.001). All buds from vegetative uprights, independently of the cultivar, differentiated floral initials. Within fruiting uprights, presence of floral initials was cultivar dependent (p-value < 0.001). In summary, no floral initials were formed in buds from fruiting uprights in Ben Lear; whereas floral initials were observed in buds from 65% of the fruiting uprights of HyRed (Figure 2.5).

2.4.2.3 Bud width. Overall, the size of apical buds was larger in HyRed than in Ben Lear. Throughout the growing season, excepting for buds of vegetative uprights in stage VIII (Sep 25th), Ben Lear buds were smaller than in HyRed (Figure 2.6 A and B). However, within each cultivar at given stages, bud size was not significantly different between fruiting and vegetative uprights before the floral initials were observed. As the floral initials expanded and differentiated into flower meristems, the buds became wider compared to those buds lacking floral initials. The differences in width between fruiting and vegetative buds was most noticeable at the end of the season (Aug, 7th and Sep 25th. Figure 2.6), corresponding to the stages VI and VIII.

For Ben Lear, weekly bud width from vegetative and fruiting uprights until July 17th (Stages IV to VI) before floral initials were observed, increased 50% more from values of approximately 280 μ M to 420 μ M (Figure 2.6, A). Bud width was similar in vegetative and fruiting uprights during stages IV and VI. After Stage VI, (Stages VII and VIII, August 7th and Sept 25th, respectively) the bud width in vegetative uprights increased in size compared to the bud width in fruiting uprights. When fruiting uprights were in stage VII, the average size of buds in these uprights was approximately only 327 μ M, compared to vegetative uprights for which bud size was 898 μ M or 175% larger. Subsequently, in stage VIII (September 25th), the average bud width for vegetative uprights was 158.38% larger (1465 μ M) than in fruiting uprights (567 μ M; Figure 2.6, A).

In HyRed, gradual increases in bud width values were observed across stages and time. Differences between the bud width from vegetative vs. the fruiting uprights in HyRed were initially observed at Stage V (July 10th; Figure 2.6, B). These differences were 138 μ M (23%) larger in buds from vegetative uprights (738 μ M) than in fruiting uprights (600 μ M). When fruiting uprights were in Stage VI and VII (July 17th to August 7th), increases in bud width were higher for buds in vegetative uprights as compared to buds in fruiting uprights. During Stage VIII (September 25th) buds from vegetative uprights had an average width of 1503 μ M (1.5mm) and in fruiting uprights the bud width was 1060 μ M (1mm; Figure 2.6, B), corresponding to 42% wider buds in vegetative uprights compared to fruiting uprights.

From the ANOVA (Table 1 and 2), the p-values showed that the collection date, and its interaction with the presence of floral initials significantly influenced bud width in both cultivars. Based on the linear regression analysis (Figure 2.6, C and D), it was observed that the increment in bud width is higher when floral initials are present, especially for Ben Lear. Buds with no floral initials in Ben Lear increased 24.6 μ M weekly, and 327.35 μ M when floral initials were formed (13 times more. Figure 2.6, C). In HyRed

bud width increased 78.42 μM every week in buds without floral initials and about two-fold (146.52 μM) with floral initials (Figure 2.6, D).

2.5 Discussion

The nature of apical buds in cranberry plays an important role in fruit production, since the ability of the uprights to induce floral initials determines the production of flowers available for pollination and fruit formation. Despite its importance, little is known about the morphology and physiology of apical buds in cranberry. In recent studies, Devetter et al. (2013) observed a positive relationship between the size of apical buds and the presence of floral initials, as well as differences in bud sizes across cultivars.

Based on our results and studies from Davenport et al. (2000) and Roper et al. (1991), we know that the growing season in cranberry is characterized by two phenomena: growth of the apical meristems and flower and fruit production as described in Figure 2.7. Once the apical buds break dormancy in late spring, they go through a period of active growth and become uprights, through all of June in the current study. In the case of fruiting uprights, their elongation is also accompanied by the development of flowers and berry set by mid-July. Parallel to berry growth, another process taking place in the apex is bud set. At this time upright elongation stops, and in the apical bud is evident the differentiation of bud scales that protect the embryonic stem and meristematic dome (Cooke et al. 2012). Bud set is also characterized by the differentiation or not

of floral initials. It is during this time when the fate of the apical buds (reproductive or vegetative) in cranberry is determined. Differences in time when the first indication of bud set occurs in HyRed and Ben Lear (July 2nd vs July 17th) suggests that these two cultivars respond differently, or have different degrees of sensitivity to environmental signals to initiate bud set. It is known that different woody species depend on different signals to induce bud set (Cooke *et al.*, 2012). For example, in some species of apples and pears low temperatures are critical to induce bud set, while in other species such as birch and poplar the plants require critical day lengths (Rhode *et al.*, 2011). In some cases for flowering to be induced in poplar, a particular nutrient balance is required (Cooke *et al.*, 2012). The understanding of the signal triggering the bud set in cranberry is beyond the scope of this study. Additional experiments involving testing the signals previously described along with light microscopy will shed light in the understanding of bud set in cranberry.

Since the phenology of a cranberry bed is a continuum where some uprights develop faster than others (even within the same genotype) and also that recently released cultivars such as HyRed develop earlier, in comparison to Ben Lear, it was important for the current study to compare Ben Lear and HyRed buds from uprights in a similar physiological stage. We used the most predominant phenological stage of uprights that were observed in Ben Lear; and this way the comparisons of apical buds from fruiting and vegetative uprights of Ben Lear and HyRed could be done at similar phenological stages.

As mentioned before, and also stated in the review by Cooke et al. (2012), and Kozlowski, (1971), growth cessation and the formation of bud scales are prerequisites for bud development. In this study, the growth of leaf structures tending to surround SAM in the cranberry uprights allowed us to infer that they were bud scales or cataphylls, which are leaves specialized in protecting the bud. However, the time when bud scales were observed for the first time varied between cultivars. HyRed developed visible bud scales approximately 2 weeks before Ben Lear, when the fruiting uprights were in Stage IV (July 2nd); while in Ben Lear, bud scales were noticed at stage VI (July 17th). These differences in the timing of bud scale formation could also be a morphological indicator for earliness in the growth and development of HyRed compared to other early selections. Moreover, the earlier bud formation of HyRed could potentially allow the upright more time to accumulate and translocate more carbohydrates; thus supporting bud development and resulting in larger buds than Ben Lear.

Ben Lear is a native selection from WI, 1901 (Fajardo et al., 2012) and exhibits a strong alternate bearing tendency (Hansen, 2014, Personal communication). This alternate bearing is also shared by other wild selections. Our results clearly support this observation of alternate bearing behavior as fruiting uprights set primarily vegetative buds, while vegetative uprights set reproductive (floral) buds. An additional tendency that has been observed in wild cultivars is the time when floral initials are first visible. In this study, the first floral initials in Ben Lear vegetative uprights were not observed until July 24th, corresponding to the phenological stage VI. Cultivars such as McFarlin have also been observed to have a delay in production of floral initials, and their appearance has been reported in July (Lendhart and Eaton, 1977; Roberts and Struckmeyer, 1943) . Similarly, Early Black and Howes do not have detectable initials until mid-

August (Lacroix, 1926). Other undefined cultivars are reported as not having initials until September 16th (Goff, 1901).

In contrast recent cultivars such as HyRed, which was released in 2003 (McCown and Zeldin, 2003) are “return bloom or rebudding cultivars”. This designation means that cultivars form reproductive buds independent of whether the upright is vegetative or fruiting each season. Our results support this capacity, but indicate that floral bud initiation is altered in vegetative upright vs. fruiting uprights of rebudding cultivars. Although, both fruiting and vegetative uprights of HyRed had visible floral initials in the apical buds, there were discernible differences in timing and size of the initials. Specifically, the vegetative uprights of HyRed set floral initials much earlier than the fruiting uprights (Stage V, July 10th). The delay of fruiting uprights of HyRed in setting floral initials (Stage VI, July 2^{4th}) was similar to the delay of vegetative uprights of Ben Lear in comparison to HyRed vegetative uprights.

Devetter et al. (2013) studied the presence of floral initials in two recently released cultivars: Crimson Queen and HyRed (Roper and Vorsa 1997; McCown and Zeldin 2003) and one traditional cultivar (Stevens). Using scanning electron microscopy techniques (SEM), they reported the first visible floral initials in all of the cultivars occurred at around the same date independent of the fate of the uprights in both years. Our results contrast with these findings as we observed distinct differences in timing. The discrepancies in results could be attributed to the difference in approaches in sampling, especially in the timing of the collection of the buds from fruiting uprights. The study of Devetter et al. (2013) and our study both collected apical buds

from vegetative and fruiting uprights, but Devetter et al. (2013) included buds from fruiting uprights with different phenological stages while we only dissected buds from vegetative uprights and from fruiting uprights in the same phenological stage. Another possible cause for the differences in our observations could be related to the actual microscopy techniques used. Even though SEM is a powerful technique that allows high resolution analysis, and due to the structure of the bud, most of the leaves and bracts surrounding SAM need to be dissected to expose the floral initials. This situation can cause an artifact if accidentally one floral initial was removed or is too small to be detected. In contrast, the fixation, slow embedding in LR Resin, and longitudinal sectioning of apical buds without prior dissection using histological techniques facilitate the capture of floral initials even before a change in the bud size is physically evident.

In many woody crops, alternate bearing processes are not completely understood. Some theories have been proposed to explain this phenomenon. One theory is the “nutritional theory” in which there is differential distribution of nutrients (carbohydrates) to sinks in the plant (i.e., buds and fruits). Under this theory, the allocation of the nutrients to the growing fruit would be favored, and in turn, the nutrients supply would be limited to the buds, impeding the development of floral meristems for the following growing season (Shalom et al., 2012; Smith and Samach, 2013; Turktas et al., 2013b).

There are several studies in cranberry that support this theory, including the work of Vanden Heuvel and Demoranville (2009) who observed that the area and weight of the leaves in fruiting uprights of three different cultivars (Steven, Howes, and Early Black) declined when fruits

initiated growth. Hagidimitriou and Roper (1994) also found differences in total carbohydrate content between vegetative and fruiting uprights in Searles and Stevens. Finally, similar results were obtained by Devetter (2013) when analyzing total carbohydrates and starch among alternate and non-alternate bearing cultivars. These studies found that although the carbohydrate content declined as the growing season progressed, vegetative uprights had higher carbohydrate content compared to the fruiting ones in all cultivars. In addition, the total carbohydrate content in fruiting uprights of an alternate bearing cultivar continued to drop during the season, while the levels of carbohydrates started to increase in fruiting uprights of non-alternate bearing cultivars subsequent to the fruit setting stage (Devetter 2013). Such differences in carbohydrate content could partially explain the difference in the capability of the uprights to grow floral initials in Ben Lear and HyRed in our study. Additionally, various studies involving labeling with ^{14}C Carbon or defoliation of fruiting uprights have also demonstrated that new growth above the fruits is the primary source of photosynthates for the growing fruits (Hagidimitriou and Roper 1994; Roper et al. 1991; Roper and Klueh 1994) and floral buds (Roberts and Struckmeyer, 1943).

In our case, it is clear that vegetative uprights of both cultivars (Ben Lear and HyRed), having no other sinks but the apical buds themselves, most likely possess a high proportion of synthesized carbohydrates that are allocated to the apex and support the growth of flower initials for next year flower production. The ability of the vegetative uprights of HyRed to sustain the growth of floral initials earlier than in fruiting uprights of Ben Lear and HyRed could be attributed to a larger store of carbohydrates in HyRed. In comparison to Ben Lear, HyRed is an earlier cultivar; thus allowing HyRed vegetative uprights to accumulate carbohydrates for a longer period of time and consequently earlier development of floral initials.

In contrast, the allocation of carbohydrates in fruiting uprights could be drastically different in Ben Lear compared to HyRed. In Ben Lear fruiting uprights, growing and maturing seeds and berries may be exhausting carbohydrates, and thus limiting the translocation of assimilates to the apical buds and subsequently preventing the development of flower initials. In HyRed, the allocation of nutrients in fruiting uprights could be initially directed to the development and growth of flowers and fruits, but after fruit set, a seasonal increase in total carbohydrate content (Devetter 2013) could allow these fruiting uprights to partition carbohydrates also into the apical bud. This seasonal increase in carbohydrates and allocation mechanism could allow HyRed to sustain differentiation and growth of floral initials in the apex while also supporting the growth and maturity of berries. This allocation of carbohydrates, however, does not seem to penalize the fruit quality and production since berries from HyRed tends to mature faster and have higher fruit quality (fruit size and color), and higher yield than Ben Lear and wild and old cultivars (McCown and Zeldin 2003).

Another theory used to explain the alternation of high and low production times in woody crops is based in an endogenous control where movement of hormones and signals are key factors in inducing or repressing the expression of genes promoting flower initiation and fruit production during ‘OFF’ and ‘ON’ years (low and high fruit production, respectively). Based on studies in *Arabidopsis*, there are four flowering pathways that have been named based on the signals that induce each of them, and they are: Photoperiod, Vernalization, Giberellic Acid (GA), and Autonomous (Hanke et al., 2012) . QTL studies in populations produced by crosses of alternate and non-alternate bearing apples, found that genes responsible to synthesize gibberellins (GAs)

were located in a QTL cluster related with alternate fruit production, which could indicate that this hormone has a negative effect in the induction of floral initials (Guitton et al. 2012).

In addition these conclusions are supported by work on mandarin trees in which consecutive applications of GA for three years interfered negatively in the development of floral buds (Goldberg-Moeller et al., 2013). It was also shown by Salazar-Garcia and Lovatt (2000) that spraying GA₃ to branches of ‘Hass’ Avocado trees with early stages of floral initials disrupted their development and stimulated production of vegetative shoots. Genes related to flowering and the synthesis of hormones such as abscissic acid (ABA) and auxins were differentially expressed among citrus buds from ‘ON’, ‘OFF’, and de-fruited trees (trees in which the fruits were removed). Flowering genes and ABA associated genes were upregulated in the ‘OFF’ and defruited trees, while auxin associated genes were upregulated in ‘ON’ trees. These results, as well as the higher number of floral buds in ‘OFF’ and de-fruited trees than in ‘ON’ trees, suggest that the hormones previously mentioned can play a signal network that affects floral initiation (Shalom et al. 2014). Our results suggest that it could be possible that in fruiting uprights in Ben Lear, signaling from growth regulators such as GAs, from the seeds of the developing fruits could be sent to the apical buds, and thus inhibit the formation of floral meristems. In contrast, the ability of fruiting uprights of HyRed to differentiate floral meristems could be due to an insensitivity of the apical meristem to inhibitory signals from seeds. Another possible explanation is that a less inhibitory signal could be sent from seeds to the apical buds of fruiting uprights of HyRed. On the other hand, vegetative uprights of Ben Lear and HyRed apical buds would not receive any inhibitory signal; and thus flower meristem formation is not halted.

Whether or not these networks of endogenous hormones, signals and gene expression could induce flower initiation in cranberry buds, still remains to be studied further.

Even though the nutritional theory, also known as carbohydrate partitioning, may partially explain some of our results, we believe that endogenous hormonal control of GA exerted differentially between alternate and non-alternate cultivars may also account for promoting or inhibiting floral induction. This explanation has been supported by molecular studies in citrus and apples (Goldberg-Moeller et al., 2013; Guitton et al., 2012; Shalom et al., 2014) and by Devlin and Demoranville in cranberry (1967). In the study by Devlin and Demoranville GA₃ was applied to developing fruits and evaluated for its effects on fruit set and yield in a commercial plantation of the Early Black cultivar. Devetter and colleagues (Devetter *et al.*, 2013b) observed differences in the formation of floral initials in Ben Lear and HyRed and also hypothesize that the production of GAs from the seeds of the developing fruits could send and inhibitory signal to the floral initiation process in the buds. In summary, whether or not endogenous GAs play a role in limiting bud set is still to be verified.

Our results showed that buds with floral initials are wider than those with no floral initials and this is also supported by observations on buds by Devetter et al. (2013a). In addition, buds from HyRed, with and without floral initials, were wider than Ben Lear buds throughout the season. We hypothesize that these differences in size between buds with and without floral initials (reproductive and vegetative buds, respectively) may be due to the increased separation between bud scales produced by the differentiation, expansion, and growth, of the floral initials into floral

meristems. This is in contrast to the more compact arrangement of bud scales observed in vegetative buds. The number of lateral bud scales observed in longitudinal sections was similar in both types of buds and cultivars; therefore, we did not consider if the scales contribute to the differences in sizes among the buds. However, further studies of transversal sections of the buds would clarify this assumption.

Taking into account the analysis of bud width data, the growth rate of buds in HyRed was still higher in reproductive buds, and it was twice the rate of the vegetative ones (146 vs 78 μM). Interestingly, this differential growth rate was even more pronounced in Ben Lear, as reproductive buds grew 13 times faster than the vegetative buds (327 vs 25 μM). One might also hypothesize that this drastic increment of growth rate in reproductive buds of Ben Lear could be the consequence of differential carbohydrate allocation; however, additional experiments would need to be performed. In the studies by Devetter (2013) she also suggests that the HyRed cultivar could have a more efficient allocation of nutrients; thus allowing it to bypass the alternate bearing phase.

In conclusion, this study supports the idea of alternate and non-alternate bearing tendencies in wild selections as Ben Lear and recent selections as HyRed, respectively, as evidenced by the differential production of uprights capable of producing floral initials. Floral initials in apical buds were found in vegetative uprights of Ben Lear and HyRed, as well as in fruiting uprights of HyRed. Vegetative uprights of HyRed differentiated floral initials two weeks earlier than fruiting uprights of HyRed and vegetative ones of Ben Lear. Additionally, bud width was positively

affected by the presence of floral initials in both cultivars. Interestingly, in Ben Lear the weekly rate of growth of the buds with floral initials was remarkably higher than buds without floral initials. This rate of growth was even higher than in HyRed. Additional histological studies of apical buds are suggested in order to determine if the differences observed in this study are generalized among earlier and recently released cultivars.

2.6 Acknowledgements

USDA-ARS (project no. 3655-21220-001-00); Ocean Spray Cranberries, Inc.; Wisconsin Cranberry Growers Association; Cranberry Institute. We want to acknowledge the help provided by members of the USDA Cranberry Genetic and Genomics Lab, especially Brandon Schlautman, Eric Wiesman, Tiffany Bougie, and Tierney Bougie who contributed in the collection of the plant material needed to carry out this research. We are also thankful to Guilherme Ludwig from the CALS Statistical Consulting Group in UW for the assistance given during the statistical analysis of the data. We also appreciate the collaboration of Nichole Hansen and Cranberry Creek Cranberries for allowing us to collect data and plant material from their marsh.

2.7 References

Cooke, J., Eriksson, M., and Juntilla, O. (2012). The dynamic nature of bud dormancy in trees: environmental control and molecular mechanisms. *Plant, Cell and Environment*, 35 (10), 1707–1728. doi:10.1111/j.1365-3040.2012.02552.x

- Davenport, J. R., Demoranville, C. J., Hart, J., and Roper, T. (2000). Nitrogen for Bearing Cranberries in North America. *EM 8741*
- Devetter, L. (2013). *Understanding Yield of Cranberry : Bud Development , Carbohydrate Allocation , and Yield Component Analysis* By Lisa Wasko DeVetter A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Horticulture. University of Wisconsin.
- Devetter, L. W., Harbut, R., and Colquhoun, J. (2013a). Bud Development , Return Bloom , and External Bud Appearance Differ among Cranberry Cultivars. *J. Amer. Soc. Hort. Sci.*, 138 (5), 338–343.
- Devetter, L. W., Harbut, R., and Colquhoun, J. (2013b). Understanding yield-contributing factors of cranberry (*Vaccinium macrocarpon* Ait.). *Journal of the American Pomological Society*, 67 (3), 147–156.
- Devlin, R., and Demoranville, I. (1967). Influence of Gibberellic Acid and Gibrel on Fruit Set and Yield in *Vaccinium macrocarpon* cv. Early Black. *Physiologia Plantarum*, 20, 587–592.
- Fajardo, D., Morales, J., Zhu, H., Steffan, S., Harbut, R., Bassil, N. *et al.* (2012). Discrimination of American Cranberry Cultivars and Assessment of Clonal Heterogeneity Using Microsatellite Markers. *Plant Molecular Biology Reporter*, 3 1(2), 264–271. doi:10.1007/s11105-012-0497-4
- Goff, E. S. (1901). Investigations of flower-buds. *Annual Report of the Agricultural Experiment Station of The University of Wisconsin*, 18, 304–319.
- Goldberg-Moeller, R., Shalom, L., Shlizerman, L., Samuels, S., Zur, N., Ophir, R., ... Sadka, A. (2013). Effects of gibberellin treatment during flowering induction period on global gene expression and the transcription of flowering-control genes in Citrus buds. *Plant Science*, 198, 46–57. doi:http://dx.doi.org/10.1016/j.plantsci.2012.09.012
- Goldschmidt, E. E. (2013). The Evolution of Fruit Tree Productivity: A Review. *Economic Botany*, 6 7(1), 51–62. doi:10.1007/s12231-012-9219-y
- Guitton, B., Kelner, J. J., Velasco, R., Gardiner, S. E., Chagné, D., and Costes, E. (2012). Genetic control of biennial bearing in apple. *Journal of Experimental Botany*, 63 (1), 131–149. doi:10.1093/jxb/err261

- Hagidimitriou, M., and Roper, T. R. (1994). Seasonal Changes in Nonstructural Carbohydrates in Cranberry, *119* (5), 1029–1033.
- Hanke, M.-V., Flachowsky, H., Hoenicka, H., and Fladung, M. (2012). Functional genomics of flowering time. In R. J. Schnell and P. M. Priyadarshan (Eds.), *Genomics of tree crops* (p. 369). New York: Springer New York Heidelberg. doi:10.1007/978-1-4614-0920-5
- Hothorn, T., Hornik, K., and Zeileis, A. (2006). Unbiased Recursive Partitioning: A Conditional Inference Framework. *Journal of Computational and Graphical Statistics*, *15* (3), 651–674. doi:10.1198/106186006X133933
- James, G., Witten, D., Hastie, T., and Tibshirani, R. (2013). *Springer Texts in Statistics An Introduction to Statistical Learning - with Applications in R*. (G. Casella, S. Fienberg, and I. Olkin, Eds.). New York: Springer. Retrieved from <http://www.jstor.org/stable/27594202>
- Kozłowski, T. . (1971). *Growth and Development of Trees: Seed Germination, Ontogeny and Shoot Growth v. 1 (Physiological Ecology)*. New York.
- Lacroix, D. S. (1926). Cranberry flower-bud investigations. *Journal of Agricultural Research*, *33* (4), 355–363.
- Lendhart, P. J., and Eaton, G. W. (1977). Cranberry flower bud initiation in British Columbia. *Fruit Var. J.*, 31–44.
- McCown, B. H., and Zeldin, E. L. (2003). “HyRed”, an Early, High Fruit Color Cranberry Hybrid. *HortScience*, *38*(2), 304–305. Retrieved from <http://hortsci.ashspublications.org/content/38/2/304.short>
- McCown, B. H., Zeldin, E. L., and Normington, P. (2003). HyRed. UNITED STATES: United States Plant Patent. Retrieved from <http://assignments.uspto.gov/assignments/q?db=patandpat=PP14225>
- R Core Team (2013). (n.d.). R: A language and environment for statistical computing. *R Foundation for Statistical Computing. Vienna, Austria*. Retrieved July 1, 2015, from <http://www.r-project.org/>.
- Roberts, R. H., and Struckmeyer, B. E. (1943). Blossom Induction of the Cranberry. *Plant Physiology*, *18*(3), 534–536.

- Roper, T. R., and Klueh, J. S. (1994). Removing New Growth Reduces Fruiting in Cranberry, 29 (3), 199–201.
- Roper, T. R., and Klueh, J. S. (1996). Movement Patterns of Carbon from Source to Sink in Cranberry. *J. Amer. Soc. Hort. Sci.*, 121 (5), 846–847.
- Roper, T. R., Stang, E. J., and Hawker, G. M. (1991). Early Season Leaf Removal Reduces Fruit Set and Size in Cranberry (*Vaccinium macrocarpon* Ait .), 1986.
- Roper, T., and Vorsa, N. (1997). Roper and Vorsa 1997 cranberry botany and horticulture.pdf. In J. Janick (Ed.), *Horticultural reviews* (pp. 215–249). New York: John Wiley and Sons, Inc.
- Rosecrance, R. C., Weinbaum, S. A., and Brown, P. H. (1998). Alternate Bearing Affects Nitrogen , Phosphorus , Potassium and Starch Storage Pools in Mature Pistachio Trees. *Annals of Botany*, 82, 463–470.
- Salazar-Garcia, S., and Lovatt, C. J. (2000). Use of GA(3) to manipulate flowering and yield of “Hass” avocado. *Journal of the American Society for Horticultural Science*, 125(1), 25–30.
- Schlautman, B., Covarrubias-Pazarán, G., Diaz-Garcia, L. a., Johnson-Cicalese, J., Iorrizo, M., Rodriguez-Bonilla, L., ... Zalapa, J. (2015). Development of a high-density cranberry SSR linkage map for comparative genetic analysis and trait detection. *Molecular Breeding*, 35(8), 177. doi:10.1007/s11032-015-0367-5
- Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat Meth*, 9 (7), 671–675.
- Shalom, L., Samuels, S., Zur, N., Shlizerman, L., Doron-Faigenboim, A., Blumwald, E., and Sadka, A. (2014). Fruit load induces changes in global gene expression and in abscisic acid (ABA) and indole acetic acid (IAA) homeostasis in citrus buds. *Journal of Experimental Botany*, 65(12), 3029–3044. doi:10.1093/jxb/eru148
- Shalom, L., Samuels, S., Zur, N., Shlizerman, L., Zemach, H., Weissberg, M., ... Sadka, A. (2012). Alternate Bearing in Citrus: Changes in the Expression of Flowering Control Genes and in Global Gene Expression in ON- versus OFF-Crop Trees. *PLoS ONE*, 7 (10), e0046930.
- Smith, H. M., and Samach, A. (2013). Constraints to obtaining consistent annual yields in perennial tree crops. I: Heavy fruit load dominates over vegetative growth. *Plant Science* :

An International Journal of Experimental Plant Biology, 207, 158–67.
doi:10.1016/j.plantsci.2013.02.014

- Turktas, M., Inal, B., Okay, S., Erkilic, E. G., Dundar, E., Hernandez, P., *et al.*, (2013a). Nutrition Metabolism Plays an Important Role in the Alternate Bearing of the Olive Tree (*Olea europaea* L.). *PLoS ONE*, 8(3). doi:10.1371/journal.pone.0059876
- Turktas, M., Inal, B., Okay, S., Erkilic, E. G., Dundar, E., Hernandez, P., *et al.*, (2013b). Nutrition metabolism plays an important role in the alternate bearing of the olive tree (*Olea europaea* L.). *PloS One*, 8(3), e59876. doi:10.1371/journal.pone.0059876
- Vanden Heuvel, J. E., and Demoranville, C. J. (2009). Competition Between Vegetative and Reproductive Growth in Cranberry, 44 (2), 322–327.
- Vorsa, N., and Johnson-cicalese, J. (2012). American Cranberry. In M. L. Badenes and D. H. Byrne (Eds.), *Fruit Breeding* (pp. 191–223). Boston, MA: Springer US. doi:10.1007/978-1-4419-0763-9
- Workmaster, B., Palta, J., and Roper, T. (1997). Terminology for Cranberry Bud Development and Growth. *Cranberries*, 61, 11–14.

Table 2. 1 Analysis of variance for bud width from vegetative and fruiting uprights of Ben Lear during the growing season 2014

	Sum sq.	Df	F –Value	Pr (>F)	
Intercept	460871	1	31.751	1.591e-07	***
Collection date	175653	1	12.101	0.0007449	***
Floral initials	2053814	1	141.495	< 2.2e-16	***
Collection date: Floral initials	2731664	1	188.195	< 2.2e-16	***
Residuals	1466027	101			

*** Significance at p-value <0.001

Table 2. 2 Analysis of variance for bud width from vegetative and fruiting uprights of HyRed during the growing season 2014

	Sum sq.	Df	F –Value	Pr(>F)	
Intercept	65801	1	1.5096	0.223631	
Collection date	643635	1	14.7661	0.000279	***
Floral initials	58821	1	1.3494	0.249623	
Collection date: Floral initials	262859	1	6.0304	0.016744	*
Residuals	2833264	65			

*** Significance at p-value <0.001

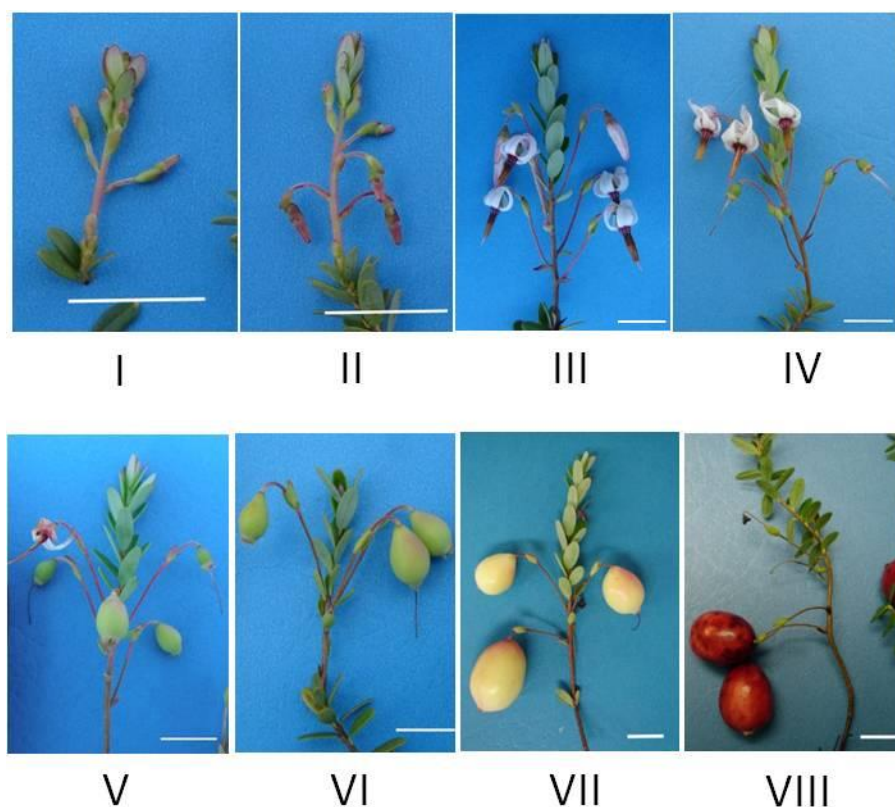


Figure 2. 1 Phenological stages of cranberry. After the bud development stages described by Workmaster et al. (1997), cranberry reproductive uprights went through different phenological stages, most of which were reached in a week. Eight distinctive phenological stages, based on the two more basal floral positions in reproductive uprights, were determined. Arabic numbers represent the phenological stage showed in each picture. Stage I: Floral bud or prehook; Stage II: Hook; Stage III: Open flower; Stage IV: Pinhead, Stages V and VI: growing berry; Stage VII: maturing berry; Stage VIII: Mature berry, ready for harvesting.

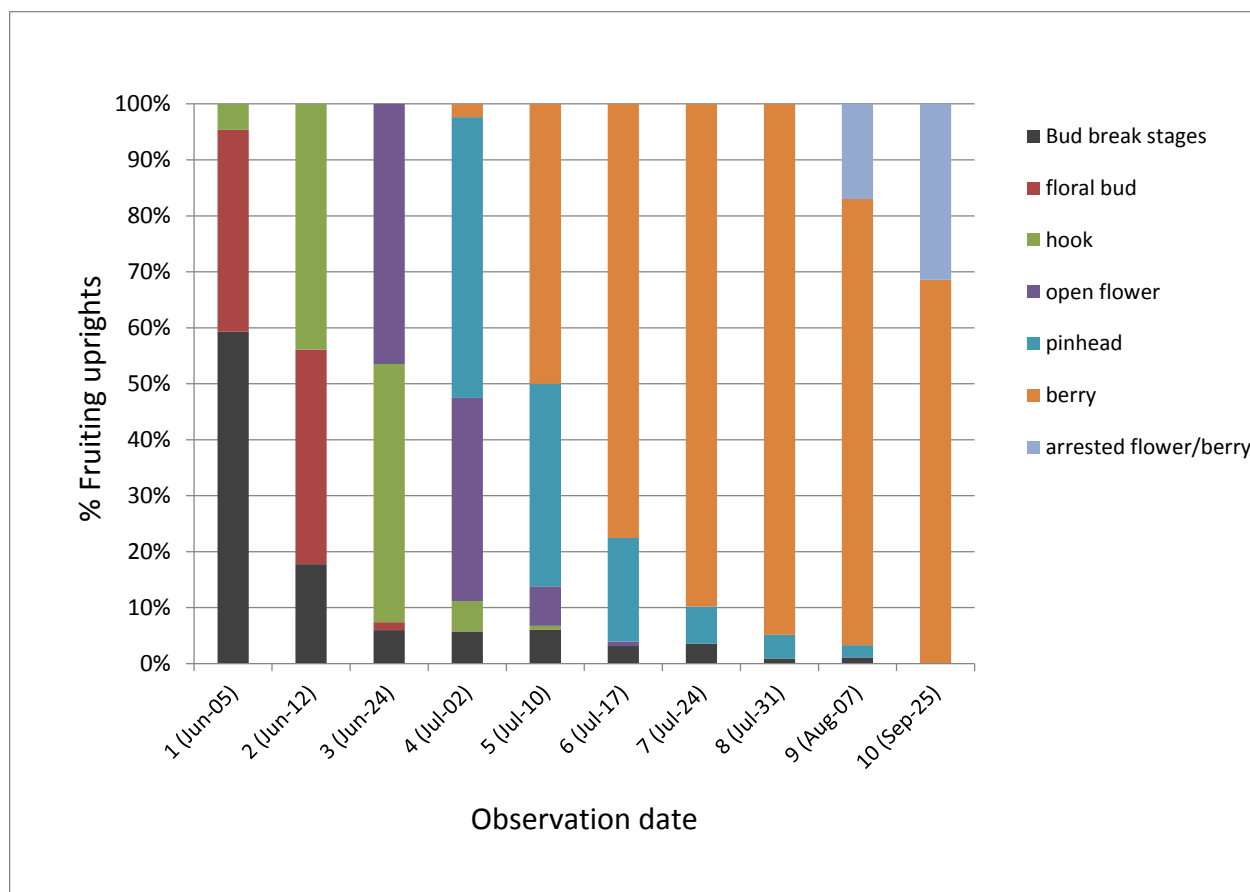


Figure 2. 2 Phenological stages of uprights derived from reproductive buds of Ben Lear. Percentage of uprights in each phenological stage was calculated based on the evaluation of 300 uprights. Six phenological stages were evaluated: Bud break, floral bud, hook, open flower, pinhead and berry. An upright was considered to be in one phenological stage based on the evaluation of the floral organs at the 2 more basal positions in the upright. Another category evaluated was the percentage of uprights with arrested berries, which was observed mainly in the last two weeks of data collection.

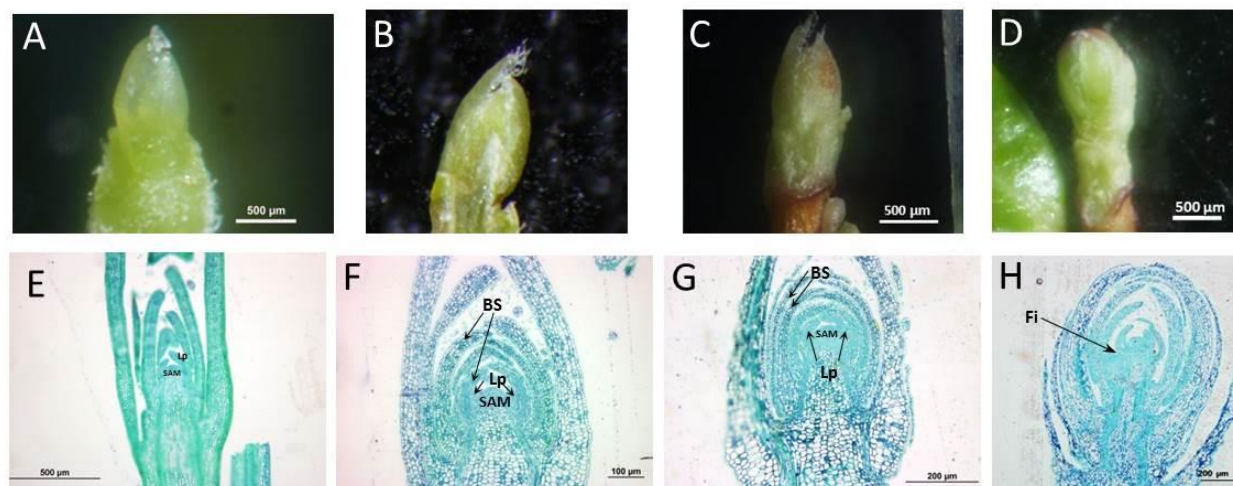


Figure 2.3 Bud formation in Cranberry. (A and B). Apical buds from reproductive uprights of Ben Lear collected from stages IV and VI (July 2nd and 17th, respectively). (C and D) Ben Lear buds from uprights in Stage VII (August 7th). (C) bud from reproductive upright. (D) bud from vegetative upright. (E, F, G, and H) micrographs of longitudinal sections corresponding to buds described above. (A and E), shoot apex in active growing phase with young leaves growing parallel to the shoot meristem (SAM). (B and F) bud scale-like structures (BS) growing over the area of the leaf primordia (Lp) and SAM). In HyRed cultivar, by July 2nd (Stage IV), it was observed bud scale-like structures as the ones observed in (F). (C and G), buds from Ben Lear and HyRed uprights were fully formed by the end of the fruit production season and before fruit harvesting with 5 to 6 pairs of lateral bud scales visible. (D and H) bud with floral initials (Fi). Due to the nature of the vertical sectioning, only lateral pairs of bud scales were shown, and therefore, evaluated.

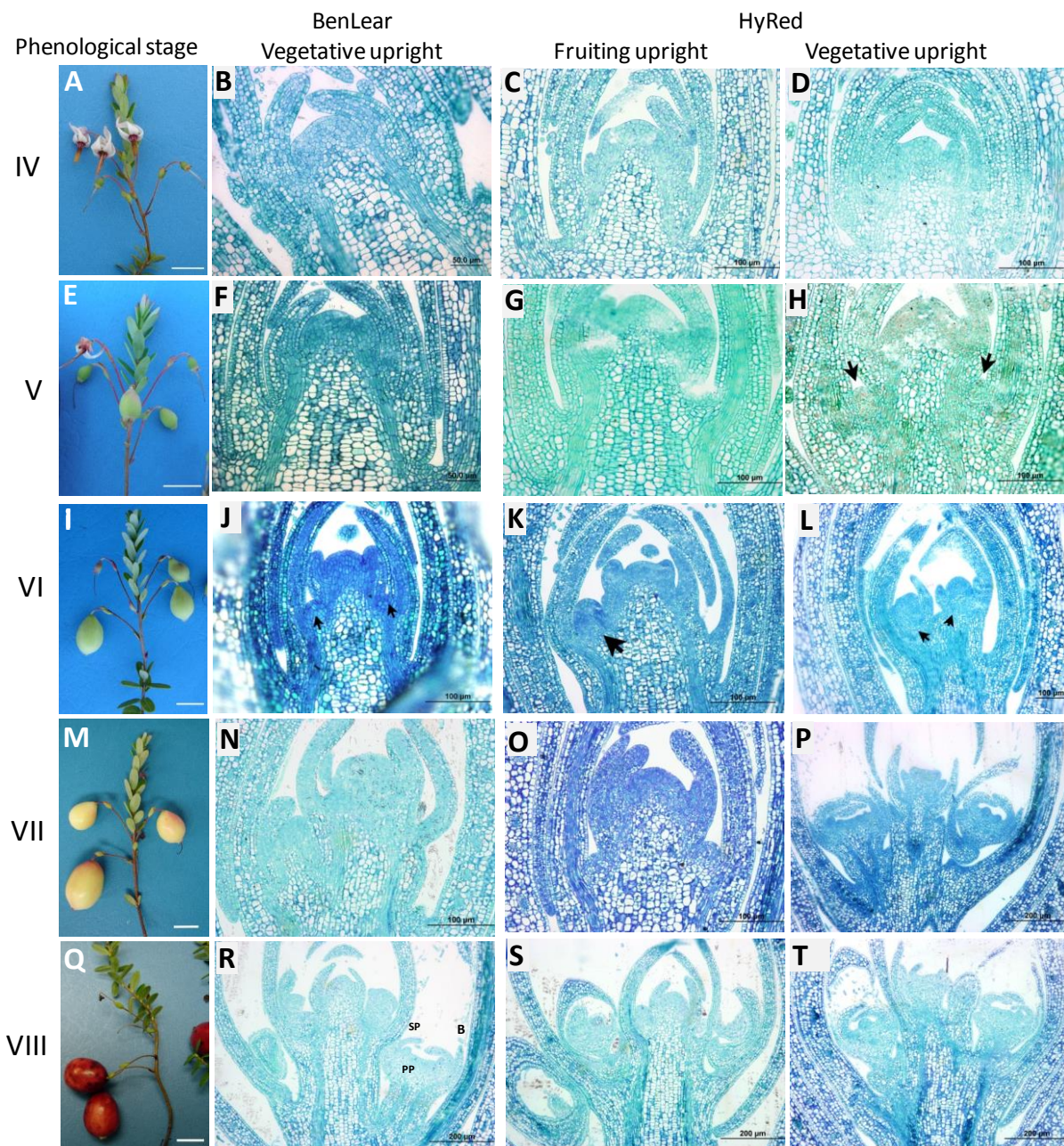


Figure 2. 4 Differentiation of floral initials in Ben Lear and HyRed cultivars. Pictures at the left side correspond to the phenological stage at which buds were collected weekly, starting with Stage IV (pinhead) to Stage VIII (mature berry). Scale bar = 1cm. First column of micrographs correspond to longitudinal sections of buds in vegetative uprights of Ben Lear. Second and third columns correspond to sections of buds from reproductive and vegetative uprights of HyRed cultivar, respectively. (I,,J) Floral initials in Ben Lear cultivar, in reproductive uprights in Stage VI (note: first appearance). (N, R) Development of floral initials into floral meristems and additional acropetal initials (R). By Stage VIII (Q) there were an array of floral meristems in different stages of development; bract, sepal and petal primordial (B, SP and PP, respectively) were noticed in the more basal meristems (R). Similar to Ben Lear, buds from reproductive uprights of HyRed, also formed floral initials at Stage VI (K) and continue its development as those in Ben Lear (O, S). In contrast, in vegetative uprights of HyRed, the

floral initials were observed two weeks earlier than in the reproductive uprights, in Stage V (H), and progressed to more advanced developmental stages of the meristems weekly compared to the initials in buds of Ben Lear and reproductive uprights of HyRed (L, P, and T). By Stage VIII, September 25th, most of the floral initials in vegetative uprights of HyRed differentiated into floral meristems. All micrographs were taken with 400 times magnification, excepting those showing later stages of floral meristem development (L, P, R, S and T) that were taken with 200x magnification.

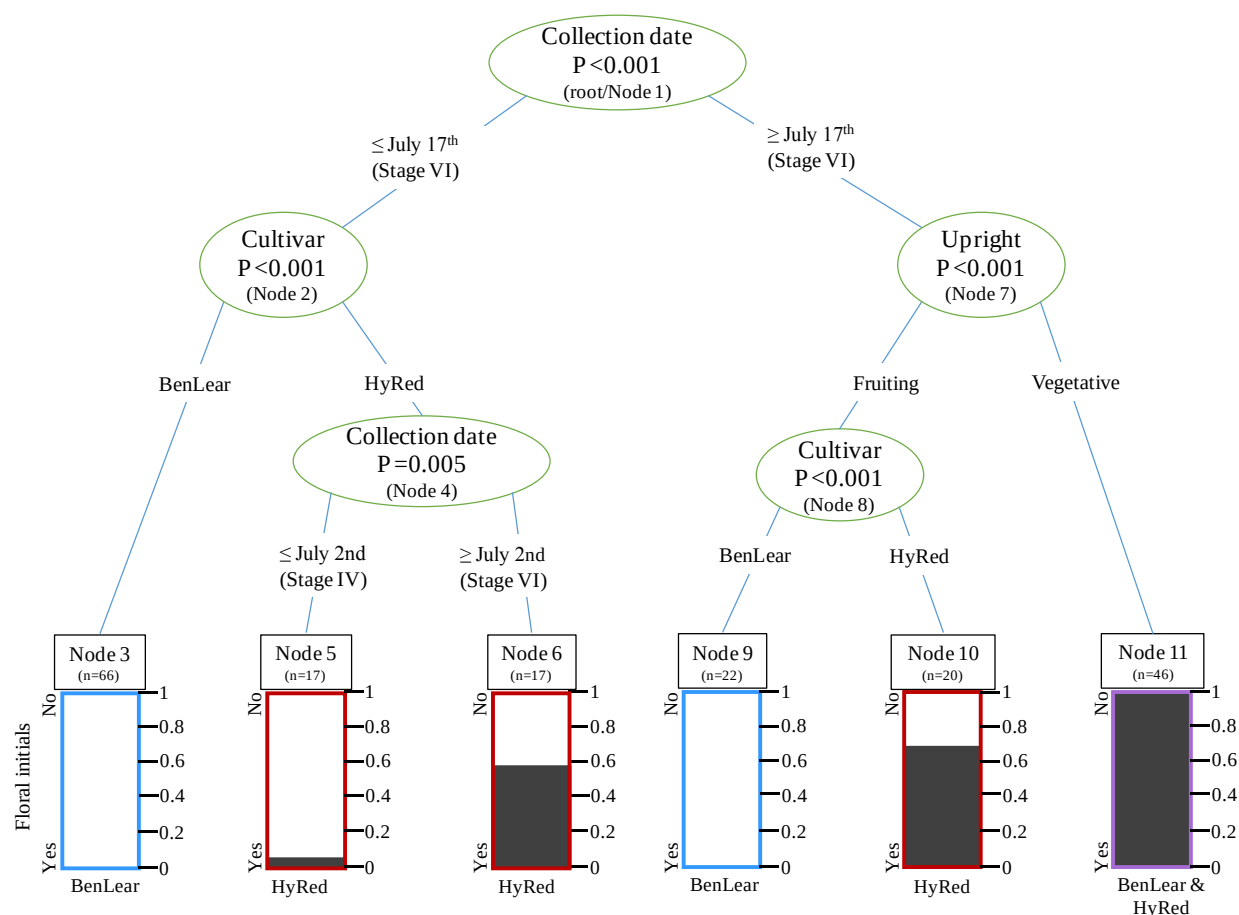


Figure 2. 5 Regression tree for presence of floral initials in cranberry buds explained by collection date (week), cultivar and upright type. The 11 nodes represent the significant data partitions according to the binary classification analysis performed using R package ‘party’. n= number of uprights in each of the most basal nodes. Bar charts in the basal nodes showed the proportion of uprights with floral initials (black) and without floral initials (white). Blue charts correspond to Ben Lear, Red for HyRed and Purple when node contains information of both, Ben Lear and HyRed. Collection date (phenological stages. Root/ node 1) was the variable that best explained the presence of floral initials. Before Stage VI (node 2), none of the Ben Lear uprights differentiated floral initials (node 3). In contrast, HyRed (node 4) began to form floral initials before uprights reached Stage IV (node 5), and more initials were observed as the uprights progressed through more advanced phenological stages (node 6, 10 and 11). After Stage IV (node7), no floral initials were observed in reproductive uprights (node 8 and 9). 65% of the reproductive uprights in HyRed Showed floral initials (node 10). All vegetative uprights from both cultivars differentiated floral initials (node 11).

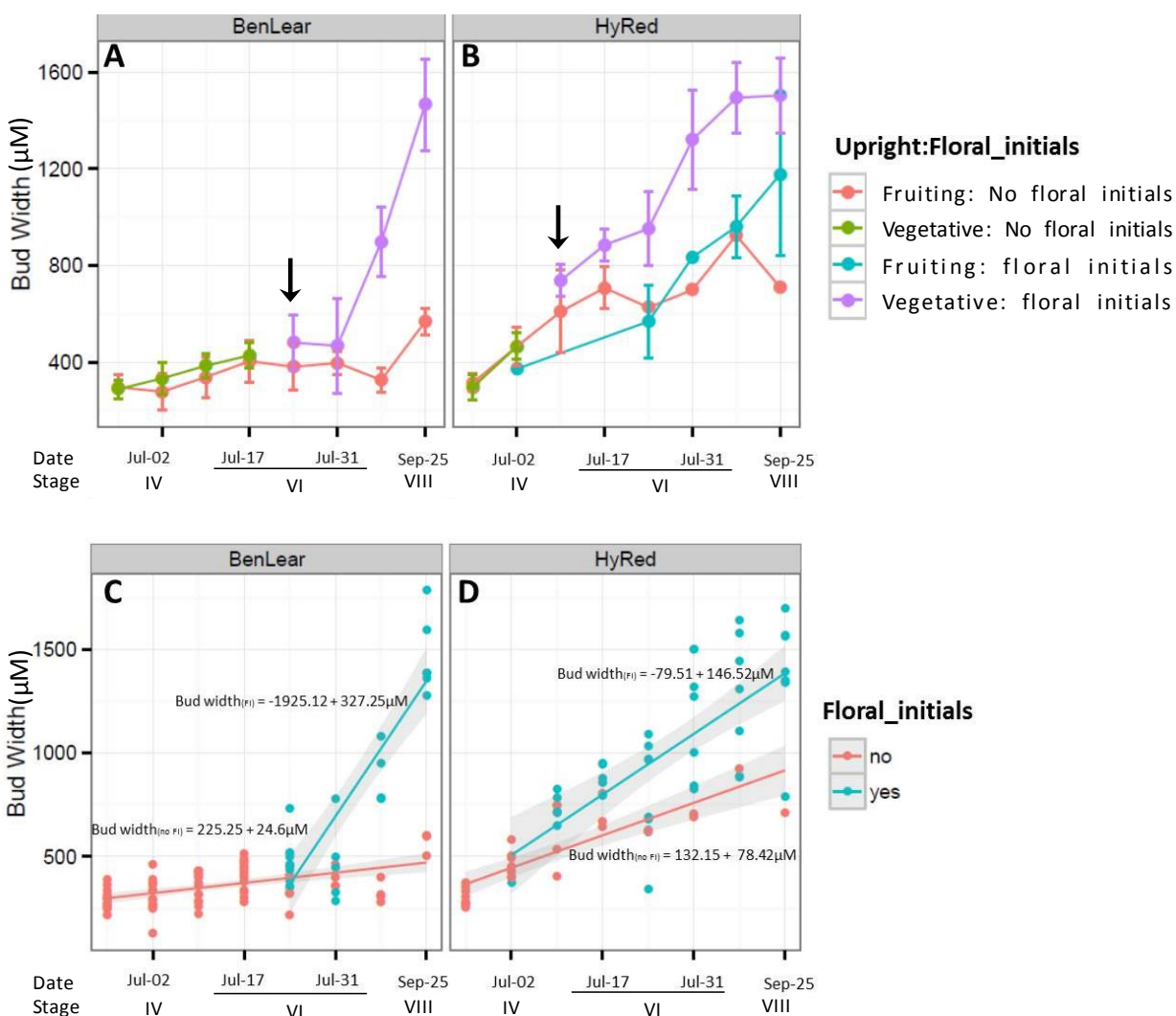


Figure 2. 6 Width of apical buds in reproductive and vegetative uprights of Ben Lear and HyRed.

Average bud width from vegetative and reproductive uprights of Ben Lear (A) and HyRed (B) throughout the growing season. Arrows indicate the time/stage of reproductive upright when floral initials were visible for the first time. Error bars indicate ± 1 deviation standard. (C and D) Linear regression analysis for bud width with (blue) and without (red) floral initials in Ben Lear (C) and HyRed (D). Shaded areas correspond to confidence intervals (95%) for the predicted regression lines. (A) The increment in bud size for Ben Lear was not significant through the season until the last two weeks of collection (August 7th and September 25th), corresponding to Stages VII and VIII. Width of buds with floral initials significantly increased compare to the width buds of reproductive uprights that did not differentiate floral initials (A). Bud width in HyRed increased weekly, and at the end of the season buds with floral initials in vegetative uprights were slightly bigger compared to the buds with floral initials in reproductive uprights (B). Regression analysis revealed that in Ben Lear, weekly width rate of buds without floral initials (red dots) was 24.6 μM , whereas buds with floral initials increased 327.35 μM per week (C). In HyRed bud width without floral initials increased 78.42 μM and with floral initials 146.52 μM every week (D).

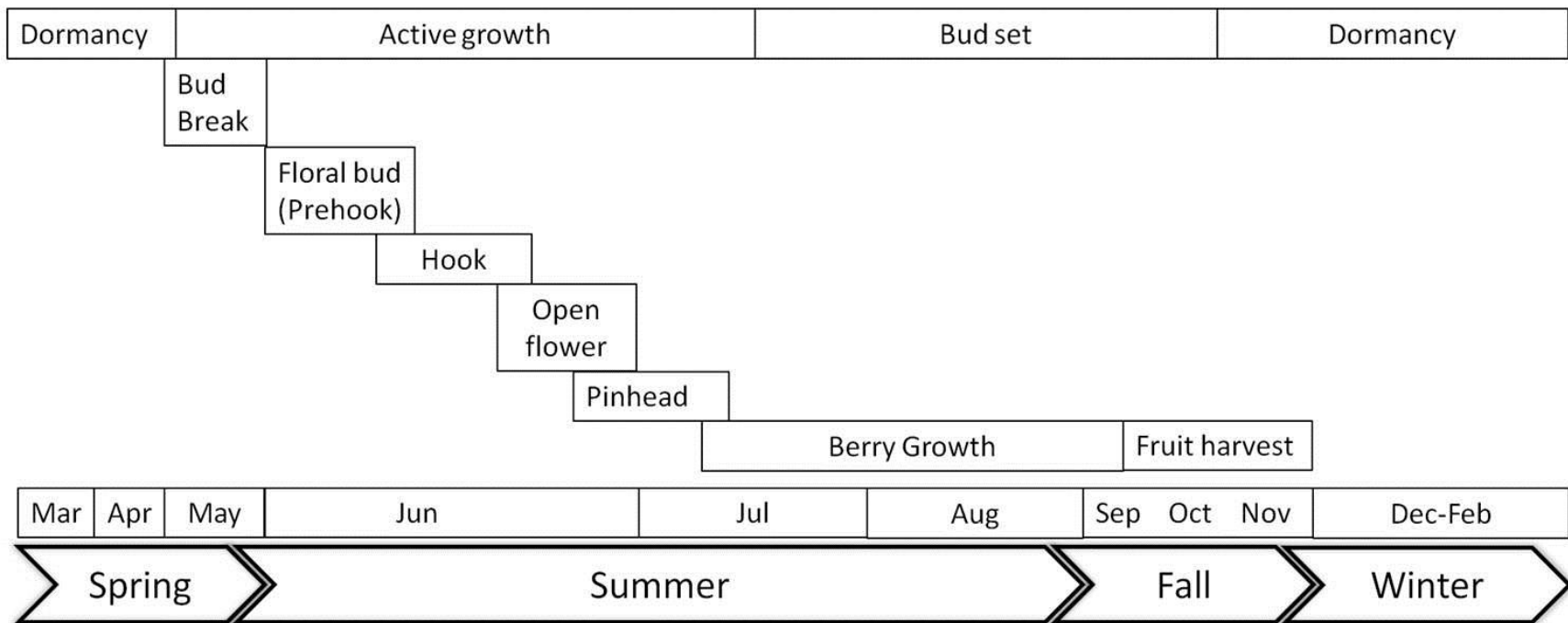


Figure 2. 7 Growing cycle in cranberry. It consists in two phenomena occurring parallel. The upper portion illustrates the growing and set cycle occurring in the apices of the uprights. At bud set time, the floral initials for the following growing season start to be differentiated. The second set of events is illustrated in the diagonal section and is occurring specifically in reproductive uprights. In an alternate bearing cultivar, during bud set, floral initials only differentiate in buds from vegetative uprights. In contrast, in non-alternate bearing cultivars the differentiation of floral initials occurs in apical buds from both types of uprights, vegetative and reproductive.

Chapter 3

**Transcriptome analysis of apical buds from fruiting and vegetative
uprights before, during and after floral initiation in an alternate
bearing cranberry**

3.1 Abstract

Alternate bearing and flowering are two correlated highly processes that affect fruit cranberry (*Vaccinium macrocarpon* Ait.) production. Fruit production in cranberry depends on the capability of the individual stems (uprights) to set apical buds with floral initials (reproductive buds) that will grow as fruiting uprights the following season. However, the molecular mechanisms behind reproductive bud production remain unknown. With the goal to determine candidate metabolic pathways, regulatory mechanisms, or plausible genes involved in floral induction in cranberry, a transcriptome was generated from reproductive and vegetative buds in three stages of floral induction (before, during, and after). The presence of genes involved in the five known flowering pathways (photoperiod, vernalization, autonomous, phase change and gibberellic acid) was analyzed. Histological analysis of buds to determine the three stages of floral induction is shown as well as the expression pattern of genes presumed to be involved in flowering. Our transcriptome analysis revealed that homologs of genes from flowering pathways were identified in cranberry transcriptome from both types of buds. Most of the genes related to these pathways were equally expressed in reproductive and vegetative buds through the floral inductions stages. Finally, enrichment analysis revealed few genes with differential expression in reproductive buds compared to vegetative buds, but suggests that the gibberellic acid pathway could be involved in flowering induction in cranberry.

3.2 Introduction

Similar to many woody fruit crops, cranberry (*Vaccinium macrocarpon* Ait.) possesses a natural tendency towards alternate bearing. This phenomenon is characterized by the alternation of high fruit production years (known as “on” years) and a low production year (“off” years). This alternate bearing tendency is highly correlated with the capability of cranberry plants to bloom. The mechanism itself is still poorly understood, although it has been found that factors such as the nutritional status (carbohydrate content) and plant growth regulators can affect the flowering process, and hence, fruit production (Rosecrance *et al.*, 1998; Smith and Samach, 2013; Yanik *et al.*, 2013).

Several techniques have been tested to reduce the alternate bearing tendencies in several crops by controlling the physiological mechanisms involved. For example, in apples and mandarins pruning, girdling or the application of growth regulators has been used to reduce alternate bearing (Samach and Smith, 2013). Unfortunately, there are no methods that can guarantee a yearly and stable fruit production in most woody fruit crops, including cranberries (Smith and Samach, 2013).

Until now, the only option to overcome alternate bearing in cranberry has been the cultivation of “re budding” varieties. These varieties set an apical bud with vegetative and floral meristems called reproductive buds (also known as mixed buds) every year. These reproductive buds will grow as fruiting uprights the next season, guaranteeing stable fruit production through the years.

In spite of the importance of rebudding, little is known about the genes that govern the flower induction and initiation process in apical cranberry buds.

The flowering process has been extensively studied in *Arabidopsis*, revealing five major pathways (photoperiodism, vernalization, gibberellic acid, autonomous, and phase transition) of genes whose expression is important for the transition of meristems to flower production (flower induction stage), and then subsequently to induce histological and morphological changes (flower initiation stage) that will culminate in flower differentiation and development (Hanke *et al.*, 2007; Koutinas *et al.*, 2010)

The genes activated and expressed to give rise to flowers have been classified in 4 main groups, based on the stage in flower development they act. These groups are: flowering regulator genes, floral pathway integrators, floral meristem identity genes, and floral organ identity genes (also called ABCDE genes). (Khan *et al.*, 2013; Tan and Swain, 2006; Wigge *et al.*, 2005; Wilkie *et al.*, 2008). Flowering regulator genes such as *CONSTANTS* (*CO*) and *FLOWERING LOCUS C* (*FLC*), positively or negatively regulate the expression of the floral pathway integrators: *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANTS 1* (*SOC1*), and *LEAFY* (*LFY*). The activation of the floral pathway integrators, induce the expression of floral meristem genes responsible for the transition from a committed vegetative meristem to an inflorescence meristem. Such genes are *APETALA 1* (*AP1*), *FRUITFUL* (*FUL*), *CAULIFLOWER* (*CAL*), *LEAFY* (*LFY*), and *SEPALLATA* (*SEP*). The flowering pathway ends with the activation of floral organ identity genes (ABCDE genes), responsible for the

differentiation and development of the floral organs (Khan *et al.*, 2013; Tan and Swain, 2006; Wigge *et al.*, 2005; Wilkie *et al.*, 2008).

In annual plants such as *Arabidopsis*, flowering occurs continuously through the flowering process from the induction stage to floral organ specification stage (page 11). In temperate perennial plants, flowering is a more complex process since the flower induction and initiation stages are separated from the flower differentiation stage by a dormancy period and also influenced by interactions among vegetative growth and flower and fruit production of previous years. Examples can be found in apple, citrus, and cranberry (Hanke *et al.*, 2012; Shalom *et al.*, 2012; Wilkie *et al.*, 2008). Specifically in cranberry, floral induction occurs in apical buds during late summer and undergoes dormancy before continuing the flowering process the following season in late spring. Cranberry buds require a minimum of 800 hours of chilling to break winter dormancy (Vorsa and Johnson-Cicalese, 2012).

In perennial plants, such as *Arabis alpina*, and woody plants, orthologs to several *Arabidopsis* floral genes have been found. For example, orthologs to *FLC*, *FLOWERING LOCUS T (FT)*, *LEAFY (LFY)*, *TERMINAL FLOWER 1 (TFL1)*, and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANTS 1 (SOC1)* have been found in *Arabis alpina*, poplar, orange, mandarin, apple, pear, and grapevine (Andres and Coupland, 2012; Hanke *et al.*, 2012; Tan and Swain, 2006). For closely related species to cranberry, few studies have been conducted to study flowering genes. Borchert *et al.* (2009) used RT-PCR analysis to identify orthologs of the *Arabidopsis* genes *AP3*, *SEP1* and *SEP2* in *Calluna vulgaris*, an ornamental species from the Ericaceae family. In

the *Vaccinium* genus, the only study of Song et al (2013) has reported the identification and functional analysis of an homolog to *FT* in blueberry (*VcFT*. Song, *et al.*, 2013). The analysis of the cranberry transcriptome through RNA sequencing (RNA-seq) will provide additional insights and more comprehensive information on gene expression during the flower induction and initiation process.

New technologies for genetic analysis developed subsequent to Sanger sequencing are now called next generation sequencing (NGS). These technologies are based on diverse techniques involving DNA or cDNA preparation, sequencing, imaging, alignment and assembly (Metzker, 2010). NGS permits efficient and economic generation and analysis of massive amounts of sequences from DNA or RNA. This data can be used to discover genes that are expressed in a specific stage of plant development or identify polymorphisms that can then be used as markers in breeding programs. Initially the application of NGS technologies in cranberry has allowed the development of a limited number of microsatellite markers (Georgi *et al.*, 2011; Zalapa *et al.*, 2012; Zhu *et al.*, 2012) that have been useful in establishing fingerprinting techniques to discriminate cranberry cultivars and to determine inter and intra-cultivar variation (Fajardo *et al.*, 2012). Subsequently, NGS studies have resulted in the reconstruction of the complete mitochondrial and plastid genomes (Fajardo *et al.*, 2013, 2014) and a nuclear genome scaffold assembly (Polashock *et al.*, 2014). These genomic resources have also been used to develop a substantial number of molecular markers for mapping studies and for analyzing genetic diversity (Schlautman, *et al.*, 2016, 2015a, 2015b).

NGS transcriptome analysis or RNA-seq has rapidly become an essential tool to efficiently analyze transcriptomes in plant species for the discovery of genes involved in different processes (Haas and Zody, 2010). Reference transcriptomes have been assembled and used as tools to identify molecular markers for breeding purposes for multiple crop species including rice (Bao *et al.*, 2015), sugar beet (Fugate *et al.*, 2014), coconut (Fan *et al.*, 2013), false flax (Liang *et al.*, 2013), and cotton (Bowman *et al.*, 2013). Transcriptomic studies have also facilitated the discovery of genes involved in stress responses in cotton and petunia (Bowman *et al.*, 2013; Villarino, 2014), dormancy in leafy spurge (Horvath *et al.*, 2008), cold acclimation in blueberry (Rowland *et al.*, 2012) and fruit development in blueberry and chili pepper (Martínez-López *et al.*, 2014; Rowland *et al.*, 2012). Regarding the Ericales, in blueberry Rowland *et al.* (2012) used RNA-seq to generate a collection of transcriptome sequences from four different stages of fruit development, buds and leaves of commercial highbush blueberry (*Vaccinium corymbosum* L.) that could be used as a base for obtaining molecular markers in blueberries and related species. Recently, a cranberry reference transcriptome has become available (Polashock *et al.*, 2014) and a transcriptome analysis of cranberry fruits was performed in order to identify genes in the flavonoid biosynthesis pathway (Sun *et al.*, 2015).

Studies on the transcriptome of woody fruit crops are limited, especially those studies aiming to understand the molecular mechanisms behind alternate bearing and flowering in general. For example, Yanik *et al.* (2013) used RNA-seq and found higher levels of expression of specific miRNAs such as miR159 and miR164 in samples of olive during “off” years than in “on” years. In the same study, other expression of other miRNAs (miR166 and miR396) showed to be high only in samples fruiting (“on” year). However, further studies need to be performed to clarify the

role of specific genes in the alternate bearing process in olive. By combining transcriptome analysis and real time RT-PCR analysis, Zhou *et al.* (2013) studied apical buds during floral induction in reblooming and non-reblooming cultivars of peony tree and identified a subset of genes orthologous to known *Arabidopsis* genes such as *FT*, *VERNALIZATION INSENSITIVE 3 (VIN3)*, *CO*, and *GIBBERELIN 20 OXIDASE (GA20OX)*, *PsVIN3*, *PsCO* and *PsGA20OX*, which could be involved in the regulation of the reblooming processes. Transcriptome analysis of young inflorescences of *Tapiscia sinensis*, an endangered tree native from China, revealed genes involved in flowering such as *SOC1*, *FLC*, *FKF1 (FLAVIN-BINDING, KELCH REPEAT, F-BOX)*, and *EARLY FLOWERING 4 (ELF4)*. Zhou *et al.*, 2015).

In order to learn about the genetic mechanisms governing floral induction in cranberry, an alternate bearing cultivar (Ben Lear) was used for transcriptome analysis of apical buds from vegetative and fruiting (reproductive) uprights in three stages of floral induction: before, during and after. We hypothesized that genes differentially expressed in reproductive buds, compared to vegetative buds, could be involved in flowering induction. The transcriptome of apical buds is presented as a reference tool for future studies as well as genes with different expression patterns throughout three floral induction stages.

3.3 Materials and methods

3.3.1 Plant Material. Using a Ben Lear cranberry commercial bed after deicing, a pilot sampling collection was performed in order to determine bud sizes for all fruiting versus vegetative

uprights within a square foot plot. Based on this sampling, buds that had a width of 0.7 mm or less were designated as narrow size buds and wide buds were determined to have a width of 1.5 mm or more. Dormant uprights with narrow or wide buds (presumably without or with floral meristems, respectively) were differentially tagged with zip ties in three areas of one square foot in each of three distinctive zones of the bed. Square foot areas were labeled from 1 to 9, from which the first three (1 to 3) corresponded to areas delimited in one side of the bed, 4 to 6 to the middle zone and 7 to 9 to the opposite side of the bed. Subsequently, 67 uprights with narrow buds and 67 with wide buds were labeled per square foot area. Approximately, 1200 individual uprights (600 with narrow bud and 600 with wide buds) in total were labeled and evaluated.

Leaf samples collected randomly from each of the square foot area per zone were fingerprinted using SSR markers (Fajardo *et al.*, 2012) to verify cultivar identity throughout the bed. With the purpose of corroborating the nature of each apical bud (with or without floral initials), a representative sample of 20 dormant buds of each type (narrow vs. wide) from the 1200 previously tagged uprights were collected and prepared for histological sectioning following the methodology previously described in Chapter 2. The resulting sections were observed under light microscopy to evaluate the absence or presence of floral initials. The remaining tagged dormant uprights were left in the field to allow apical buds to grow as uprights from which phenotypical data and new buds were collected over one month during development.

3.3.2 Phenology. Once the remaining labeled buds grew as new uprights, phenological data was collected weekly from 315 fruiting uprights (35 per square foot) from June 5th to August 7th and

one additional data collection date before fruit harvesting (Sep 11, 2014). Each data collection date was labeled as weeks 1 to 10 as follows: Week 1: June 5th, week 2: June 12th, week 3: June 24th, week 4: July 2nd, week 5: July 10th, week 6: July 17th, week 7: July 24th, week 8: July 31st, week 9: August 7th, and week 10: Sep 11th.

The phenological data was recorded as the number of uprights that were in each of the following phenological stages: Stage I: prehook, Stage II: hook, Stage III: open flower, Stage IV: pinhead, Stage V: berry (green and red berries). Additionally, percentage of vegetative and fruiting uprights developed from narrow or wide buds was recorded to confirm percentage fate as vegetative or fruiting uprights. These data provided insights regarding the homogeneity of dormant buds samples collected at the beginning of the experiment.

In order to confirm the alternate bearing nature of Ben Lear cultivar, 200 vegetative and 200 fruiting uprights were evaluated for bud size at the time of harvest. Uprights were also evaluated for the type of upright they were in the previous growing season based on the presence or absence of pedicels.

3.3.3 Data analysis. All statistical analysis was performed using R environment (R Core Team, 2013). In order to determine if there were differences in the development of the uprights throughout the Ben Lear bed, and the data was analyzed using generalized additive model (GAM). The model used to analyze the data was:

$$\text{Log}(x) = f_{\text{site } 1}(\text{week}) + \dots + f_{\text{site } 9}(\text{week})$$

Where the Log (x) represents the Log of counts of uprights per phenological stage, $f_{\text{site}}(\text{week})$ corresponds to the smoothing function of the data collected per each site (9 sites) across the weeks.

Pairwise comparisons (with Bonferroni corrections) among all the 9 squares delimited in the bed were performed for each of the 5 phenological stages evaluated. In each comparison, the overlapping of the confidence intervals was evaluated, and a value of proportion on non-overlapping area was obtained. This value is an approximation to a p-value; therefore, it was considered that the uprights from two squares were significantly different in development if the pairwise comparison produced a non-overlapping area value lower than 0.05. Complete overlapping of the curves was considered when the value was 1.

3.3.4 Collection and selection of bud sets samples for transcriptome analysis. From preliminary experiments done to determine the minimum number of buds to extract at least 1ug of total RNA for transcriptome analysis, it was determined that a minimum of 25 mg, corresponding to 25 buds with 1 mm of length (from the tip of the most external and upper bud scale) was sufficient to extract good quantity and quality of total RNA.

Once flowering was the most prominent phenological stage in the fruiting uprights, apical buds (1mm length from the tip to the bud down) were collected weekly from 25 fruiting and 25 vegetative uprights across the foot squares per zone of the bed. In order to collect bud samples that were developmentally similar, buds were collected from only the fruiting uprights that were in the most predominant phenological stage evaluated in each week. Only buds from vegetative uprights that grew exclusively from the apical bud (not from axillary buds) were harvested.

Each set of 25 buds derived from each zone was considered a biological replicate, and a total of three biological replicates were collected. Each replicate (25 buds) was kept in RNA later® (Invitrogen) in a 1.5ml centrifuge tube at room temperature immediately after collection and subsequently kept overnight at 4°C. After that, and in order to facilitate tissue grinding, buds were removed from RNA later, blotted to remove the excess solution and immediately transferred to new tubes, immersed in liquid nitrogen and stored at -80°C until the RNA was extracted.

An extra sample of 10 apical buds per upright type per week of collection was harvested and used in the microscopy studies discussed in Chapter 2. These analyses helped to verify the classification of the three sets of buds per upright type from which the RNA was extracted and the RNA-seq analysis performed. From the morphological study in Chapter 2, the floral initials were observed only in buds from vegetative uprights collected in week 5 (July 10th). Based on these results and with the purpose of identification of genes upregulated during floral initiation,

three sets of buds per upright type were selected for RNA-seq analysis. The sets selected were from: week 2 (before floral induction), 4 (during floral induction), and 6 (after floral induction).

3.3.5 RNA extraction and experimental design. RNA extraction was performed using a modified protocol from Yockteng et al (2013). We used only 25mg (coming from the 25 buds) per set rather than 100mg; all the original volumes of the reagents suggested by Yockteng et al (2013) were used. The quantity and quality of the RNA extracted was measured using Experion (BioRad Technologies) following manufacturer instructions. Samples with a RIN number equal to 7 or higher were used for further analysis.

The experimental design was a balanced incomplete block multiplexing, with three biological repetitions (zones in the bed) per time of bud collection (before, during and after floral induction) and type of upright (vegetative and fruiting), for a total of 18 samples analyzed (Figure 3.1) This design was similar to Auer and Doerge (2010). Each biological replicate per upright type (vegetative and fruiting) and per time of harvesting (before, during, after) was constituted of 25 buds from uprights growing in each of three distinctive zones in the cranberry field. A total of 18 samples were collected and analyzed through RNA-seq.

3.3.6 Library construction. *rRNA depletion and quality control.* Total RNA obtained from each of the 18 samples was sent to Cofactor Genomics (<http://cofactorgenomics.com>, Saint Louis, Missouri, USA) for sequencing and further analysis. Briefly, rRNA-probes were hybridized to total RNA for removal of ribosomal RNA from the sample. Ribo-depleted RNA was then

fragmented prior to cDNA synthesis using random primers. Double-stranded cDNA was end-repaired and polyA-tailed to prepare for adaptor ligation. Indexed adaptors were ligated to DNA, and the adaptor-ligated DNA was amplified by PCR. cDNA libraries were DSN treated. Library size and quality was assessed on an Agilent Bioanalyzer and library yield was quantified by qPCR using the Kapa Biosystems (Willmington, MA) library quantification kit. RNA-seq was performed in 2 lanes from a flow cell using Illumina NextSeq 500. The samples were distributed in the cell such that one biological replicate of one floral induction stage was represented in at least one of the lanes, (Auger and Doerge 2010).

Raw sequence data were assessed for quality in Fastq format (FastQC, <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and ribosomal RNA content, and then adapter- and quality-filtered. Phred-like quality score (Qscore) was generated by Real Time Analysis software (RTA) during the sequencing. All libraries had a Qscore >30 and length >50bp (Q30L50).

3.3.7 Transcriptome assembly. *De novo* assembly of the transcriptome was carried out on a DSN treated library of pooled samples using Trinity Suite (trinityrnaseq_r20140413p1., Grabber *et al.*, 2011). The paired end reads were adapter trimmed with Trimmomatic using TruSeq v3 adapter filtering. Trinity was run with the following parameters in addition to the defaults: --SS_lib_type RF, --trimmomatic, --min_kmer_cov 2, --JM 200G, resulting in transcript models for alternatively spliced isoforms. (<https://github.com/trinityrnaseq/trinityrnaseq/wiki>).

Alignment. Samples were next aligned to the assembly with NovoAlign (Version 3.02.05. Novocraft, <http://novocraft.com>) using the following parameters: -i 150 30 -t 20,3 -a -r all -e 100 --hlimit 8. Parameters were trained to maximize sensitivity while maintaining the highest specificity for this dataset. To allow unbiased representation of alternative isoform expression, alignments were allowed to map to multiple transcripts

Functional Annotation. For annotation, Gene Ontology (GO) analysis was performed using Blast2GO (Conesa *et al.* 2005) and Cluster of Orthologous Groups (COG) with an e-value $< 1 \times 10^{-10}$.

Differential expression analysis. Each transcript's read counts were normalized to the length of the transcript to allow equal comparison of expression levels between transcripts. To allow equal comparison of expression levels between samples, each sample's counts for a given transcript were linearly normalized to the mean total reads across all samples. This normalized expression data is the basis for the expression comparison.

To performed Differential expression analysis was performed to determine how the expression of the genes changed through the floral induction stages. Statistical differential expression between consecutive floral induction stages was determined. For buds from both fruiting and vegetative uprights, comparisons between before vs. during, and during vs. after, were computed. For each comparison, in order to determine if the expression levels of a gene were significantly different

between two floral induction stages, with a false discovery rate (FDR) of 0.2, we calculated the $\log_2(B/A)$, where B was the following stage after stage A. In addition, when B was smaller than A, the result of $\log_2(B/A)$ was set negative, and the opposite when B was larger. After this, each gene had two numerical values explaining the dynamic changes in expression during bud development.

Subsequently, the expression changes of each gene was classified in nine pattern expression categories (according with the two calculated values) as follows: SS, SI, SD, ID, IS, II, DS, DI, DD, where the first letter denotes the change from before to during floral induction, and the second letter denotes the change from during to after floral induction. Letters I, S and D indicate increase, steady (for non-significant change) and decrease, respectively. The pattern of gene expression categories were as follows: SS, steady between the three floral induction stages; SI, steady before and during floral induction and then increased in ‘after’ floral induction; SD, steady in the first two floral induction stages (before and during) and decreased after floral induction; ID, increased during floral induction, compared to its expression before floral induction, and then decreased after floral induction; IS, increased in the first two stages of floral induction and steady in the third stage; II, increased through the three induction stages; DS, decreased from before to during induction stage and steady from during and after floral induction; DI, decreased from before to during induction stage and increased after flower induction; and DD, decreased throughout the three stages of floral induction.

Additionally, we made an overall comparison between reproductive and vegetative buds using all the stages as repetitions (F-test). In this case, we proceeded to calculate the $\log_2(A/B)$ where A was the mean expression of reproductive buds, and B the mean expression of the vegetative buds. The previous formula was computed only when statistical differences were found. If no differences it was set to 0 (or S). Similar to the use of patterns identified for differential expression, an S was used to denote non-difference in expression between vegetative and reproductive buds, an I to denote an increment in expression in reproductive compared to vegetative buds, and a D to denote a decrease in expression.

Once the genes were classified in the described categories based on their expression patterns and in their upright origin (vegetative or fruiting uprights), we used a basic local alignment search tool (BLASTx) to compare the cranberry genes with 179 *Arabidopsis* genes known to be involved in the different flowering pathways in order to identify orthologues of flowering genes in the cranberry transcriptome. Parameters as percentage of similarity higher than 95%, length of similarity equal or bigger than 100 base pairs (bp) and an e-value smaller than 1×10^{-10} were used to find the orthologues. A comprehensive list of *Arabidopsis* flowering genes names and their respective roles in the flowering pathways was found at Max Planck Institute for Plant Breeding Research (http://www.mpipz.mpg.de/14637/Arabidopsis_flowering_genes). 75 of the genes in the list are known to be involved in the photoperiod pathway, 49 in the autonomous and vernalization pathway, 25 in the gibberellic acid pathway, 18 in phase change, and 28 genes in meristem response and development. Additionally, flowering gene sequences have previously been identified and are available from the National Center for Biotechnology Information (NCBI), for many additional species: *Arabis alpina* (11 genes), apple (40 genes), Citrus (16

genes), pear (7), peach (5), mango (7), kiwi (40), rhododendron (4) and grape (10). These genes were also used for BLAST searches.

3.4 Results

3.4.1 Verification of Ben Lear cultivar in the bed.

In order to confirm that all the plant material used in both field experiments and for microscopy studies were true to cultivar, leaf samples from each square foot area were evaluated to confirm the genotype of the cultivar (Ben Lear). All fingerprinting results confirmed that the plant materials labeled and subsequently collected were true-to-predicted genotype.

3.4.2 Microscopy of wide and small dormant buds tagged in the field.

Longitudinal sections of apical buds revealed that the two types of buds (vegetative and reproductive) have a similar number of bud scales, and significant differences in bud size- based on the presence or absence of floral meristems. All the narrow buds sectioned presented a pointy and compact arrangement of bud scales protecting the shoot apical meristem (SAM), and they were difficult to hand-section. In wider buds, which tended to be rounded, floral meristems differentiated in between bud scales and were protected by bracts (Figure 3.2). None of the sections of narrow buds showed visible floral meristems, and in contrast, at least one floral meristem was observed in longitudinal sections of wide buds.

3.4.3 Phenology.

Once the buds grew through 5 phenological stages of bud development, as described by Workmaster *et al.* (1997), the fruiting uprights were evaluated (Figure 3.3). The phenological stages were determined based on the developmental stage of the floral buds from the first two positions, acropetally counted. *Stage I* also called floral bud or prehook, was characterized by the upward orientation of the floral buds in the first two positions. When floral buds became oriented downward due to elongation and bending of the pedicel, the upright progressed to the hook stage (*Stage II*). Subsequently, the floral buds developed into fully open flowers (*Stage III*), with petals expanded and upside bended and visible androecium ring surrounding the gynoecium. Once the flowers passed the fertile stage and the petals and androecium abscised, the uprights reached the pinhead stage (*Stage IV*). If the flowers were pollinated and successfully fertilized, the ovary began to swell and transition to fruit growth and ultimately maturation (*Stage V*). It was observed that the fruiting uprights moved from stages I to IV (prehook, hook, open flower, and pinhead) weekly, and the remaining of the season the uprights held berries (*Stage V*) until maturation and fruit harvesting (Figure 3.3).

In order to determine if the uprights of Ben Lear growing in different zones of the bed developed similarly, pairwise comparisons among the square areas were done. The results gave insights about the different developmental and physiological stages of the apical buds among biological replicates. In general, there were not significant differences among the developmental stages of the uprights within the 3 zones in the bed of Ben Lear (approximate p-value > 0.5).

Based on the counting of reproductive uprights in each phenological stage, two phenological stages predominated weekly up to week 6 of observations (July 17th). Thus, for week 1, most of the uprights were in prehook stage (Stage I), by the following week (week 2) prehook and hook stages (Stages I and II. Figures 3.4 and 3.5). During week 3, the predominant stages were shared between Stages II and III (hook and open flower. Figures 3.5 and 3.6). In week 4, open flower and pinhead (Stages III and IV) predominated (Figures 3.6 and 3.7); whereas, by week 5 pinhead and berry were the most representative stages (Stages IV, and V. Figures 3.7 and 3.8). The following weeks (week 6 to 10) Stage V (berry) prevailed throughout the bed (Figure 3.8).

The confidence intervals per individual square foot area in each phenological stage, generated from the analysis, is represented as a grey area around the curves (Figures 3.4 to 3.8). In general, confidence intervals were narrow; however, in few occasions expanded. This result agrees with the approximate p-values observed in the matrix generated from the pair comparisons for each phenological stage. (Supplementary Tables 3.1 to 3.5). Approximate p-values higher than 0.05 indicated no significant difference in the phenological stage evaluated in a pairwise comparison. Thus, in this study, approximate p-values oscillated between 0.4 and 0.9 indicating that there were no significant differences in the phenological stages among square foot areas and also across zones in the beds.

Even though there were no significant differences in the number of uprights in each of the phenological stages evaluated, since the approximate p-value was 0.5 or higher; there were still some differences between the zones. When prehook stage (Stage I) was predominant, the

approximate p-values revealed that uprights from the square area 7 (zone 3) of the bed, was slightly different than the uprights sample in the other 8 square foot areas. (Supplementary Table 3.1)

In the following phenological stages, the pairwise comparisons for the hook stage (Stage II) , suggests that the square 1 from zone 1, and square 6 from zone 2 were slightly different from the other areas (Supplementary Table 3.1 and 3. 2) with approximate p-values between 0.4 and 0.6. For uprights flower stage (Stage III), the lowest approximate p-value, 0.6, was found only when the square 2 was compared to square 7 (Supplementary Table 3.3). When uprights were in the pinhead stage, phonological stages were slightly different in square 5 than the remaining squares (approximate p-values=0.5); otherwise, all square foot areas were highly similar (approximate p-values between 0.8 and 0.96. Supplementary Table 3.4). For the comparisons when berry stage (Stage V) was predominant, the lowest approximate p-values oscillated between 0.62 and 0.68 when square 2 was compared to squares 3 to 7 (Supplementary Table 3.5).

Previous year upright type and current bud set. Data collected from the 200 vegetative and 200 fruiting uprights, showed that 85% of the fruiting uprights were vegetative and just 15% were fruiting in the previous season. In contrast, 85% of the vegetative uprights were reproductive the previous season and 12% were vegetative. Regarding bud set, most of the fruiting uprights (60%) set narrow buds for the following year; whereas vegetative uprights (50%) set wide buds.

In 17% and 10% of the fruiting and vegetative uprights, respectively, apical buds had a medium size and not possible to categorize as wide buds (presumably with floral initials) or narrow buds. Some uprights (8 and 10%) showed damage in the apical bud, therefore it was not possible to classify in bud size categories.

3.4.4 Floral induction stages for transcriptome analysis.

Transcriptome analysis of buds from vegetative and reproductive uprights in three stages of floral induction was performed. The floral induction stages (before, during and after) were determined based on histological studies compiled in Chapter 2, and depicted in Figure 3.9.

The selection of the stages was done based on the date when floral initials were observed for the first time in longitudinal section of buds from vegetative uprights. Previously, when initials were observed, July 24th, fruiting uprights were in berry stage (Stage V). Taking this into consideration along with the rationale that the floral induction probably occurred before signs of the initials were evident, buds were collected a week before, July 18th, and represented the ‘during floral induction stage’. Additional buds samples were collected 2 weeks before (July 2nd) to represent the ‘before floral induction stage’ and buds collected 2 weeks later (July 31st) represented the “after floral induction stage”. By the time the buds for stages ‘before’ and ‘after’ floral induction were selected, fruiting uprights were predominantly in pinhead stage (Stage IV) and berry (Stage V. Figure 3.9).

3.4.5 Transcriptome sequencing and *de novo* assembly.

We constructed 18 cDNA libraries from RNA extracted from buds of vegetative and fruiting uprights from three floral induction stages and three biological replicates. The total number of reads generated per sample was between 28,121,116 and 38,722,038 nucleotides; from which the total clean reads fluctuated between 12 and 36 million per sample. Within the mRNA reads obtained, 84% to 93% were successfully aligned (Table 3.1)

The software Trinity assembled a total of 344,334 contigs, representing 278.72 Mbp with a GC percentage of 41.27. The N50 was 1.423kb, whose median and average length was of 428 base pair (bp) and 809.46bp, respectively. From the total transcripts (344,334), there were 171,337 expressed in only one of the 18 total biological replicates (3 floral induction stages x 2 upright types x 3 biological rep per stage =18). Therefore, these transcripts were excluded from the gene expression comparisons. The remaining 172,997 transcripts had a mean length of 1.212 Kbp and median length of 813 bp (almost two times bigger than contigs before the filtering), representing 209 MB. Using only the largest isoform per gene, there were 99,866 unigenes.

3.4.6 Transcriptome Functional annotation

To identify putative functions and over representation of specific pathways or regulatory mechanism, the unigenes (only the most prevalent isoform per gene) were compared, using BLASTX, against the Cluster of Orthologous Groups (COG) database (Huerta-Cepas *et al.*,

2015), which contains 25 general clusters. Additionally, all the contigs (multiple isoforms per gene) were annotated with their gene ontology terms (GO) using Blast2GO.

COG annotation resulted in 41,973 unigenes assigned to 23 out of the 25 functional groups. The two groups not represented included “Cell motility” (N), and “General functional prediction only” (R. Figure 3.10). The largest group was “Unknown function” (S) with 13500 unigenes, representing 32% of the annotated unigenes. “Signal transduction mechanism (T) was the next most abundant with 4188 unigenes (10%). Additional groups with significant representation included: O (“Post-translational modification, protein turnover, chaperones”) with 3745 genes (9%), K (“Transcription”) with 3139 genes (7.5%) and G (“Carbohydrate transport and metabolism”) with 2728 genes (6.5%).

Other groups represented by fewer than 2000 unigenes, yet higher than 1000 unigenes included J (“Translation”) with 1800 unigenes (4.3%), U (“Intracellular trafficking and secretion”) with 1735 (4.1%), Q (“Secondary structure”) and C (“Energy production and conversion”) each contained about 1500 unigenes (3.6 %), A (“RNA processing and modification”) with 1304 unigenes (3.1%). Groups P (“Metabolism”), I (“Lipid metabolism), and E (“Amino acid metabolism and transport”) contained 1200 unigenes each (representing 3.6% of the genes in each group), and group L (“Replication and repair”) with 1156 genes (2.8%).

Grouping fewer than 1000 unigenes were also observed. Groups Z (“Cytoeskeleton”), M (“Cell wall/membrane biogenesis”) and D (“Cell cycle control, cell division and chromosome partitioning”), contained 732, 602, and 532 unigenes, respectively (corresponding to 1.2, 1.4, and 1.7%). Last, 1% or less of the annotated unigenes were assigned to groups H (“Coenzyme metabolism”, 459 unigenes, representing 1%), B (“Chromatin structure and dynamics”, 396 unigenes, 0.9%), F (“Transport”, 346 unigenes, 0.82%), V (“Defense mechanisms”, 329 unigenes, 0.8%), W (“Extracellular structures”, 136 unigenes, 0.32%), and Y (“Nuclear structure”, 100 unigenes, 0.24%) .

Related to the GO annotation, 82,847 transcripts (out of 172,997) were annotated with one or more GO terms. As observed in Figure 3.11, the more predominant subcategories in the biological processes were cellular, metabolic, single-organism process, followed by response to stimulus and biological regulation. Within molecular function category, binding and catalytic activity were represented with 19% of genes in these subcategories; and in cellular components, cell, organelle and membrane were the most predominant subcategories with 52, 41 and 22 % of the genes, respectively.

3.4.7 Differential expression analysis in vegetative and fruiting uprights

Based on the gene expression in buds from vegetative and fruiting uprights, genes were classified in three main categories: steady, increased and decreased. Since the comparisons were

made between consecutive stages, an additional type of pattern, N (for unknown), was added for the genes which had incomplete data for pairs at contiguous stages. For example, if a gene had information for the stages before and after, but not for during, comparisons were not performed. In addition, for the overall comparison between reproductive and vegetative uprights, the same criterion was used. Most of the genes COG-annotated (31,911, equivalent to 75%) did not show any difference in expression in vegetative and fruiting uprights (overall comparison), and were denoted as S (steady) (Figure 3.12). The following category was formed by genes with decreased expression in buds from vegetative uprights compared to fruiting uprights (D) (4632 unigenes) and represented 11% of the annotated genes. Also, 3739 unigenes (9%) whose expression was not able to be estimated, due to lack of data in some samples, were placed in the unknown category (N). The smallest category belongs to the 2311 genes (5%) upregulated in buds of vegetative uprights (I) (Figure 3.12).

3.4.8 Changes in gene expression through three floral induction stages in buds from vegetative and fruiting uprights

3.4.8.1 Vegetative uprights. From the 9 possible gene expression categories, 8 were represented in this study. A total of 56% of the genes (23,496 unigenes) had no differences in expression through the floral induction stages, therefore they were grouped in the category SS (steady-steady). The pattern NN was the following most represented category with 7627 unigenes (18%. Figure 3.13A).

Descending in the expression categories, based on the number of genes assigned, the categories IS contained 6587 genes (16%) and DS with 3604 genes (8.6%). The remaining categories represented 1% or less of the annotated genes. Thus, SD had 446 genes (1%); meanwhile, ID category grouped 81 genes (0.2%), and SI category with 123 genes (0.29%. Figure 3.13A). In contrast, no genes were found in the category II, and just 9 genes (0.02%) showed a DI pattern.

3.4.8.2 Fruiting uprights. In contrast to buds from vegetative uprights, genes expressed in buds from fruiting uprights were assigned to four categories out of the possible 9. The highest number of genes (32860 unigenes, 78%) had a constant expression and was grouped in the SS category. It was followed by the unknown category with 7939 genes (0.19%), IS category with 977 unigenes (2.33%) and DS with 197 genes (0.5%. Figure 3.13B).

3.4.9 Comparative distribution of gene expression in floral induction stages between buds from vegetative uprights and fruiting uprights

The comparison in the change of gene expression categories from buds of vegetative uprights and fruiting uprights is illustrated in Table 3.2. There were no genes found in buds from either types of uprights (vegetative and fruiting) that represented the expression categories II or its opposite DD. In contrast, the majority of the genes (88 to 100%) expressed in buds from vegetative uprights and grouped in the different expression patterns, was kept in the SS category in buds from fruiting uprights.

The 6587 genes expressed in the category IS in buds from vegetative uprights were represented in 4 categories in fruiting uprights; 478 genes (7%) were similarly expressed in fruiting uprights; 5 genes (0.1%) in DS category, 176 genes (3%) were in the unknown category, and the majority of the genes (5928 or 90%) fell in the SS category in fruiting uprights. 10 unigenes (12.4%) and 71 unigenes (88%) expressed in ID category in vegetative uprights fell in the IS and SS categories, respectively, in fruiting uprights. From the SI category in vegetative uprights, 115 unigenes (94%) were SS in fruiting uprights, and 4 unigenes (3%) in DS and unknown categories in fruiting uprights.

Genes from the SS category in vegetative uprights were present in 4 categories in fruiting uprights, such as, IS (444 unigenes, 2%), SS (21763, 93%), DS (134 unigenes, 0.57%), and unknown (1155 unigenes, 5%). Similar categories were represented in fruiting uprights in unigenes showing expression patterns of DS and unknown in vegetative uprights, thus genes with expression pattern of DS in vegetative uprights had expression patterns of IS (8, .2%), SS (3422 genes, 96%), DS (45 genes, 1.3%), and Unknown (109 genes, 3%) in fruiting uprights. Genes in the unknown category in vegetative uprights had expression patterns of IS (9 genes, 0.12%), SS (1118 genes, 15%), DS (9 genes, 0.12%), unknown (6491 genes, 85%). All 9 genes with DI expression pattern in vegetative uprights had an expression pattern of SS in fruiting uprights (Table 3.2).

3. 4.10 Enrichment analysis.

Even though there were genes in the expression patterns of ID, SI, SD, DI, in the transcriptome of buds from vegetative uprights, no COG functional classes were significantly overrepresented ($p\text{-value} < 0.05$, hypergeometric distribution) in these categories. COG functional classes were enriched in 4 categories: IS, SS, DS, and unknown in buds from both, vegetative and fruiting uprights (Table 3.3). In most of the cases, it was observed that two pathways were assigned to one gene.

3.4.11 Expression analysis of flowering genes in buds from vegetative and fruiting uprights.

Flowering genes of *Arabidopsis* and perennial species such as *Arabis alpina*, fruit crops (apple, pear, peach, grape, and kiwi) and rhododendron were BLAST against the transcriptome generated in this study in order to identify homologous to flowering genes in cranberry and determined the expression of them during the three stages of floral induction.

Unigenes with homologous sequences to 45 genes (60%) involved in the photoperiod pathway, 24 genes (49%) in the autonomous and vernalization pathways, 7 genes (28%) in the gibberellic pathway, and 7 genes (38%) in the phase change pathway were identified. In addition, homologous unigenes to 8 genes (29%) known to be involved in meristem response and development were identified (Figure 3.14). From the BLAST using flowering genes of perennial and woody species, homologous unigenes were identified for only one flowering gene of each apple (*GalLDH*, coding for L-galactono-1,4-lactone dehydrogenase), citrus (coding for translation initiation factor), and rhododendron (*RpACT*, coding for actin) species.

Based on the gene expression patterns through three stages of floral induction, 30 from the 45 unigenes with homologous in photoperiod, 6 from 7 in the aging, 13 from 24 genes in the autonomous, and 5 from 7 genes in gibberellic pathways showed constant expression across floral induction (SS) in both vegetative and fruiting uprights, with COG pathways J, K, L, O, S and T. Among those genes are: *GIGANTEA (GI)*, *PHYTOCHROME AND FLOWERING TIME REGULATOR (PTF1)*, *PHYTOCHROME A, B, C and E (PHYA, PHYB, PHYC, PHYE)*, *TARGET OF EARLY ACTIVATION TAGGED 2, 3 (TOE2, TOE3)*, *APETALA (AP2)*, *SCHNARCHZAPFEN (SNZ)*, *SCHLAFMUTZE (SMZ)*, *GIBBERELLIN 2-OXIDASE 1, 2, and 8 (ATGA2OX1, ATGA2OX2, ATGA2OX8)* among others. Regarding meristem response genes homologous to *LEAFY (LFY)*, *AGAMOUS-LIKE 20 (AGL20)*, *SEPALATA 3* were constantly expressed. Under this expression pattern category, homologous for one gene of apple, and for rhododendron were observed (Supplementary Table 3.6)

When the expression in buds from fruiting uprights was constant (SS), unigenes homologous to photoperiod pathway genes were identified: *LATE ELONGATED HYPOCOTYL (LHY1)*, *CYCLING DOF FACTOR 5 (CDF5)*, *CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)*, *CONSTANS-LIKE 5 (COL5)*; Autonomous/vernalization pathway: *ASH1 HOMOLOG 2 (ASHH2)*, *RELATIVE OF EARLY FLOWERING 6 (REF6)*, *FY*, *ATX1 (ARABIDOPSIS TRITHORAX 1)*, and gibberellic pathway: *GIBBERELIC ACID INSENSITIVE (GAI)* that showed a decrease in expression from ‘before’ to ‘during’ floral induction stages and then kept steady from ‘during’ to ‘after’ floral induction (DS) in buds from vegetative uprights. No genes

related to aging pathway were represented in this expression pattern. Homologous related to meristem response genes like *SHORT VEGETATIVE PHASE (SVP)*, and *TERMINAL FLOWER 1 (TFL1)* were also in this expression pattern. Additionally, homology for one gene of citrus was observed (Supplementary Table 3.7). In the opposite expression pattern in buds from vegetative uprights (SD), and SS in fruiting uprights, only a unigene homologous to *NUCLEAR TRANSCRIPTION FACTOR Y SUBUNIT B-1/HEME ACTIVATED PROTEIN 3A (NFYB1/HAP3A)* was observed (Supplementary Table 3.8).

In the category where the expression pattern in buds from vegetative uprights was IS but SS in fruiting uprights, a lower number of homologues were identified from photoperiod pathway, such as *CRYPTOCHROME 2 (CRY2)*; from both photoperiod and aging pathway: *TARGET OF EAT 1 (TOE1)*; from autonomous/vernalization pathway: *UBIQUITIN PROTEIN CARRIER 1 (ATUBC1)*, *HISTONE MONO-UBIQUITINATION BINDING 2 (HUB2)*, *EARLY FLOWERING 8 (ELF8)*, *UBIQUITIN-SPECIFIC PROTEASE 26 (UBP26)*; and from gibberellic acid pathway: *GIBBERELLIN 20-OXIDASE 1 (GA20ox)*. No genes related to meristem response fell in this expression pattern (Supplementary Table 3.9).

For the gene expression pattern SS vegetative uprights but DS in fruiting upright, only one homologous to a photoperiod *Arabidopsis* gene, *RAV2* was detected. No other homologous for genes involved in the other flowering pathways were observed (Supplementary Table 3.10). When the gene expression in buds from vegetative uprights was SS and IS in buds from fruiting uprights, there were homologous for one gen related to photoperiod pathway: *CULLIN4 (CUL4)*;

one gene related to both, photoperiod and aging pathway: *SMZ*; two genes related to vernalization/autonomous pathway: *BRASSINOID INSENSITIVE 1 (BRI1)*, and *ENHANCER OF AGAMOUS-4 2 (HUA2)*. No homologous were found in the gibberellic acid pathway nor genes related to meristem response (Supplementary Table 3.11).

Even though homologous sequences to genes related to different pathways: photoperiod, *ABA-RESPONSIVE KINASE SUBSTRATE 1 (AKS1)*; vernalization/autonomous, *REDUCED VERNALIZATION RESPONSE2 (VRN2)*; and meristem response, *AGAMOUS-LIKE 8*, as well as one flowering gene in tomato, no information about pattern expression in the three floral induction stages within buds from each upright type (vegetative and fruiting) were identified due to the lack of information in at least 2 of the biological replicates per floral stage per upright type (Supplementary Table 3.12).

3.5 Discussion

In perennial fruit woody crops, the ability of the plants to induce flowers will directly influence the fruit production for the following season. Despite the fact that the flowering process is widely understood in plant species such as *Arabidopsis*, woody crops face additional layers of complexity for this process, including the alternate bearing are significant juvenility. In addition, fruit crop plants often must go through dormancy to overcome the winter period. In fruit species such as apples, pears, plums and cranberries, among others, the flowering process is briefly arrested as initiation occurs during the previous year and is interrupted by the dormancy period.

Once the environmental conditions are favorable to reassume growth, the flowering process reassumes and ends with floral differentiation (Hanke *et al.*, 2012).

Cranberry fruit production depends on the ability of the uprights to set reproductive buds that contain both, vegetative and floral meristems. Alternate bearing occurs to different degrees in specific cranberry cultivars. Early selections (from late 1800s and earlier 1900s) such as Ben Lear, Early Black, and Howes show strong alternate bearing tendencies, where fruiting uprights set a vegetative bud for the following year, and in contrast, vegetative uprights set a reproductive bud (Vorsa and Johnson-cicalese, 2012). Alternatively, cultivars such as Stevens (a cultivar released in mid 1900s) show a reduced alternate bearing behavior; and they can display both alternate and non-alternate bearing throughout the years (Vorsa and Johnson-cicalese, 2012). Second and third breeding cycle cultivars such as HyRed, show a remarkably capacity to set reproductive buds every year independent of the fate of the upright the previous year. This characteristic guarantees a constant production over the years, and in conjunction with increased plant vigor and fruit traits, make these new released cultivars an attractive option for commercial production of cranberry.

Although alternate bearing is highly influenced by flowering, this latter process is still poorly understood in cranberries. From the evidence collected in field studies and the analyses of carbohydrates, theories such as nutrient partitioning and hormonal control are often used to explain this phenomenon (Rosecrance, *et al.*, 1998; Turktas *et al.* 2013; Roper and Klueh 1996; Hagidimitriou and Roper 1994; Roper *et al.*,1991; Devetter, 2013). However, studies on olives

and citrus, have found that genes related to the flowering processes are also responsible for the alternate bearing phenomenon (Shalom *et al.*, 2012; Yanik *et al.*, 2013) .

Studies related to molecular aspects of the flowering process in cranberry are clearly lacking. This study is the first attempt to understand the expression of floral induction genes in cranberry. In order to accomplish this, and keeping in mind that numerous buds had to be collected in specific phenological stages to extract sufficient total RNA to pursue the transcriptome analysis, histological analysis of apical buds and phenological analysis of uprights in different areas of the Ben Lear plantation (bed) were performed. The analysis began when buds were still in dormancy. The histological analysis from dormant small and wide buds revealed that no floral initials were present in small buds but in wide buds. This finding concurs with previous studies in other cultivars, where differences in bud size coincided with the presence or absence of floral initials (Devetter, 2013). Additional, evidence was given by field evaluation of the uprights grown from field labeled buds from which data was collected through the growing season. Most narrow buds grew as vegetative uprights, whereas wider buds developed into fruiting uprights.

The study of phenological stages in Ben Lear, revealed that in general after reinitiation of growth in the spring, there were two phenological stages present in the field until week 6 when holding berries (Stage V) were observed to be the only phenological stage until fruit harvest. Additionally, fruiting uprights moved from one phenological stage to the following weekly. Similar phenological stages in fruiting uprights of Stevens were determined by Brown and McNeil (2006) in order to determine the influence of flower position and pollinators correlation

to fruit production. Demoranville (1992) studied the nutrients content in uprights at different phenological stages in Early Black. However, previous to the present study, no evaluation of the phenological stages in fruiting uprights through time in a growing season has been reported. Our observations in the uniformity thus are unique and provide evidence of the rapid growing and development of fruiting uprights during a growing season.

Pairwise comparisons through square foot areas and three zones across a Ben Lear plantation revealed great uniformity in development across the bed. While it is true that not all the uprights were in the same phenological stage, two phenological stages were reached weekly by the majority of fruiting uprights across the bed. In fact, no significant differences were found across comparisons and in concordance with Figures 3.4 to 3.8, the curves in each phenological stage follow a similar pattern across zones and foot square areas. These results indicate that the three biological replicates per each type of upright and floral initiation collected for transcriptome analysis are in a similar physiological stage.

In order to understand the processes that occurs in buds competent to differentiate into floral meristems (reproductive buds) versus vegetative buds, this study used a strong alternate bearing cultivar, with a predictable majority of vegetative uprights able to set reproductive buds and most, if not all, fruiting uprights able to set up vegetative apical buds. Such predictable alternative bearing behavior in Ben Lear was confirmed by the histology of dormant buds, where only wide bud contained floral meristems. Another piece of evidence was the histology analysis through the growing season, which as fully described in Chapter 2, as well as the high

percentages of vegetative uprights that set wide buds and fruiting uprights that set a narrow bud for the following growing season.

Recently, two cranberry transcriptomes have been published. The first transcriptome (from leaves and shoot tips) along with genome sequencing, were used to confirm the taxonomic position of cranberry, as well as to identify genes associated with disease resistance and defense (Polashock *et al.* 2014). The second transcriptome published was from fruit in two stages of maturation (white and red) in order to identify pigmentation and flavonoid biosynthesis pathway genes, and to compare gene expression between the two stages of fruit maturation (Sun *et al.*, 2015). From the transcriptomes previously mentioned and comparing them with the transcriptome in this study obtained, the number of cDNAs (in the case of Polashock *et al.*, 2014) or unigenes identified were different. In the study of Polashock *et al.* (2014) 48,271 cDNAs were assembled; whereas 105,000 contigs were grouped in 57,331 unigenes in the fruit transcriptome (Sun *et al.*, 2015), and in the present study, 172,997 transcripts represented 99,866 unigenes. This discrepancies can be attributed to the inherent difficulty of the *de novo* assembly itself, and therefore to the techniques, parameters and software used for the assembly (Cresko Lab, n.d.). Other important consideration is that the plant material from which the RNA was extracted is different among the three studies, then the genes, and the number of genes expressed differ among cranberry cultivars (Ben Lear vs. Bergman) and within plants.

Based on the knowledge that vegetative uprights produced buds with floral initials, from now on called reproductive bud, and that fruiting uprights produce buds lacking floral initials, from now

on called vegetative bud, the discussion of the results in changes in gene expression were described in terms of the bud type (reproductive vs vegetative buds) rather than the upright type where the bud was set (vegetative vs. fruiting uprights).

In order to determine genes that were differentially expressed we used a FDR of 0.2, higher than what is usually recommended (Cresko Lab n.d), with the purpose to allow the detection of unigenes that had homology with flowering genes from *Arabidopsis* and some perennial plants and had some degree of change in expression through the three floral induction stages studied. Even with this parameter, most of the unigenes annotated fell in the category with no change in expression (Steady). It is important to keep in mind that genes that were presumably to be differentially expressed and suggested for further analysis would need to be re- evaluated with smaller FDR (0.5).

In general, and as mentioned above, the majority of the unigenes expressed in both reproductive and vegetative buds were classified into the expression category labeled as SS. Within such category, it is assumed that those genes have a similar function in both types of buds throughout the floral induction stages. In buds from vegetative uprights, genes were classified in more COG expression categories than in fruiting uprights, which could indicate that there were processes occurring in reproductive buds (from vegetative uprights) were not detected or revealed in the same proportions in vegetative buds. Additionally, there were no genes in both types of buds, whose expression continuously increased or decreased across the floral induction stages.

Flowering process is a complex network that involves the upregulation and downregulation of genes through each of the flowering stages (induction, initiation and differentiation). Most of the knowledge in flowering has come from detailed studies in *Arabidopsis* (Amasino and Michaels, 2010; Andres and Coupland, 2012; Khan *et al.*, 2013), and recently from emerging studies in woody plants such as apple (Dadpour *et al.*, 2011; Hanke *et al.*, 2007), citrus (Shalom *et al.*, 2014), and few fruit crops (Nakagawa *et al.*, 2012). Five pathways have been distinguished based on the signal perceived by the plant and responsible to start the process, such pathways are: photoperiod, vernalization, autonomous, aging, and gibberellic acid (Amasino and Michaels, 2010; Hanke *et al.*, 2012; Khan *et al.*, 2013). Here, we have BLAST cranberry unigenes against the sequences of a list of 169 genes of *Arabidopsis* known or presumed to be involved in one or more flowering pathways in order to determine candidate genes in the flowering process in cranberry and to hypothesize what flowering pathway cranberry could be involved. Based on the literature review, this is the first study to explore the molecular control of flowering in cranberry.

BLAST analysis revealed that most of the genes in the photoperiod pathway were represented in the transcriptome from buds. Such finding was expected, since photoperiod pathway has the highest number of genes identified, compared to the other pathways. Moreover, the expression of unigenes with homology to most of the genes in each flowering pathway shown to be unchanged (steady) through the floral induction stages in both reproductive and vegetative buds. In addition to this expression pattern category, homologous to flowering genes were found in other six categories: DS-SS, SD-SS, IS-SS, SS-DS, SS-IS, and unknown.

The photoperiod pathway in long days is activated by the perception of the light by phytochromes (Phytochrome A) and cryptochromes such as cryptochrome 2 (CRY2). Once the light is perceived, major regulator genes are activated such *GIGANTEA (GI)*, *FLAVIN KELCH F BOX 1(FKF1)*, *CONSTANS (CO)* and *FLOWERING LOCUS T (FT)* (Andres and Coupland, 2012). Their expression and interactions regulate downstream transcription factors involved in flowering. Thus, *CO* needs to be activated and stabilized, so that *CO* protein can interact and activate *FT* transcription. All these interactions occur in the leaves and the *FT* protein resulting, should travel via phloem to the SAM, in order to floral induction occur (Andres and Coupland, 2012). The activation of *CO* and stabilization of *CO* protein resulted from the action of the complex *GI* and *FKF1* in degrading the transcriptional repressor *CYCLING DOF FACTORS (CDFs)*. In addition degradation of *CO* protein by *CONSTITUTIVE MORPHOGENIC 1 (COP1)* and *SUPPRESSOR OF PHYTOCHROME A (SPA1)* is inhibited by the photoreceptors *PHYTOCHROME A (PHYA)* and *CRYPTOCHROME 2 (CRY2)*.

Cranberry is a plant that flowers in long days; however, from the gene expression patterns it appears that genes with homologies to genes related to photoperiod, including: *CO*, *PHYA*, *CRY1*, *GI*, *SPA1*, *SPA2*, *SPA4*, *FKF1*, *CDF3*, and other genes interacting with *CO* such as *HAP5C*, as well as other transcription factors were categorized as SS in both reproductive and vegetative buds. In addition *CONSTANS-LIKE 5 (COL5)* was downregulated in cranberry reproductive buds from the ‘before’ and ‘during’ floral induction stages and kept steadily low in “after” floral induction stage (expression pattern DS) while their expression was steady (SS) in vegetative uprights. *LATE ELONGATED HYPOCOTYL (LHY)* is a MYB DNA-binding protein whose expression has shown to be important in circadian clock and influence flowering

time in *Arabidopsis* (Schaffer *et al.*, 1998) by controlling the expression of *GI*, *CO*, and *FT*. The unigene homologous to *LHY*, had an expression pattern similar as *COL5* with a DS pattern in reproductive buds, and a SS in vegetative buds. No homologous unigenes to *FT* were found in neither vegetative nor reproductive buds. This result contrasts with the study by Song *et al.* (2013) who was able to isolate a *FT-like* gene from blueberry and overexpressed it in tobacco.

The observations that none of the known activators of photoperiod flowering showed an increased expression in reproductive buds compared to vegetative buds, suggest that flowering process in cranberry could not be activated by this pathway. In addition the constant expression of the activators in both types of buds could also suggest that they could be involved in other responses as it was suggested for the role of *CO* in dormancy process in poplar, and reviewed by Amasino and Michaels (2010), and for the upregulation of various genes in the photoperiod pathway, that also form part of the circadian clock, when studying the transcriptome of crown buds of leafy spurge (*Euphorbia esula* L) at different stages of dormancy (Horvath *et al.*, 2008). However, these assumptions need to be verified for cranberry with more experimental evidence.

The phase change pathway is a relatively recently proposed flowering pathway and is based on the control of the transitions between juvenile and adult stages. This is distinguished by changes in expression of two micro RNAs (miRNAs): miR156 and miR172 (Khan *et al* 2013). miR156 inhibits the flowering process by regulating negatively the expression of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* genes, specially *SPL3*. *SPL3* activates floral meristem identity genes including, *LEAFY (LFY)*, *FRUITFUL (FUL)* and *APETALA 1 (AP1)*.

Meanwhile, flowering is promoted by miR172, independent of CO, by targeting transcripts of flowering repressors such as *AP2*, *SCHLAFMUTZE (SMZ)*, *SCHNARCHZAPFEN (SNZ)*, *TARGET OF EAT 1(TOE1)*, *TOE2* and *TOE3* (Khan *et al.* 2013).

In this study, unigenes homologous to flowering repressors targeted by the phase change pathway were identified, yet no unigenes homologous to *SPL* genes were detected. Although one might expect repressors to be downregulated in reproductive buds in at least one of the floral induction stages (such as decreased-steady, DS, or steady-decreased, SD) compared to vegetative buds. Other possible expression pattern that could be expected would be that the expression of the repressors would remain steady in reproductive buds (SS) and increased in vegetative buds (IS).

Only *SCHLAFMUTZE (SMZ)* showed the latter pattern of expression, steady in reproductive buds, and increased in reproductive buds between the stages of ‘before’ and ‘during’ and then ‘steady’ (IS). None of the remaining genes, *AP2*, *SCHNARCHZAPFEN (SNZ)*, *TARGET OF EAT 1(TOE1)*, *TOE2* and *TOE3* had homologous unigenes in the category of DS for reproductive buds when SS in vegetative buds. Instead, all of them had a constant (steady) expression through the floral induction stages in both types of buds. From all the unigenes analyzed in this study, only *TOE1* had homology with two unigenes, c149845 and c157750, while c157750 had a steady pattern expression (SS) in both vegetative and reproductive buds; c149845 showed an expression pattern of IS in reproductive buds and SS in vegetative buds. Even though, there were homologous genes to the flowering repressors, no downregulation of

them was observed for which we could not suggest that aging pathway could influence flowering in cranberry. Reviews by Spanudakis and Jackson (2014), and by Yamaguchi and Abe (2012) described cellular details of the process in *Arabidopsis*, but there is still no information involving this process in flowering in woody species.

The vernalization pathway is based in the induction of flowering due to long exposures to low temperatures ($<10^{\circ}\text{C}$) and has been identified in winter and biennial plants (Amasino and Michaels, 2010). *FRIGIDA (FRI)* is a gene whose biochemical function is still unknown; however, it is known that its interaction with *FLC* promotes highly expression of *FLC*, and hence repress flowering. The function of *FRI* requires transcriptional components such as Paf complex and chromatin remodeling activities described by Kahn *et al.* (2013). Cold temperatures perceived by the apical meristem, induce the expression of genes such as *VERNALIZATION 1 (VRN1)* and *VERNALIZATION INSENSITIVE 3 (VIN3)*, which encodes plant specific proteins responsible for changes in DNA methylation resulting in repression of the expression of *FLC* (Amasino and Michaels, 2010; Hanke *et al.*, 2012).

Although, there were no unigenes with homology to *FLC* and *FRI*, some genes needed to favor the upregulation of *FLC* through *FRI* were found. Genes that influence negatively or positively *FLC* expression were found in the different expression pattern categories. Thus, in the expression pattern where no changes in gene expression were found throughout the floral induction stages (SS), in vegetative and reproductive buds, there were homologous to genes regulating negatively the expression of *FLC* such as *CSTF77*, *CSTF6*, and *CURLY LEAF (CF)*, and *SET DOMAIN*

GROUP 26 (SDG26), and others required for FLC activation (*PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1, PIE1*) or to keep the repressing action of FLC after cold exposure (*ARABIDOPSIS THALIANA HOMEODOMAIN 1, ATH1*). As expected, homologous to genes activating the expression of *FLC*, which inhibits flowering, followed a decreased-steady pattern (DS) in reproductive buds and remained constant in vegetative buds. However, other genes enhancing FLC activation (*UBP26: UBIQUITIN-SPECIFIC PROTEASE 26; ELF8: EARLY FLOWERING 8; HUB2: HISTONE MONOUBIQUITINATION 2; and UBC1: UBIQUITIN CONJUGATING ENZYME 1*) had homology with unigenes with increased-steady (IS) expression in reproductive buds and kept steady (SS) in vegetative buds.

Similarly to the epigenetic control exerted over *FLC* in the vernalization pathway, in the autonomous pathway the expression of *FLC* is repressed by a group of genes called autonomous floral-promotion pathway (AP genes; Veley and Michaels, 2008). Among the AP genes are *FLOWERING TIME CONTROL PROTEIN ALPHA (FCA)*, *FPA*, *FLOWERING LOCUS K*, and *FY*, which encode proteins with RNA binding domains and regulate *FLC* transcripts. These genes have also been suggested to regulate *FLC* via chromatin remodeling (Veley and Michaels, 2008). Other AP genes code for histone deacetylation factors like *FVE*, *FLOWERING LOCUS D (FLD)* and *RELATION OF EARLY FLOWERING 6 (REF6)* (Amasino and Michaels, 2010; Hanke *et al.*, 2012; Khan *et al.*, 2013). It has also been shown by Veley and Michaels (2008) that AP genes are also involved in cellular organization and normal growth in Arabidopsis. From this pathway all of the genes, excepting *FY*, represented here such as *FVE*, *FPA*, *FLK*, *PLD* and *LD*, did not show differences in expression across the floral induction stages in both bud types. *FY* and *REF6* showed a decreased in expression in reproductive buds from ‘before’ to ‘during’ floral

stages, and remained constant in vegetative buds. AP genes have been known to play roles in flowering and also can regulate themselves post-transcriptionally, as is the case of *FCA* and *FY* (Veley and Michaels, 2008). It is possible that these genetic interactions are responsible for the lack of differences in the expression patterns of these genes across floral induction stages in vegetative and reproductive buds. Moreover, the roles that AP genes have in plant growth and development may mask their role in flowering.

Another flowering pathway proposed is the gibberellic acid (GA), in this case, GA is synthesized in the leaves and transported to competent meristems, leading to positive regulation of *LFY* directly or through the activation of the floral integrator *SOC1* (Hanke *et al.*, 2012). In *Arabidopsis*, high concentrations of LFY have been shown to induce the expression of GA catabolism gene (*ELA1*), and GA degradation can lead to the activation of DELLA proteins (GIBBERELIC ACID IINSENSITIVE 1, GA1; REPRESSOR OF GA1-3, RGA; RGA-LIKE 1. RGL1). These proteins have been shown to be recruited by *SPL* and *LFY* to activate the expression of *AP1* and trigger flowering (Khan *et al.*, 2013; Park *et al.*, 2013; Yamaguchi *et al.*, 2014).

The results of this study demonstrated that most genes associated with GA biosynthesis shown steady expression throughout the observed floral induction stages. An exception is *GIBBERELLIN 20 OXIDASE 1 (GA20ox1)*, as its expression increased in reproductive buds between ‘before’ and ‘during’ flower induction stages, yet remained constant in vegetative buds. In addition, *GA1*, displayed decreased expression between ‘before and ‘during’ floral induction

stages in reproductive buds, yet remained constant in vegetative buds. These results agree with Yamaguchi *et al.*, (2014) and Park *et al.*, (2013) who found that during GA biosynthesis DELLA proteins, like GA1 are downregulated. Additionally, similar to the model of Yamaguchi *et al.* (2014) dual GA activity in *Arabidopsis*, could regulate flowering in cranberry. However, further studies with RT-PCR and *in situ* hybridization could give more insights to this hypothesis.

The lack of flowering buds developing in cranberry plantations when GA was applied (Devlin and Demoranville, 1967; Eady and Eaton, 1972) could be explained by the findings of (Goldberg-Moeller *et al.*, 2013), who studied the expression of flowering genes in citrus buds after treatments with GA application. When external GA was applied, mRNA levels of *FT*, *AP*, *SEP1*, *SEP2*, *PI*, were reduced and *LFY* mRNA increased. Additional experiments as the expression analysis described before as well as studies of flowering covering more floral induction stages, as well as field experiments with applications of gibberellins and anti-gibberellins could clarify the participation of GAs in the flowering process in cranberry

It was expected that integrator and some identity genes would be upregulated in reproductive buds, mainly in the transition of stages ‘during’ and ‘after’ floral initiation. However, the expression of most known genes associated with meristem development such as *SOC1*, *LFY*, *LFY3*, *SEP3*, *AGAMOUS-LIKE 9 (AGL9)*, *AGL20* were constant during the observed floral induction stages. However, *TERMINAL FLOWER 1*, and the floral repressor *SHORT VEGETATIVE PHASE (SVP)*, showed decreases in expression in ‘before’ and ‘during’ floral induction stages (DS) in reproductive buds, and were constant (SS) in vegetative buds. This

could be due to the time the stages were selected. As revealed in Figure 3.9, no physical evidence of meristems was observed until the buds reached the “after” stage of floral induction (Figure 3.9, C). This condition could account for such gene expression and it could be possible that in later developmental stages of the buds, sufficient transcripts from those genes could accumulate to detectable levels.

From the BLAST of a total of 140 genes from other perennial species such as apple, citrus, pear, peach, mango, and kiwi, among others, only one gene, HM59672, from trifoliate orange had homology with a single cranberry unigene, c158898_g1, which showed a decreased pattern of expression between ‘before’ and ‘during’ flower induction in reproductive buds. HM59672 is a regulatory gene encoding an eukaryotic translation initiation factor protein, with a predicted similar function to a putative *FLC* gene (EU497676) in trifoliate orange (Zhang *et al.*, 2011). From the transcriptome analysis in floral buds of Japanese pear (Bai *et al.*, 2013), *FLC* and *FT-like* genes were observed and were not downregulated after dormancy released. Homologous genes in the vernalization pathway, such as *PsFRI*, *PsVIN3*, and *PsFT*, were expressed in peonies floral buds, similarly as it is expressed in *Arabidopsis* (Zhou *et al.*, 2013). Additional to these genes, the authors also found genes involved in the GA pathway. Similar to our observations, *PsGA20ox* in peonies was downregulated during reblooming induction, and in contrast, *PsGID* was upregulated. After induction *PsGA20ox* was upregulated, which suggested that GA could play a role later in the differentiation process (Zhou *et al.*, 2013). The transcriptome analysis of inflorescences of the tree *Tapiscia sinensis*, revealed the upregulation of homologous genes involved in the photoperiod and circadian clock in *Arabidopsis*: *SOC1*, *FLC*, *EARLY FLOWERING 4 (ELF4)* and *FLAVIN-BINDING, KELCH REPEAT, F-BOX (FKF1)*;

Zhou *et al.*, 2015). In our study, the lack of evidence of up-regulation of homologous genes in cranberry reproductive buds to genes known to be involved in the activation of the flowering pathway as well as those genes important for floral induction in species like *Arabidopsis*, and some perennial plants as previously described, suggests that the flowering stages studied missed the time when all the induction process did occur. Additional studies related specifically to the expression of activator and integrator genes in earlier stages of bud development would help to understand flowering induction process in cranberry.

In conclusion, the studies performed in this chapter verify that wide buds effectively grew as fruiting uprights; and their counterpart, narrow buds grew as vegetative uprights. In addition, no differences in development, via the study of phenological stages, were found in fruiting uprights growing in different sectors of the bed, which allowed us to collect apical buds from different zones of the bed and count them as biological replicates for transcriptome analysis. Additionally, we developed a reference transcriptome from apical buds of cranberry (Ben Lear cultivar). Enrichment analysis revealed that even though, most of the genes were similarly expressed in vegetative and reproductive buds through the floral induction stages evaluated, there were some differentially expressed in reproductive buds from the gibberellic pathway. These could be potential candidates for further studies related to flowering in cranberry.

3.6 References

Amasino, R. M., and Michaels, S. D. (2010). The Timing of Flowering. *Plant Physiology*, 154(2), 516–520. doi:10.1104/pp.110.161653

- Andres, F., and Coupland, G. (2012). The genetic basis of flowering responses to seasonal cues. *Nat Rev Genet*, 13(9), 627–639. Retrieved from <http://dx.doi.org/10.1038/nrg3291>
- Auer, P. L., and Doerge, R. W. (2010). Statistical Design and Analysis of RNA Sequencing Data. *Genetics*, 185(2), 405–416. doi:10.1534/genetics.110.114983
- Bai, S., Saito, T., Sakamoto, D., Ito, A., Fujii, H., and Moriguchi, T. (2013). Transcriptome Analysis of Japanese Pear (*Pyrus pyrifolia* Nakai) Flower Buds Transitioning Through Endodormancy. *Plant and Cell Physiology*, 54(7), 1132–1151. doi:10.1093/pcp/pct067
- Bao, Y., Xu, S., Jing, X., Meng, L., and Qin, Z. (2015). De novo assembly and characterization of *Oryza officinalis* leaf transcriptome by using RNA-seq. *BioMed Research International*, 2015, 1–7. doi:10.1155/2015/982065
- Borchert, T., Eckardt, K., Fuchs, J., Krüger, K., and Hohe, A. (2009). “Who’s who’ in two different flower types of *Calluna vulgaris* (Ericaceae): morphological and molecular analyses of flower organ identity. *BMC Plant Biology*, 9(1), 148. doi:10.1186/1471-2229-9-148
- Bowman, M. J., Park, W., Bauer, P. J., Udall, J. A., Page, J. T., *et al.* (2013). RNA-Seq Transcriptome Profiling of Upland Cotton (*Gossypium hirsutum* L.) Root Tissue under Water-Deficit Stress. *PLoS ONE*, 8(12), e82634. doi:10.1371/journal.pone.0082634
- Brown, A. O., and McNeil, J. N. (2006). Fruit production in cranberry (Ericaceae: *Vaccinium macrocarpon*): a bet-hedging strategy to optimize reproductive effort. *American Journal of Botany*, 93 (6), 910–916. doi:10.3732/ajb.93.6.910
- Cresko Lab. (n.d.). RNA-seqlopedia. *University of Oregon*. Retrieved November 11, 2015, from <http://rnaseq.uoregon.edu>
- Dadpour, M. R., Movafeghi, a., Grigorian, W., and Omid, Y. (2011). Determination of floral initiation in *Malus domestica*: A novel morphogenetic approach. *Biologia Plantarum*, 55(June 2009), 243–252. doi:10.1007/s10535-011-0035-5
- Demoranville, C. J. (1992). *Cranberry nutrients, phenology, and N-P-K fertilization*. University of Massachusetts.
- Devetter, L. (2013). *Understanding Yield of Cranberry : Bud Development , Carbohydrate Allocation , and Yield Component Analysis By Lisa Wasko DeVetter A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Horticulture)*. University of Wisconsin.
- Devlin, R., and Demoranville, I. (1967). Influence of Gibberellic Acid and Gibrel on Fruit Set and Yield in *Vaccinium macrocarpon* cv. Early Black. *Physiologia Plantarum*, 20, 587–592.
- Eady, F. ., and Eaton, W. (1972). The role of gibberellic acid and gibberellin-like substances in the dormancy of the cranberry. *Canadian Journal of Botany*, 52(3), 263–271. doi:10.1016/j.plantsci.2011.04.016
- Fajardo, D., Morales, J., Zhu, H., Steffan, S., Harbut, R., *et al.*, (2012). Discrimination of American Cranberry Cultivars and Assessment of Clonal Heterogeneity Using Microsatellite Markers. *Plant Molecular Biology Reporter*, 31(2), 264–271.

doi:10.1007/s11105-012-0497-4

- Fajardo, D., Schlautman, B., Steffan, S., Polashock, J., Vorsa, N., and Zalapa, J. (2014). The American cranberry mitochondrial genome reveals the presence of selenocysteine (tRNA-Sec and SECIS) insertion machinery in land plants. *Gene*, 536(2), 336–43. doi:10.1016/j.gene.2013.11.104
- Fajardo, D., Senalik, D., Ames, M., Zhu, H., Steffan, S. a., *et al.* (2013). Complete plastid genome sequence of *Vaccinium macrocarpon*: Structure, gene content, and rearrangements revealed by next generation sequencing. *Tree Genetics and Genomes*, 9(2), 489–498. doi:10.1007/s11295-012-0573-9
- Fan, H., Xiao, Y., Yang, Y., Xia, W., Mason, A. S., Xia, Z., *et al.* (2013). RNA-Seq Analysis of *Cocos nucifera*: Transcriptome Sequencing and De Novo Assembly for Subsequent Functional Genomics Approaches. *PLoS ONE*, 8(3), e59997. doi:10.1371/journal.pone.0059997
- Fugate, K. K., Fajardo, D., Schlautman, B., Ferrareze, J. P., Bolton, M. D., *et al.* (2014). Generation and Characterization of a Sugarbeet Transcriptome and Transcript-Based SSR Markers. *The Plant Genome*, 7(2). doi:10.3835/plantgenome2013.11.0038
- Georgi, L., Herai, R. H., Vidal, R., Carazzolle, M. F., Pereira, G. G., Polashock, J., and Vorsa, N. (2011). Cranberry microsatellite marker development from assembled next-generation genomic sequence. *Molecular Breeding*, 30(1), 227–237. doi:10.1007/s11032-011-9613-7
- Goldberg-Moeller, R., Shalom, L., Shlizerman, L., Samuels, S., Zur, N., *et al.* (2013). Effects of gibberellin treatment during flowering induction period on global gene expression and the transcription of flowering-control genes in Citrus buds. *Plant Science*, 198, 46–57. doi:10.1016/j.plantsci.2012.09.012
- Haas, B. J., and Zody, M. C. (2010). Advancing RNA-Seq analysis. *Nature Biotechnology*, 28(5), 421–3. doi:10.1038/nbt0510-421
- Hanke, M.-V., Flachowsky, H., Hoenicka, H., and Fladung, M. (2012). Functional genomics of flowering time. In R. J. Schnell and P. M. Priyadarshan (Eds.), *Genomics of tree crops* (p. 369). New York: Springer New York Heidelberg. doi:10.1007/978-1-4614-0920-5
- Hanke, M.-V., Flachowsky, H., Peil, A., and Hättasch, C. (2007). No Flower no Fruit – Genetic Potentials to Trigger Flowering in Fruit Trees. *Genes, Genomes and Genomics*, 1(1), 1–20.
- Horvath, D. P., Chao, W. S., Suttle, J. C., Thimmapuram, J., and Anderson, J. V. (2008). Transcriptome analysis identifies novel responses and potential regulatory genes involved in seasonal dormancy transitions of leafy spurge (*Euphorbia esula* L.). *BMC Genomics*, 9(1), 536. doi:10.1186/1471-2164-9-536
- Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., *et al.* (2015). eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Research*. doi:10.1093/nar/gkv1248
- Khan, M. R. G., Ai, X.-Y., and Zhang, J.-Z. (2013). Genetic regulation of flowering time in annual and perennial plants. *Wiley Interdisciplinary Reviews: RNA*, n/a–n/a. doi:10.1002/wrna.1215

- Koutinas, N., Pepelyankov, G., and Lichev, V. (2010). Flower induction and flower bud development in apple and sweet cherry. *Biotechnology and Biotechnological Equipment*, 24(1), 1549–1558. doi:10.2478/V10133-010-0003-9
- Liang, C., Liu, X., Yiu, S.-M., and Lim, B. L. (2013). *De novo* assembly and characterization of *Camelina sativa* transcriptome by paired-end sequencing. *BMC Genomics*, 14(1), 146. doi:10.1186/1471-2164-14-146
- Martínez-López, L. a, Ochoa-Alejo, N., and Martínez, O. (2014). Dynamics of the chili pepper transcriptome during fruit development. *BMC Genomics*, 15, 143. doi:10.1186/1471-2164-15-143
- Metzker, M. (2010). Sequencing technologies- the next generation. *Nature Reviews*, 11. 31-46. doi: 10.1038/nrg2626
- Nakagawa, M., Honsho, C., Kanzaki, S., Shimizu, K., and Utsunomiya, N. (2012). Isolation and expression analysis of *FLOWERING LOCUS T*-like and gibberellin metabolism genes in biennial-bearing mango trees. *Scientia Horticulturae*, 139, 108–117. doi:10.1016/j.scienta.2012.03.005
- Park, J., Nguyen, K. T., Park, E., Jeon, J.-S., and Choi, G. (2013). DELLA proteins and their interacting RING Finger proteins repress gibberellin responses by binding to the promoters of a subset of gibberellin-responsive genes in *Arabidopsis*. *The Plant Cell*, 25(3), 927–43. doi:10.1105/tpc.112.108951
- Polashock, J., Zelzion, E., Fajardo, D., Zalapa, J., Georgi, L., Bhattacharya, D., and Vorsa, N. (2014). The American cranberry: first insights into the whole genome of a species adapted to bog habitat. *BMC Plant Biology*, 14(1), 165. doi:10.1186/1471-2229-14-165
- R Core Team (2013). (n.d.). R: A language and environment for statistical computing. *R Foundation for Statistical Computing. Vienna, Austria*. Retrieved July 1, 2015, from <http://www.r-project.org/>.
- Rosecrance, R. C., Weinbaum, S. A., and Brown, P. H. (1998). Alternate Bearing Affects Nitrogen , Phosphorus , Potassium and Starch Storage Pools in Mature Pistachio Trees. *Annals of Botany*, 82, 463–470. Retrieved from <http://aob.oxfordjournals.org/content/82/4/463.full.pdf>
- Rowland, L. J., Alkharouf, N., Darwish, O., Ogden, E. L., Polashock, J. J., Bassil, N. V, and Main, D. (2012). Generation and analysis of blueberry transcriptome sequences from leaves, developing fruit, and flower buds from cold acclimation through deacclimation. *BMC Plant Biology*, 12(1), 46. doi:10.1186/1471-2229-12-46
- Samach, A., and Smith, H. M. (2013). Constraints to obtaining consistent annual yields in perennials. II: Environment and fruit load affect induction of flowering. *Plant Science : An International Journal of Experimental Plant Biology*, 207, 168–76. doi:10.1016/j.plantsci.2013.02.006
- Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carré, I. a., and Coupland, G. (1998). The late elongated hypocotyl mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. *Cell*, 93(7), 1219–1229. doi:10.1016/S0092-8674(00)81465-8

- Schlautman, B., Covarrubias-Pazarán, G., Díaz-García, L. a., Johnson-Cicalese, J., Iorizzo, M., Rodríguez-Bonilla, L., ... Zalapa, J. (2015). Development of a high-density cranberry SSR linkage map for comparative genetic analysis and trait detection. *Molecular Breeding*, 35(8), 177. doi:10.1007/s11032-015-0367-5
- Schlautman, B., Covarrubias-Pazarán, G., Fajardo, D., Steffan, S., and Zalapa, J. (2016). Discriminating power of microsatellites in cranberry organelles for taxonomic studies in *Vaccinium* and *Ericaceae*. *Genetic Resources and Crop Evolution*, *Accepted*.
- Schlautman, B., Fajardo, D., Bougie, T., Wiesman, E., Polashock, J., *et al.* (2015). Development and Validation of 697 Novel Polymorphic Genomic and EST-SSR Markers in the American Cranberry (*Vaccinium macrocarpon* Ait.). *Molecules*, 20(2), 2001–2013. doi:10.3390/molecules20022001
- Shalom, L., Samuels, S., Zur, N., Shlizerman, L., Doron-Faigenboim, A., Blumwald, E., and Sadka, A. (2014). Fruit load induces changes in global gene expression and in abscisic acid (ABA) and indole acetic acid (IAA) homeostasis in citrus buds. *Journal of Experimental Botany*, 65 (12), 3029–3044. doi:10.1093/jxb/eru148
- Shalom, L., Samuels, S., Zur, N., Shlizerman, L., Zemach, H., *et al.* (2012). Alternate Bearing in Citrus: Changes in the Expression of Flowering Control Genes and in Global Gene Expression in ON- versus OFF-Crop Trees. *PLoS ONE*, 7(10), e46930. Retrieved from <http://dx.doi.org/10.1371/journal.pone.0046930>
- Smith, H. M., and Samach, A. (2013). Constraints to obtaining consistent annual yields in perennial tree crops. I: Heavy fruit load dominates over vegetative growth. *Plant Science : An International Journal of Experimental Plant Biology*, 207, 158–67. doi:10.1016/j.plantsci.2013.02.014
- Song, G., Walworth, A., Zhao, D., Jiang, N., and Hancock, J. (2013). The *Vaccinium corymbosum* FLOWERING LOCUS T-like gene (VcFT): a flowering activator reverses photoperiodic and chilling requirements in blueberry. *Plant Cell Reports*, 32(11), 1759–1769. doi:10.1007/s00299-013-1489-z
- Spanudakis, E., and Jackson, S. (2014). The role of microRNAs in the control of flowering time. *Journal of Experimental Botany*, 65(2), 365–380. doi:10.1093/jxb/ert453
- Sun, H., Liu, Y., Gai, Y., Geng, J., Chen, L., Liu, H *et al.* (2015). *De novo* sequencing and analysis of the cranberry fruit transcriptome to identify putative genes involved in flavonoid biosynthesis, transport and regulation. *BMC Genomics*, 16(1), 652. doi:10.1186/s12864-015-1842-4
- Tan, F.-C., and Swain, S. M. (2006). Genetics of flower initiation and development in annual and perennial plants. *Physiologia Plantarum*, 128(1), 8–17. doi:10.1111/j.1399-3054.2006.00724.x
- Turktas, M., Inal, B., Okay, S., Erkilic, E. G., Dundar, E., *et al.* (2013). Nutrition Metabolism Plays an Important Role in the Alternate Bearing of the Olive Tree (*Olea europaea* L.). *PLoS ONE*, 8(3). doi:10.1371/journal.pone.0059876
- Veley, K. M., and Michaels, S. D. (2008). Functional Redundancy and New Roles for Genes of the Autonomous Floral-Promotion Pathway. *Plant Physiology*, 147(2), 682–695.

doi:10.1104/pp.108.118927

- Villarino, G. H. (2014). *Transcriptomic analysis of Petunia hybrida in response to salt stress using high throughput RNA sequencing*. Cornell University.
- Vorsa, N., and Johnson-cicalese, J. (2012). American Cranberry. In M. L. Badenes and D. H. Byrne (Eds.), *Fruit Breeding* (pp. 191–223). Boston, MA: Springer US. doi:10.1007/978-1-4419-0763-9
- Wigge, P. A., Kim, M. C., Jaeger, K. E., Busch, W., Schmid, M., Lohmann, J. U., and Weigel, D. (2005). Integration of Spatial and Temporal Information During Floral Induction in *Arabidopsis*. *Science*, 309(5737), 1056–1059. doi:10.1126/science.1114358
- Wilkie, J. D., Sedgley, M., and Olesen, T. (2008). Regulation of floral initiation in horticultural trees. *Journal of Experimental Botany*, 59(12), 3215–28. doi:10.1093/jxb/ern188
- Workmaster, B., Palta, J., and Roper, T. (1997). Terminology for Cranberry Bud Development and Growth. *Cranberries*, 61, 11–14.
- Yamaguchi, A., and Abe, M. (2012). Regulation of reproductive development by non-coding RNA in *Arabidopsis*: to flower or not to flower. *Journal of Plant Research*, 125(6), 693–704. doi:10.1007/s10265-012-0513-7
- Yamaguchi, Nobutoshi, Winter, C., Wu, M.-F., Kanno, Y., Yamaguchi, A., Seo, M., and Wagner, D. (2014). Gibberellin acts positively then negatively to control onset of flower formation in *Arabidopsis*. *Science*, 344(May), 638–641.
- Yanik, H., Turktas, M., Dundar, E., Hernandez, P., Dorado, G., and Unver, T. (2013). Genome-wide identification of alternate bearing-associated microRNAs (miRNAs) in olive (*Olea europaea* L.). *BMC Plant Biology*, 13(1), 10. doi:10.1186/1471-2229-13-10
- Yockteng, R., Almeida, A. M. R., Yee, S., Andre, T., Hill, C., and Specht, C. D. (2013). A Method for Extracting High-Quality RNA from Diverse Plants for Next-Generation Sequencing and Gene Expression Analyses. *Applications in Plant Sciences*, 1(12), 1300070. doi:10.3732/apps.1300070
- Zalapa, J. E., Cuevas, H., Zhu, H., Steffan, S., Senalik, D., et al. (2012). Using next-generation sequencing approaches to isolate simple sequence repeat (SSR) loci in the plant sciences. *American Journal of Botany*, 99(2), 193–208. doi:10.3732/ajb.1100394
- Zhang, J. Z., Ai, X. Y., Sun, L. M., Zhang, D. L., Guo, W. W., Deng, X. X., and Hu, C. G. (2011). Molecular cloning and functional characterization of genes associated with flowering in citrus using an early-flowering trifoliolate orange (*Poncirus trifoliata* L. Raf.) mutant. *Plant Molecular Biology*, 76(1-2), 187–204. doi:10.1007/s11103-011-9780-2
- Zhou, H., Cheng, F.-Y., Wang, R., Zhong, Y., and He, C. (2013). Transcriptome comparison reveals key candidate genes responsible for the unusual reblooming trait in tree peonies. *PLoS One*, 8(11), e79996. doi:10.1371/journal.pone.0079996
- Zhou, X.-J., Wang, Y.-Y., Xu, Y.-N., Yan, R.-S., Zhao, P., and Liu, W.-Z. (2015). De Novo Characterization of Flower Bud Transcriptomes and the Development of EST-SSR Markers for the Endangered Tree *Tapiscia sinensis*. *International Journal of Molecular Sciences*, 16(6), 12855–12870. doi:10.3390/ijms160612855

Zhu, H., Senalik, D., McCown, B. H., Zeldin, E. L., Speers, J., *et al.* (2012). Mining and validation of pyrosequenced simple sequence repeats (SSRs) from American cranberry (*Vaccinium macrocarpon* Ait.). *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, 124(1), 87–96. doi:10.1007/s00122-011-1689-2

Table 3.1 Summary of reads per sample generated after *de novo* assembly from apical buds of cranberry.

Collection week	Upright type	Biological replicate (Name)	Total clean reads*	Aligned Reads	Percentage of aligned reads (%)
2	Fruiting	1 (R1-3-2)	22,056,310	20,035,952	90.84
		2 (R4-6-2)	27,158,732	22,786,176	83.9
		3 (R7-9-2)	12,102,293	10,921,109	90.24
	Vegetative	1 (V1-3-2)	15,508,619	14,021,342	90.41
		2 (V4-6-2)	24,658,062	22,811,173	92.51
		3 (V7-9-2)	28,024,592	25,149,269	89.74
4	Fruiting	1 (R1-3-4)	36,309,646	33,629,994	92.62
		2 (R4-6-4)	31,198,205	28,508,920	91.38
		3 (R7-9-4)	36,624,440	33,786,046	92.25
	Vegetative	1 (V1-3-4)	27,560,885	23,991,750	87.05
		2 (V4-6-4)	27,535,494	24,187,178	87.84
		3 (V7-9-4)	25,834,226	23,875,992	92.42
6	Fruiting	1 (R1-3-6)	35,482,020	32,586,687	91.84
		2 (R4-6-6)	32,289,900	28,754,156	89.05
		3 (R7-9-6)	29,094,578	26,790,287	92.08
	Vegetative	1 (V1-3-6)	32,198,484	29,815,796	92.6
		2 (V4-6-6)	22,429,149	19,224,024	85.71
		3 (V7-9-6)	31,149,288	28,261,749	90.73

* Reads from mRNA only

Table 3.2 Comparative expression of genes in cranberry buds from vegetative uprights (upper) and fruiting uprights (left side) throughout three stages of floral induction: before, during, and after. Columns and rows with letters correspond to the pattern expression categories through floral induction stages as described in page 85. Numbers indicate the total number of genes in each category.

Expression pattern		VEGETATIVE UPRIGHTS										total
		I I	I S	I D	S I	S S	S D	D I	D S	D D	Unknowr	
FRUITING UPRIGHTS	I I	0	0	0	0	0	0	0	0	0	0	0
	I S	0	478	10	0	444	28	0	8	0	9	977
	I D	0		0	0	0	0	0	0	0	0	0
	S I	0	0	0	0	0	0	0	0	0	0	0
	S S	0	5928	71	115	21763	414	9	3442	0	1118	32860
	S D	0	0	0	0	0	0	0	0	0	0	0
	D I	0	0	0	0	0	0	0	0	0	0	0
	D S	0	5	0	4	134	0	0	45	0	9	197
	D D	0	0	0	0	0	0	0	0	0	0	0
	Unknown	0	176	0	4	1155	4	0	109	0	6491	7939
total		0	6587	81	123	23496	446	9	3604	0	7627	41973

Table 3.3 Enrichment analysis of genes with expression patterns increased steady (IS), steady-steady (SS), decreased-steady (DS) as described in page 85. Enrichment was done with COG analysis. Genes were involved in one or more pathways as shown by the combination of letters. Each letter represents one category assigned from COG database ($p < 0.001$).

Expression pattern in buds from fruiting uprights	Expression pattern in buds from vegetative uprights		COG pathways
	Expression pattern	# genes	
IS	IS	478	DKT,G,GMO,GOT,JO,MO,T
SS		5928	AT,DT,IJ,IQ,K,TZ
DS		5	DZ,Q
Unknown		176	Z, S
IS	SS	444	A,BDL,C,CH,DK,DP,DUZ,DZ,E,EF,EG,EI,EO,FH, G,GI,GM,GMO,GMW,GQ,I,IT,JO,KLO,KO,KY,MT VW,O,OU,P,TV,U,UY,Z
SS		21763	A,AB,AD,AJ,AK,AT,ATY,B,BD,BDL,BDLT,BK,BL ,BT,C,CE,CG,CH,CIQ,CO,CU,D,DKLT,DO,DT,DU ,DZ,E,EG,EGO,EH,EIOV,EJ,F,FH,FP,G,GMW,G OT,GT,H,I,IJ,IM,IOT,IQ,IU,J,JK,JP,JUY,K,KL,KO, KOT,KT,KU,L,LT,M,MOT,MOU,MW,O,OP,OT,OT U,OW,P,PQ,Q,S,T,TU,TZ,U,UY,V,W,Y,Z
DS		134	A,B,CG,DL,GM,IMO,JU,K,S
Unknown		1155	DT,G,P,PQ,PT,Q, D,TU,Z
IS	DS	8	E,K
SS		3442	AD
DS		45	CG,EG,H
Unknown		109	S, O, I,K
IS	Unknown	9	OW,Q
SS		1118	S, AD, AK, BDLT, CG, EO, IMO, IU, JK, L, OP, PQ, PT, Q, T, BDLT,IOT,KL,L,OP,S
DS		9	CG, TZ, L,S
Unknown		6491	BT, DO, GMW, IOT, T, TU, TZ, Q, EF, GMWEI, CG, IJ, L, DKLT, IJ, IU, J, L, OW, P, Q, UZ, Z, C, EIOV, FQ, J, L, MTVW, O, PT, QAK, G

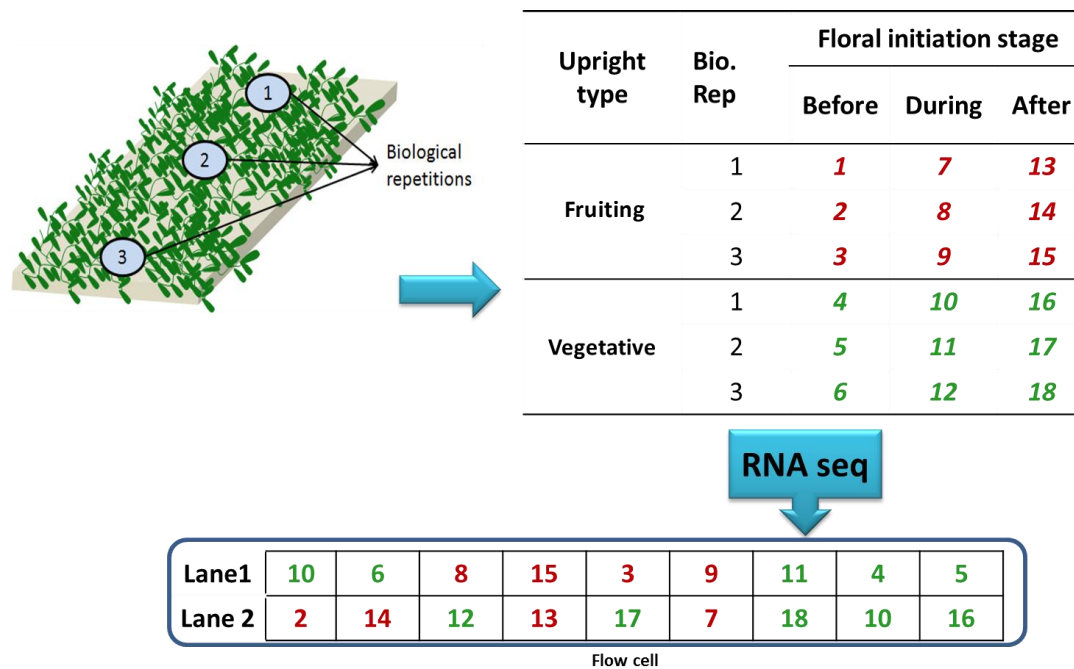


Figure 3.1 Experimental design and bud sample collections from a Ben Lear plantation. Buds from vegetative and fruiting uprights in three floral induction stages: before, during, and after were collected for transcriptome analysis. Three biological replicates, collected from three distinctive zones in a Ben Lear plantation (bed) per upright type (vegetative and fruiting upright) and floral induction stage were included in the transcriptome analysis. Each biological replicate consisted of a bulk of 25 buds collected from in one zone of the Ben Lear bed. A total of 18 biological replicates were analyzed

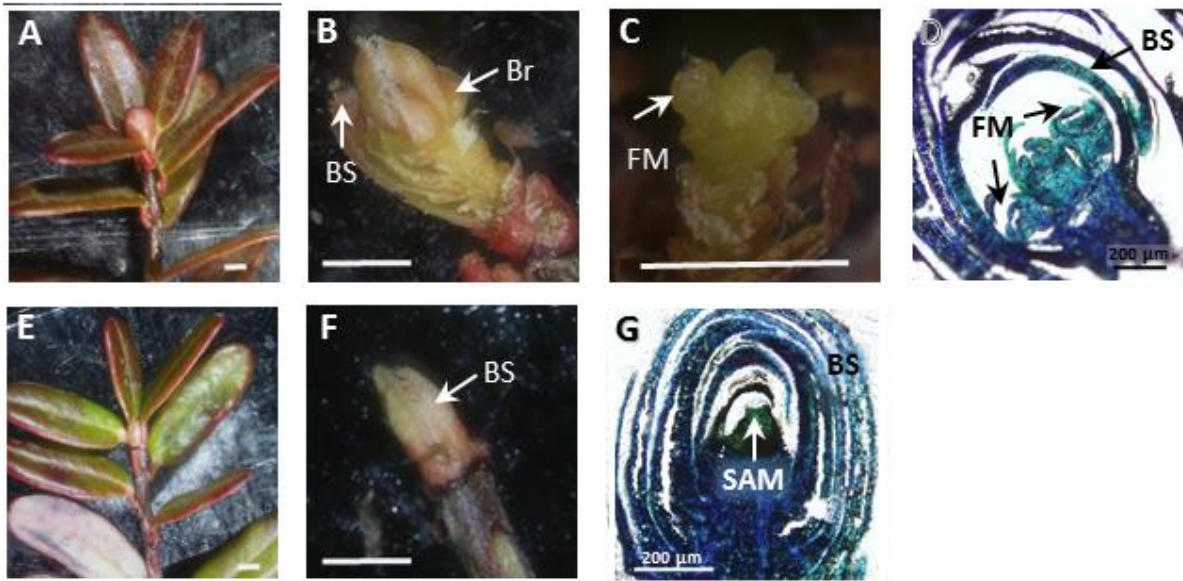


Figure 3.2. Wide and narrow dormant buds of Ben Lear. Upper row (A to D) corresponds to the wide buds, and lower row (E to G) to the narrow buds. Wide buds tend to be also rounded (A), and floral meristems are visible after removal of protective bud scales and bracts (B and C). Under light microscopy, the floral meristems are clearly visible (D). Narrow buds are pointed (E) and more difficult to hand section (F). Light microscopy revealed the compact arrangement of bud scales. No lateral meristems were visible (G). BS= bud scale, Br= Bract, FM= floral meristem, SAM = shoot apical meristem. Scale bar = 1mm, unless specified.

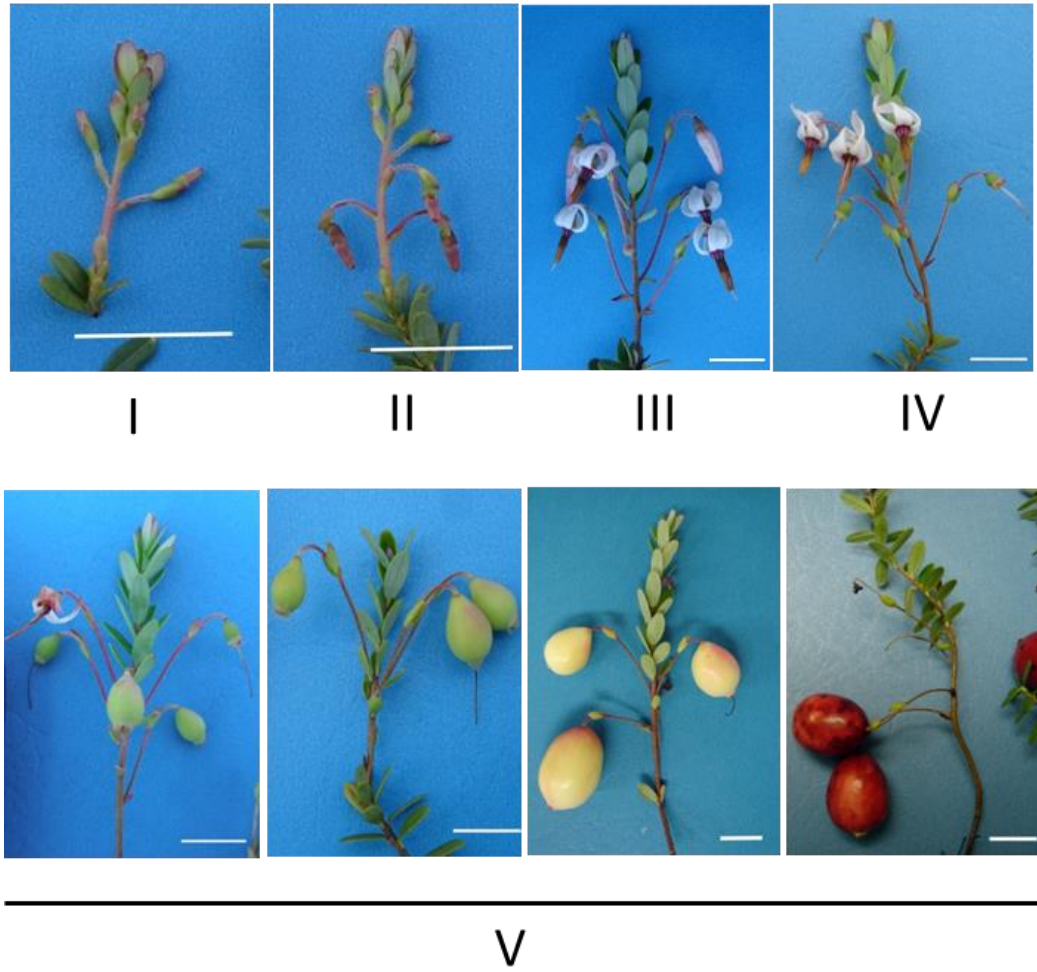


Figure 3.3. Phenological stages in reproductive uprights of Ben Lear. These stages were named from 1 to 5, and the roman numbers correspond to the Stage depicted in the picture above each number. Stage I: floral bud or prehook; Stage II: hook; Stage III: open flower; Stage IV: pinhead; Stage V: berry (includes berry growth and maturation stages).

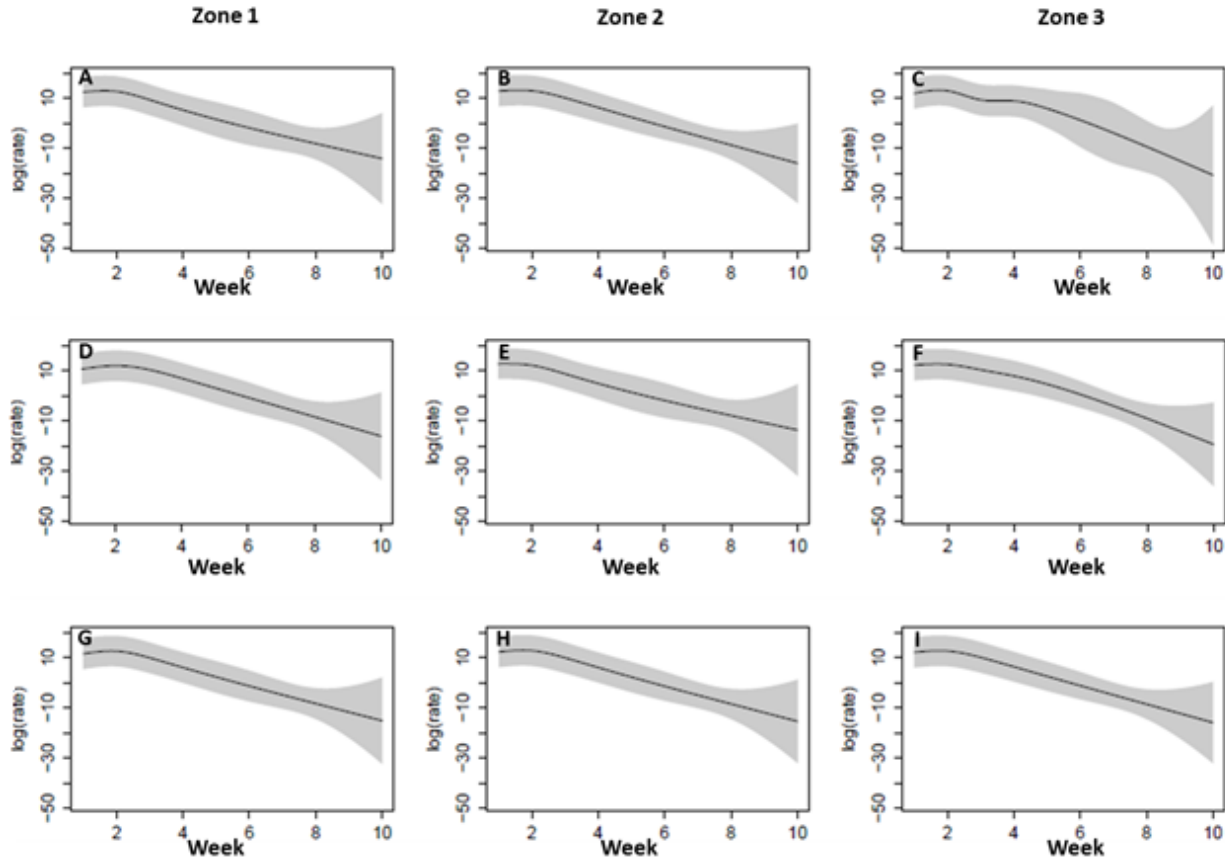


Figure 3.4. Prehook count (Stage I). Plots generated after generalized additive model (GAM) analysis of the data. Each plot corresponds to the data collected from each square foot area marked in the field. 9 plots represent three zones from a single plantation (bed) of Ben Lear cultivar. Zone 1, is one side of the bed and represented by the A, D, G plots. Zone 2 corresponded to the middle area in the bed and represented by B, E, and H. Zone 3, corresponded to the other side of the Ben Lear bed and represented by C, F and I. Gray areas around the curves represent confident intervals. Stage I was predominant in week 1 and 2 of data collection. (June 5th and 12th, respectively). Notice that confidence intervals were narrow in most of the square areas throughout the zones, excepting for squares C, from the zone 3.

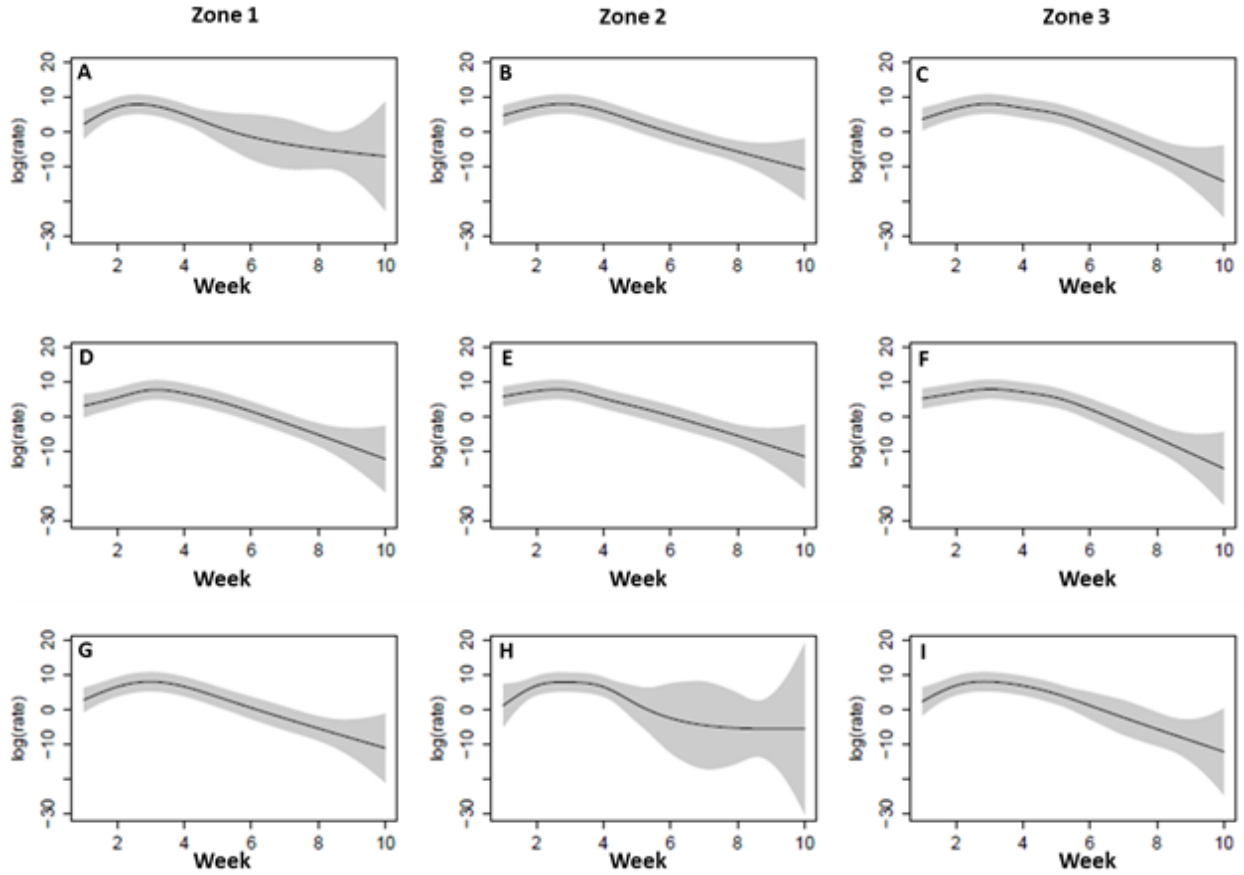


Figure 3. 5. Hook count (Stage II). Plots generated after generalized additive model (GAM) analysis of the data. Each plot corresponds to the data collected from each square foot area marked in the field. 9 plots represent three zones from a single plantation (bed) of Ben Lear cultivar. Zone 1, is one side of the bed and represented by the A, D, G plots. Zone 2 corresponded to the middle area in the bed and represented by B, E, and H. Zone 3, corresponded to the other side of the Ben Lear bed and represented by C, F and I. Gray areas around the curves represent confident intervals. Notice that confidence intervals were narrow in most of the square areas throughout the zones, excepting for squares A and H were the interval as expanded. Those particular areas showed also lower significance values observed in Table 1. In week 3, hook was the phenological stage more predominant around the bed.

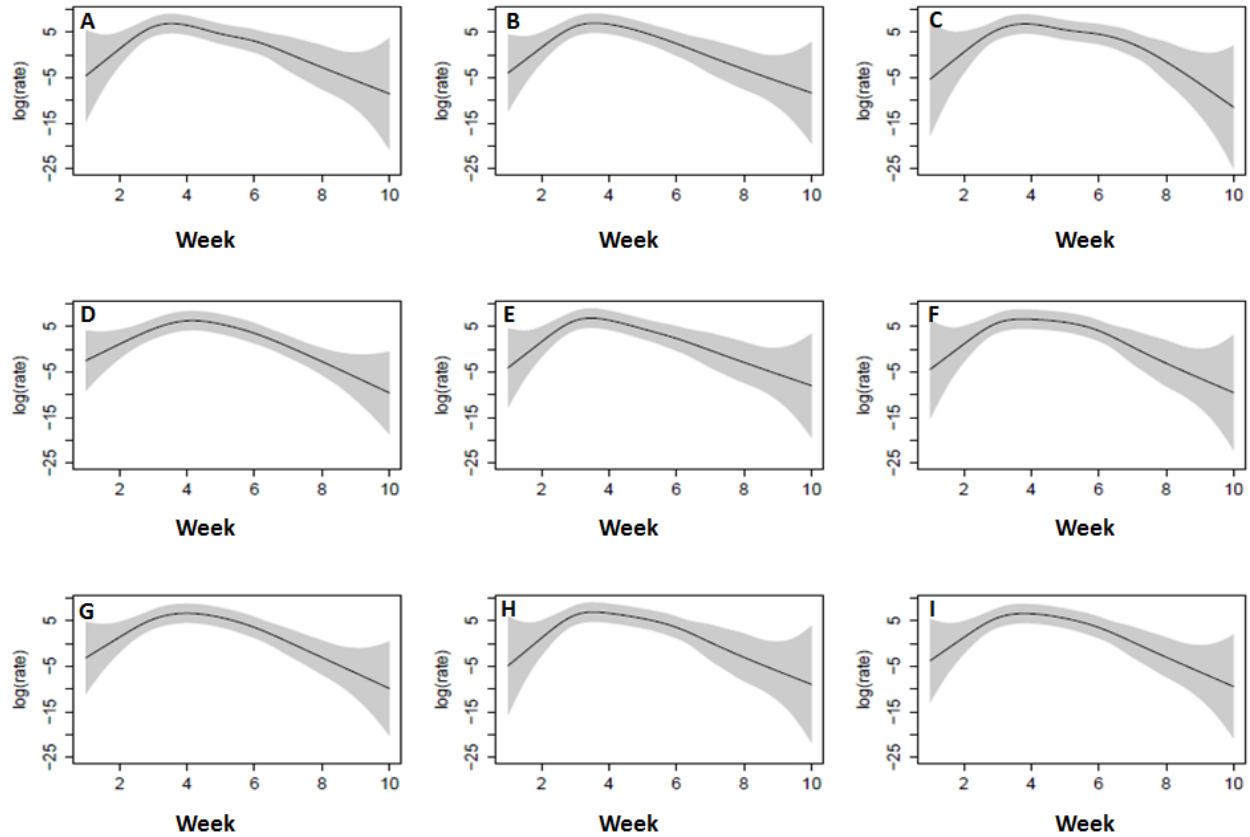


Figure 3.6. Flower count (Stage III). Plots generated after generalized additive model (GAM) analysis of the data. Each plot corresponds to the data collected from each square foot area marked in the field. 9 plots represent three zones from a single plantation (bed) of Ben Lear cultivar. Zone 1, is one side of the bed and represented by the A, D, G plots. Zone 2 corresponded to the middle area in the bed and represented by B, E, and H. Zone 3, corresponded to the other side of the Ben Lear bed and represented by C, F and I. As the time progressed and the uprights continued their development, it was observed that most of the uprights across the bed were in Stage III, at week 4 (July 2nd)

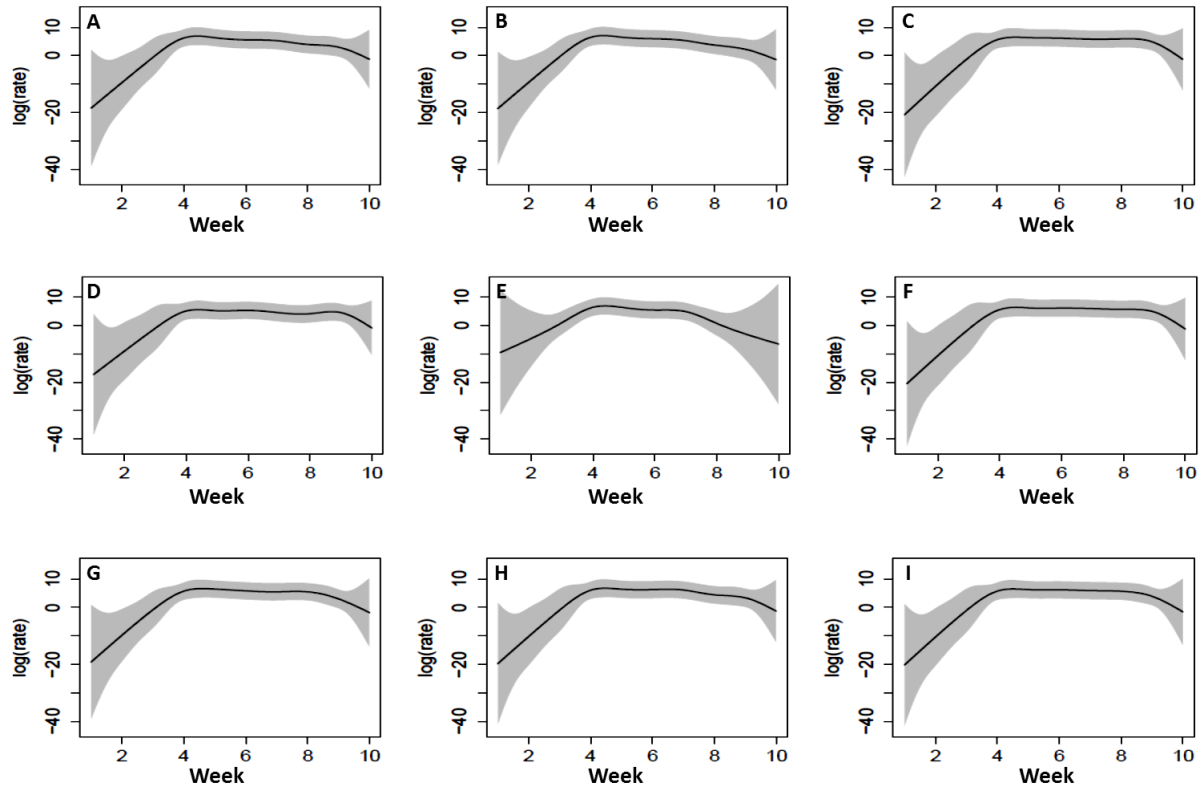


Figure 3. 7. Pinhead stage (Stage IV). Plots generated after generalized additive model (GAM) analysis of the data. Each plot corresponds to the data collected from each square foot area marked in the field. 9 plots represent three zones from a single plantation (bed) of Ben Lear cultivar. Zone 1, is one side of the bed and represented by the A, D, G plots. Zone 2 corresponded to the middle area in the bed and represented by B, E, and H. Zone 3, corresponded to the other side of the Ben Lear bed and represented by C, F and I. As the time progressed and the uprights continued their development, it was observed that most of the uprights across the bed were in Stage III, at week 4 (July 2nd)

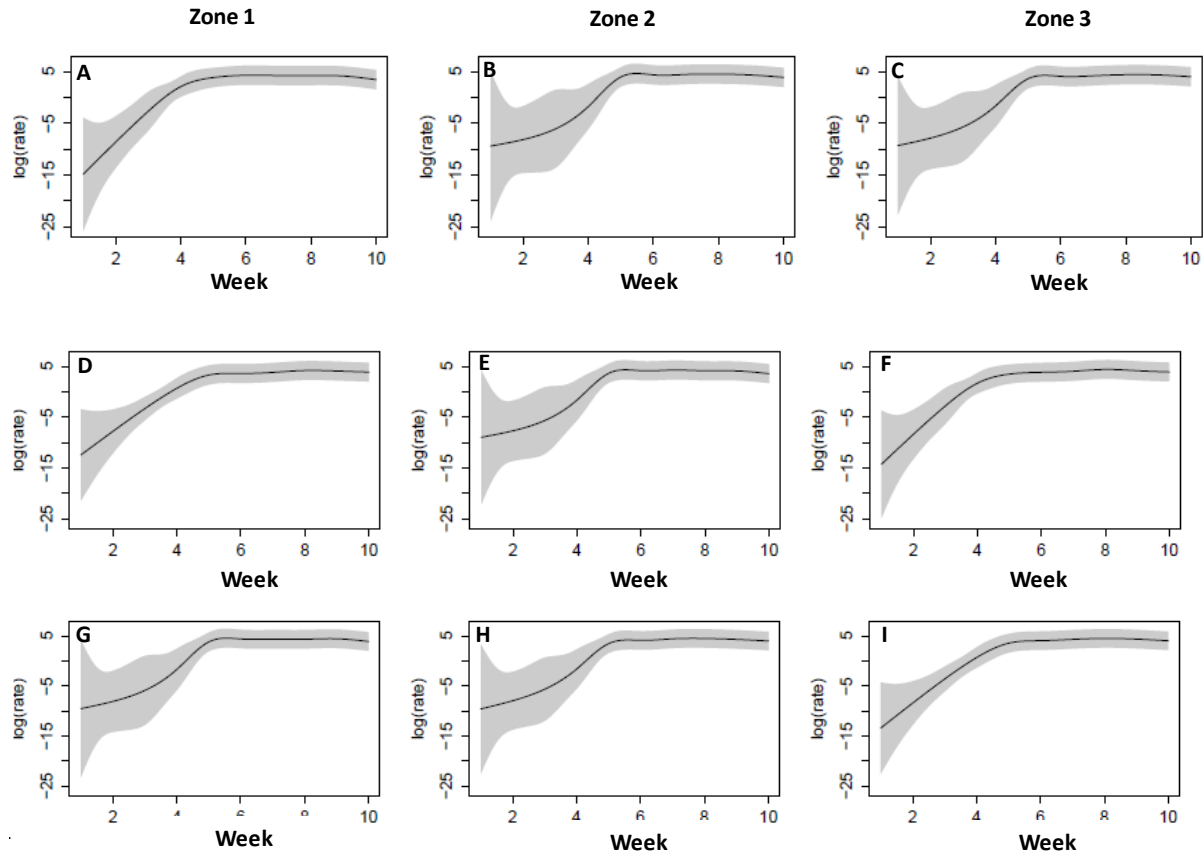


Figure 3.8. Berry count (Stage V). Plots generated after generalized additive model (GAM) analysis of the data. Each plot corresponds to the data collected from each square foot area marked in the field. 9 plots represent three zones from a single plantation (bed) of Ben Lear cultivar. Zone 1, is one side of the bed and represented by the A, D, G plots. Zone 2 corresponded to the middle area in the bed and represented by B, E, and H. Zone 3, corresponded to the other side of the Ben Lear bed and represented by C, F and I. As the time passed, the number of uprights with berries increased to week 5, and from there the number remained constant until the time of harvesting.

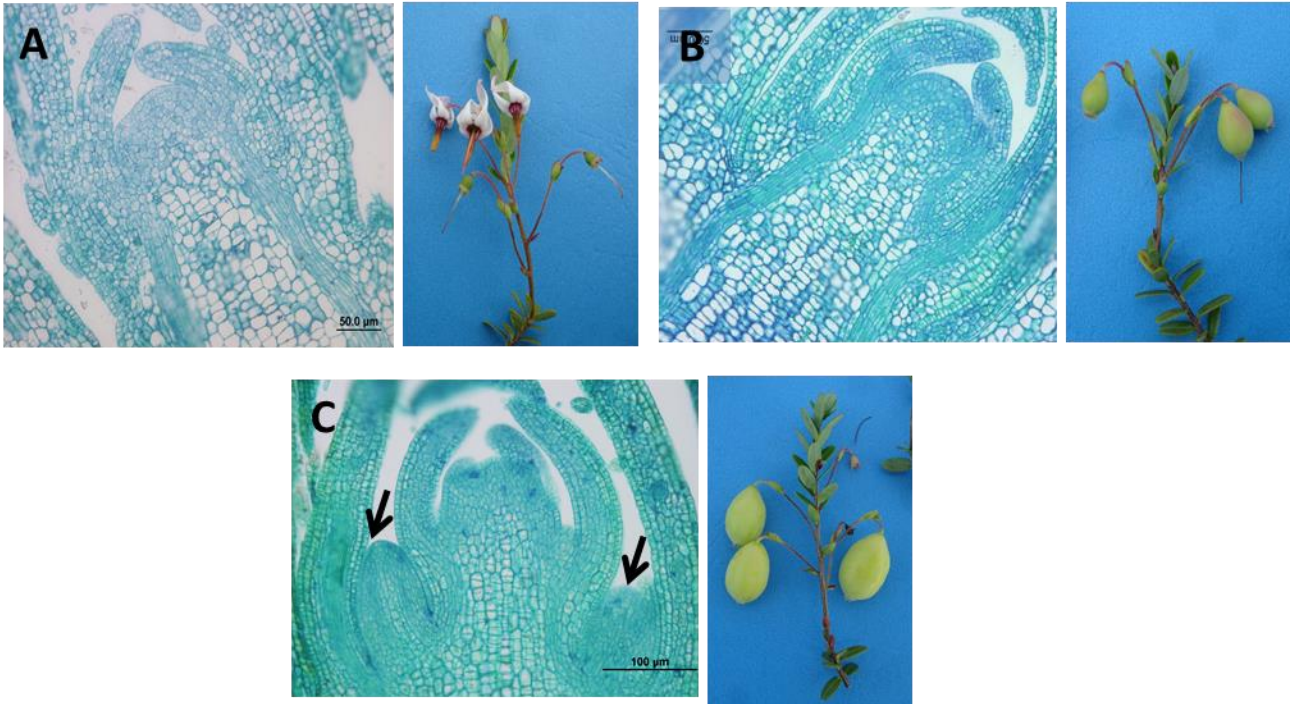


Figure 3.9. Floral induction stages in apical buds of cranberry analyzed by RNA-seq.. Micrographs of apical buds from vegetative uprights showing the SAM and leaf primordia. Pictures to the right of each micrograph depict the phenological stage of the fruiting uprights at the time the apical buds were collected. **A.** Apical bud at the stage of 'Before' floral induction when fruiting uprights were at pinhead stage (Stage IV). **B.** Apical bud 'during' floral induction, one week later first floral initials were differentiating. Fruiting upright were in berry stage (Stage V) at the moment of the bud collection. **C.** Apical bud 'after' floral induction. Arrows point to floral initials developing from axyls of leaf primordia. By that time fruiting upright was in Stage V, berries were still in actively growing

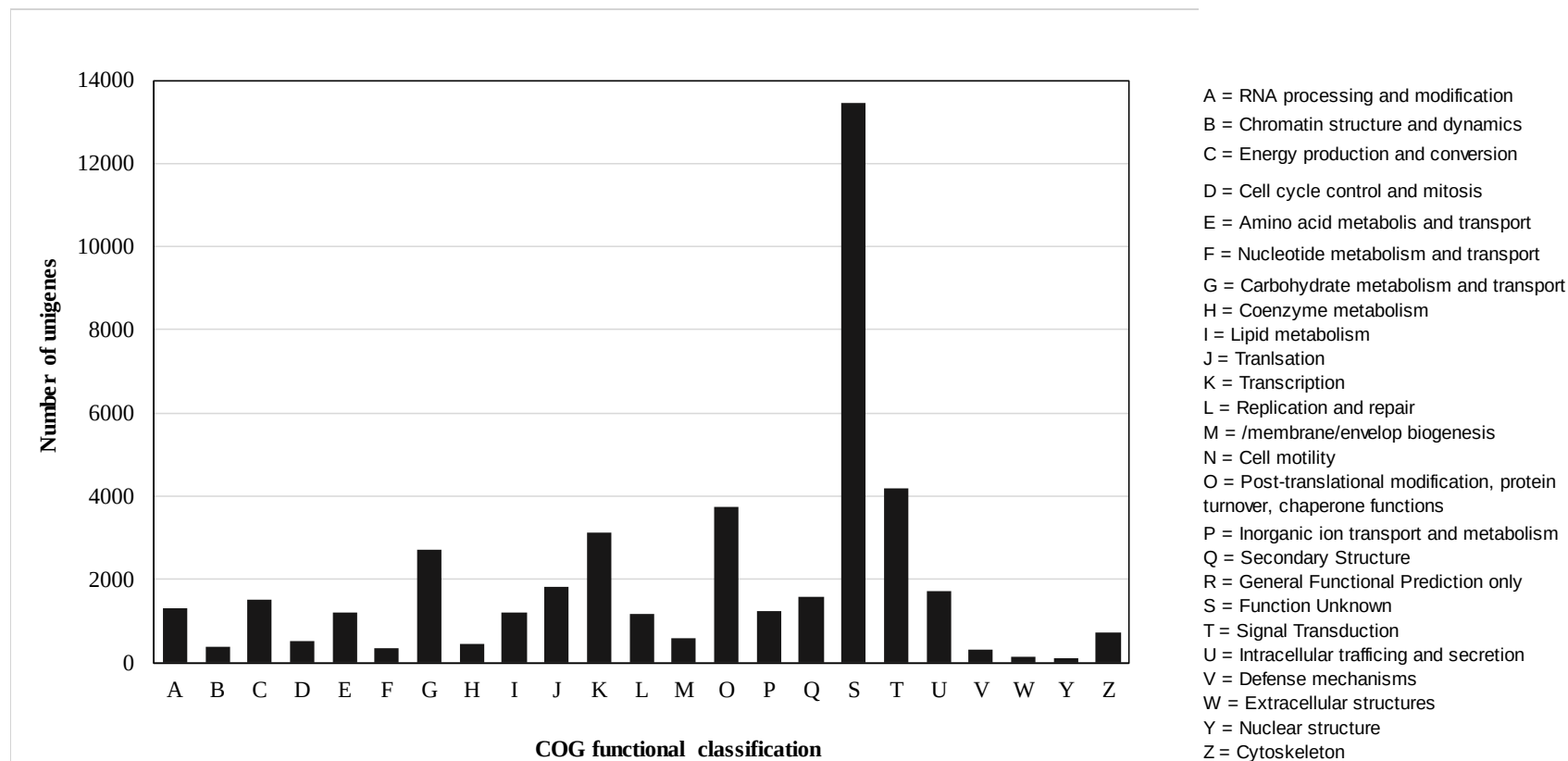


Figure 3.10 Cluster of orthologous groups (GO) classification. From the 172997 transcripts, 41973 unigenes were annotated in 23 of the 25 GO functional classifications. The highest number of unigenes, 13500, was assigned to the unknown function category (S). Other functional classes enriched were T (Signal transduction) with 4188 unigenes, O (Post-translational modification) with 3745 unigenes, K (Transcription), with 3139 genes, and G (Carbohydrate metabolism) with 2728 unigenes.

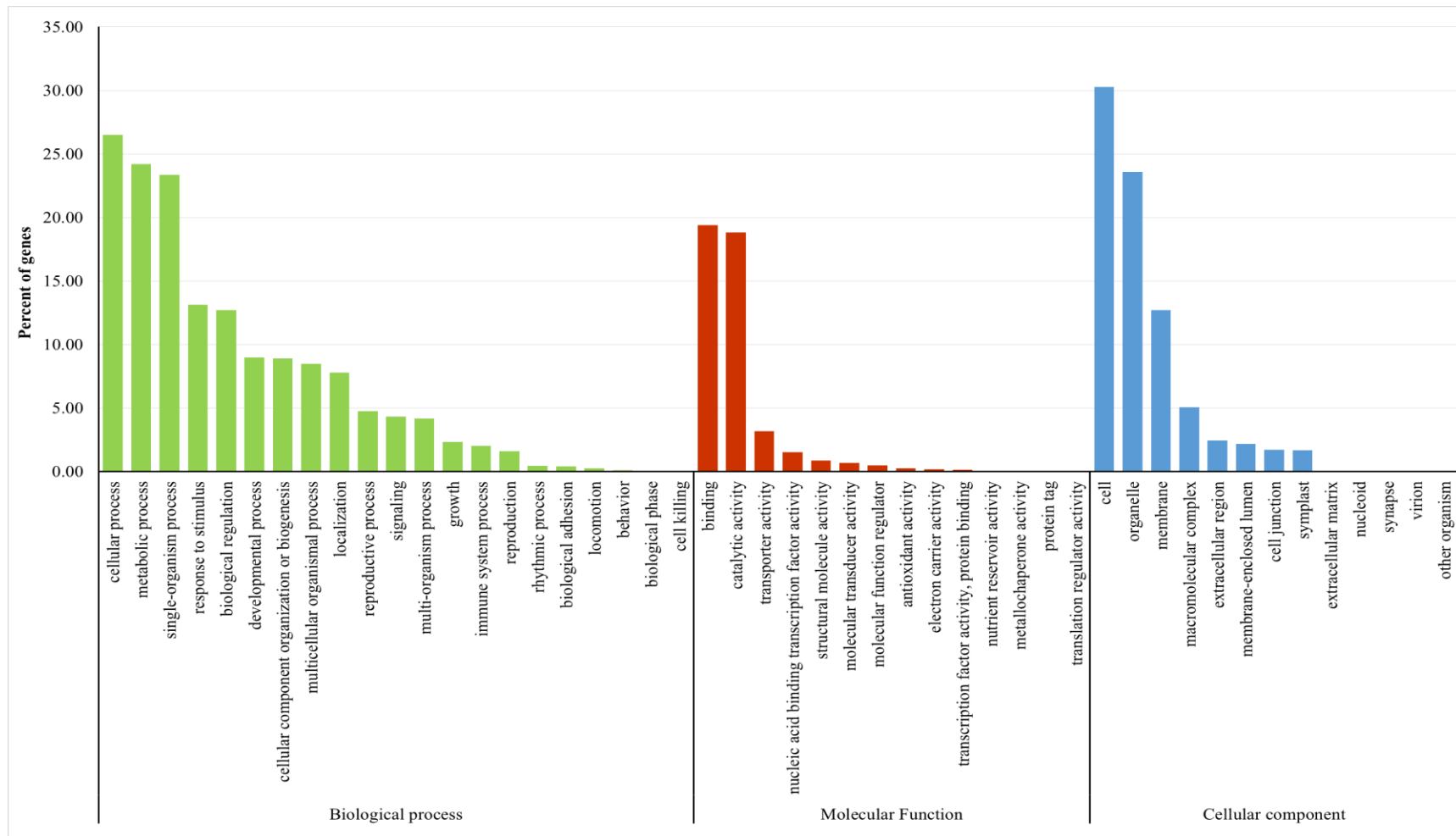


Figure 3.11 Gene ontology classification. Genes from apical buds were classified in three main categories: Biological process, Molecular Function and Cellular Components, and 48 subcategories. y- axis indicates the percentage of annotated genes in each GO category (x-axis). The biological process category was more represented than the other two categories.

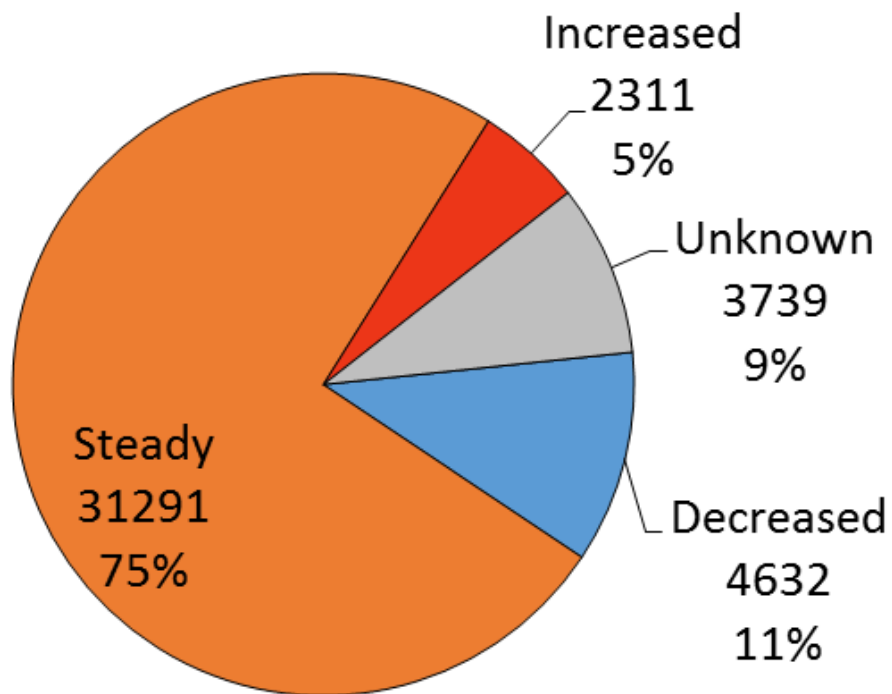


Figure 3.12 Number of genes upregulated in apical buds of vegetative uprights compared to buds from fruiting uprights. Depending on their expression, genes were classified in 4 groups: steady, increased, decreased and unknown. The highest number of genes (31291) did not show differences in expression and represented 75% of the annotated genes. Following this category, the genes with decreased expression in vegetative uprights represented 11% of the genes. Only 5% of the genes showed increased expression in buds from vegetative uprights.

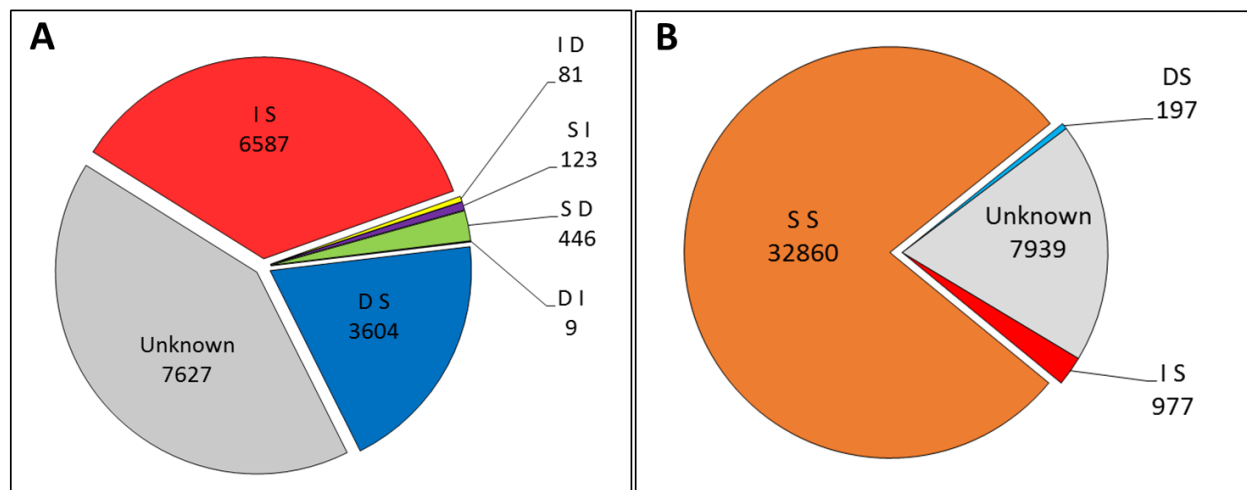


Figure 3.13 Number of genes with differential expression through three floral induction stages in cranberry apical buds from vegetative and fruiting uprights. **A.** Gene expression in buds from vegetative uprights. More than 50% of the genes (23496) were classified in the Steady-Steady (SS) category in vegetative uprights and are not presented in the figure to allow visibility of the other categories **B.** Gene expression in fruiting uprights. Expressed genes were classified in only three gene expression patterns: SS, IS and DS. (IS = Increased-Steady; DS = Decreased-Steady; ID = Increased-Decreased; SI = Steady-Increased, SD = Steady-decreased, DI = Decreased-Increased, DS = Decreased-Steady).

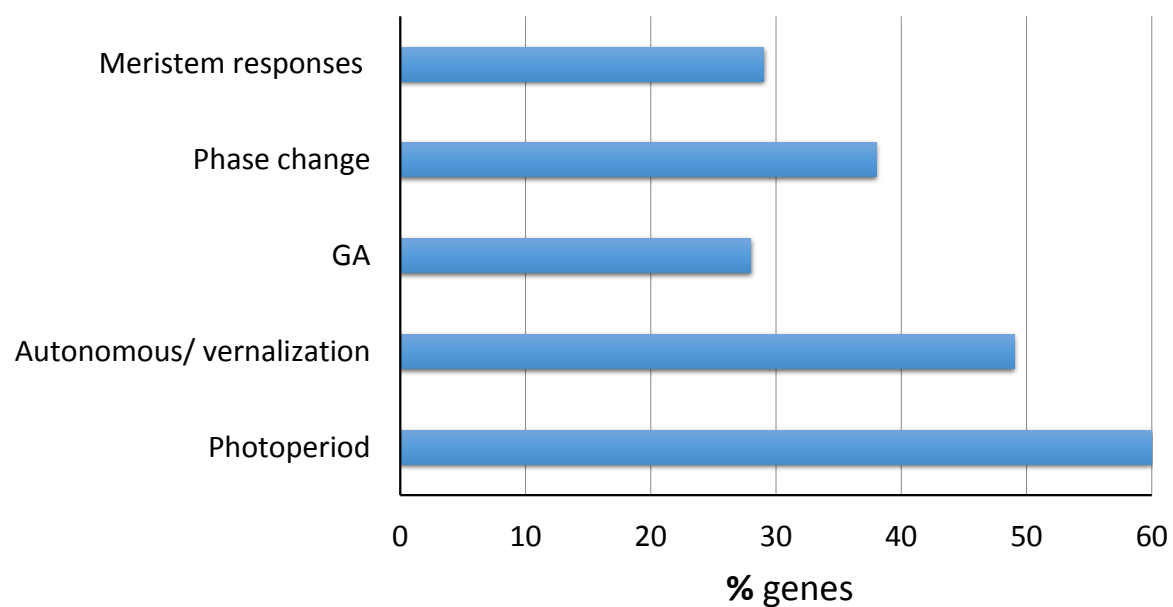


Figure 14. Percentage of *Arabidopsis* genes per flowering pathway with homologous in unigenes from apical buds of cranberry

Supplement Table 3.1. Pairwise comparisons with Bonferroni corrections for the counting of fruiting uprights of cranberry in prehook stage in nine areas of one square foot (S1 to S9) distributed in three zones of a cranberry bed (Ben Lear cultivar) (n=315 fruiting uprights, represented by 35 uprights evaluated per square foot)

		Zone 1			Zone 2			Zone 3		
		S1	S2	S3	S4	S5	S6	S7	S8	S9
Zone 1	S1		0.8939	0.9357	0.8803	0.9714	0.9195	0.7013	0.8251	0.8952
	S2			0.9417	0.8976	0.8785	0.9259	0.6836	0.8847	0.9222
	S3				0.9215	0.9155	0.9653	0.6804	0.8606	0.9457
Zone 2	S4					0.8645	0.9535	0.644	0.8744	0.9724
	S5						0.8984	0.69	0.8053	0.8756
	S6							0.6658	0.8673	0.9718
Zone 3	S7								0.6726	0.6572
	S8									0.8844
	S9									

Supplement Table 3.2. Pairwise comparisons with Bonferroni corrections for the counting of fruiting uprights of cranberry in hook stage in nine areas of one square foot (S1 to S9) distributed in three zones of a cranberry bed (Ben Lear cultivar) (n=315 fruiting uprights, represented by 35 uprights evaluated per square foot)

		Zone 1			Zone 2		Zone 3		
	S1	S2	S3	S4	S5	S6	S7	S8	S9
Zone 1	S1	0.6053	0.6533	0.6037	0.6162	0.6699	0.62	0.5987	0.7448
	S2		0.8914	0.8068	0.8085	0.433	0.8606	0.8047	0.7805
	S3			0.8597	0.8412	0.4619	0.8296	0.778	0.8265
Zone 2	S4				0.9165	0.4206	0.7613	0.75	0.729
	S5					0.4221	0.7546	0.7592	0.7129
	S6						0.4585	0.459	0.5466
Zone 3	S7							0.9296	0.8273
	S8								0.8013
	S9								

		Zone 1			Zone 2		Zone 3		
	S1	S2	S3	S4	S5	S6	S7	S8	S9
Zone 1	S1	0.8055	0.7296	0.7118	0.7383	0.7358	0.7285	0.9313	0.826
	S2	0	0.6519	0.6199	0.6786	0.6872	0.6737	0.8553	0.9059
	S3			0.9456	0.9474	0.9384	0.9649	0.7295	0.6818
Zone 2	S4				0.9041	0.8994	0.9188	0.7037	0.651
	S5					0.9542	0.9656	0.7319	0.6953
	S6						0.9649	0.7523	0.7225
Zone 3	S7							0.7436	0.7037
	S8								0.8659
	S9								

Supplementary Table 3. 6 Cranberry Unigenes with expression pattern SS in apical buds from vegetative and fruiting uprights (BD = transition from floral induction stages 'before' and 'during'; DA= transition from floralinduction stages 'before' and 'during'). Fold change ≥ 2 .

Pathway	unigene	VEGETATIVE UPRIGHTS		FRUITING UPRIGHTS		COG category	BLAST RESULTS						
		V_pattern	V_BD_fc_hange	V_DA_fc_hange	F_pattern		F_BD_fc_hange	F_DA_fc_hange	Gene Model	Symbol	Description	Protein function	
Photoperiod	c92602_g1	SS	0.463959	0.37102	SS	0.46396	0.371017	K	AT1G08970	HAP5C, NF-YC9	nuclear factor Y, subunit C9. Interacts with CO in yeast	CCAAT-box binding transcription factor	0
	c162301_g6	SS	0.108373	0.00776	SS	0.10837	0.007756	K	AT1G09570	PHYA, FHY2, FRE1, HY8	phytochrome A	Red or far-red light photoreceptor	Light perception
	c45817_g1	SS	0.13237	0.13439	SS	0.13237	0.134386	S	AT1G12910	ATAN11, LWD1	Transducin. Along with LWD2 interact with MYB and BHLB proteins	WD40 repeat	photoperiod, circadian clock
	c160066_g3	SS	0.702024	0.30909	SS	0.70202	0.309086	S	AT1G22770	GI, FB	gigantea protein (GI)	-	photoperiod, circadian clock
	c163938_g4	SS	0.639718	0.28592	SS	0.63972	0.285921	K	AT1G25540	PFT1	phytochrome and flowering time regulatory protein (PFT1)	Mediator Complex Subunit 25	Light quality perception acts downstream PHYB. Includes flowerign in suboptimal conditions
	c164422_g3	SS	0.409367	0.26816	SS	0.40937	0.268163	J	AT1G53090	SPA4	SPA1-related 4	WD-40 and protein kinase-like domain	Photoperiod
	c165923_g1	SS	-0.18267	0.04099	SS	-0.1827	0.040986	K	AT1G56170	HAP5B, ATHAP5B, NF-YC2	nuclear factor Y, subunit C2	CCAAT-box binding transcription factor	0
	c161211_g1	SS	-0.74268	-0.08853	SS	-0.7427	-0.088534	O	AT1G68050	FKF1, ADO3	flavin-binding, kelch repeat, f box 1	E3 ubiquitin ligase, blue light photoreceptor	photoperiod, light perception
	c90585_g1	SS	-0.14395	0.14214	SS	-0.1439	0.142137	K	AT2G18790	PHYB, HY3, OOP1	phytochrome B	Red/Far-red light photoreceptor	Light perception
	c246430_g1	SS	1.358842	0.82574	SS	1.35884	0.825735	O	AT2G18915	LKP2, ADO2	LOV KELCH protein 2	E3 ubiquitin ligase	photoperiod, circadian clock
	c241491_g1	SS	-0.36608	0.27396	SS	-0.3661	0.273964	S	AT2G21070	FIO1	methyltransferases	unknown (nuclear localized)	photoperiod, circadian clock
	c157750_g1	SS	0.406433	-0.22619	SS	0.40643	-0.226188	K	AT2G28550	RAP2.7, TOE1	related to AP2.7	AP2-domain transcription factor	Photoperiod
	c164641_g3	SS	0.252662	0.12839	SS	0.25266	0.128389	K	AT2G39250	SNZ	Integrase-type DNA-binding superfamily protein	AP2-domain transcription factor	Photoperiod
	c167067_g1	SS	0.071501	-0.00779	SS	0.0715	-0.007788	K	AT2G42280	ABA-RESPONSIVE KINASE SUBSTRATE 3,	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	helix-loop-hhelix (bHLH)Transcription factor- activator of CO	photoperiodism, flowering, regulation of transcription, DNA-templated, transcription, DNA-templated
	c143379_g1	SS	0.125004	-0.1801	SS	0.125	-0.1801	K	AT2G42280	ABA-RESPONSIVE KINASE SUBSTRATE 3,	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	helix-loop-hhelix (bHLH)Transcription factor- activator of CO	photoperiodism, flowering, regulation of transcription, DNA-templated, transcription, DNA-templated
	c250829_g1	SS	0.311098	0.19436	SS	0.3111	0.194362	J	AT2G46340	SPA1	SPA (suppressor of phyA-105) protein family	WD-40 and protein kinase-like domain	Photoperiod
	c168481_g1	SS	-0.45513	0.49442	SS	-0.4551	0.49442	K	AT2G46790	APRR9, PRR9, TL1	pseudo-response regulator 9	unknown	photoperiod, circadian clock
	c140650_g1	SS	-0.42054	-0.52698	SS	-0.4205	-0.526984	T	AT3G04910	WNK1, ZIKA, ATWNK1	with no lysine (K) kinase 1	0	photoperiod, circadian clock
	c143096_g1	SS	-0.38983	0.5593	SS	-0.3898	0.559302	K	AT3G47500	CDF3	cycling DOF factor 3	DOF transcription factor	Photoperiod
	c173733_g1	SS	0.201459	0.02922	SS	0.20146	0.029217	T	AT3G60250	CKB3	casein kinase II beta chain 3	subunit of CK2 kinase	photoperiod, circadian clock
	c112994_g1	SS	0.401404	0.25683	SS	0.4014	0.256825	LT	AT4G08920	CRY1, BLU1, HY4, OOP2, ATRCY1	cryptochrome 1	blue light photoreceptor	photoperiod, circadian clock
	c167265_g1	SS	0.237627	0.21639	SS	0.23763	0.21639	K	AT4G09180	FBH2, FLOWERING BHLH 2	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	helix-loop-hhelix (bHLH)Transcription factor- activator of CO	regulation of transcription, photoperiod, circadian clock
	c162456_g2	SS	0.262506	0.20779	SS	0.26251	0.207787	J	AT4G11110	SPA2	SPA1-related 2	WD-40 and protein kinase-like domain	Photoperiod
	c133180_g2	SS	-0.21236	0.29129	SS	-0.2124	0.291287	K	AT4G14540	NF-YB3	nuclear factor Y, subunit B3	CCAAT-box binding transcription factor	Photoperiod
	c188321_g1	SS	0.204207	0.04206	SS	0.20421	0.042062	K	AT4G18130	PHYE	phytochrome E	red/far red light photoreceptor	photoperiod, circadian clock
	c160596_g2	SS	0.172829	-0.25335	SS	0.17283	-0.253352	K	AT4G34530	CIB1	cryptochrome-interacting basic-helix-loop-helix 1	bHLH transcription factor	Light signaling
	c165864_g1	SS	-0.5885	0.36027	SS	-0.5885	0.360268	K	AT5G15840	CO_66	B-box type zinc finger protein with CCT domain	Zinc finger transcription factor	Photoperiod
	c159496_g2	SS	-0.01967	0.48278	SS	-0.0197	0.482784	K	AT5G35840	PHYC	phytochrome C	red/far red light photoreceptor	photoperiod, circadian clock
	c170787_g2	SS	0.856826	0.611	SS	0.85683	0.611003	O	AT5G57360	ZTL, LKP1, ADO1, FKL2	Galactose oxidase	E3 ubiquitin ligase, blue lught photoreceptor	Circadian clock and photoperiod
	c171460_g2	SS	0.937233	0.07095	SS	0.93723	0.070953	S	AT5G60120	TOE2	target of early activation tagged (EAT) 2	AP2-domain transcription factor	photoperiod
Aging	c157750_g1	SS	0.406433	-0.22619	SS	0.40643	-0.226188	K	AT2G28550	RAP2.7, TOE1	related to AP2.7	AP2-domain transcription factor	Photoperiod
	c164641_g3	SS	0.252662	0.12839	SS	0.25266	0.128389	K	AT2G39250	SNZ	Integrase-type DNA-binding superfamily protein	Encodes a AP2 domain transcription factor that can repress flowering. SNZ and its paralogue gene, SCHLAFMUTZE (SMZ), share a signature with partial complementarity to the miR172 microRNA, whose precursor is induced upon flowering.	Photoperiod
	c156966_g2	SS	0.166473	0.28995	SS	0.16647	0.289952	K	AT4G36920	AP2, FLO2, FL1	Integrase-type DNA-binding superfamily protein	a member of the AP2/EREBP (ethylene responsive element binding protein) class of transcription factors and is involved in the specification of floral organ identity, establishment of floral meristem identity, suppression of floral meristem indeterminacy, transcription factor	cell differentiation, flower development, meristem maintenance, ovule development, regulation of transcription, DNA-templated, seed development, sexual reproduction, specification of floral organ identity, transcription, DNA-templated
	c171460_g2	SS	0.937233	0.07095	SS	0.93723	0.070953	S	AT5G60120	TOE2	target of early activation tagged (EAT) 2		photoperiod
	c202208_g1	SS	0.195166	0.19715	SS	0.19517	0.197148	K	AT5G67180	TOE3	target of early activation tagged (EAT) 3	AP2-domain transcription factor	0 AP2-domain transcription factor
Autonomous/Vernalization	c172741_g3	SS	0.261095	-0.07136	SS	0.2611	-0.071365	A	AT1G17760	CSTF77, ATCSTF77	Tetratricopeptide repeat (TPR)-like superfamily protein	Component of RNA 3'-processing complex. Silence FLC by 3' processing FLC antisense transcripts	Vernalization
	c163352_g2	SS	-0.00065	-0.13021	SS	-0.0007	-0.130208	A	AT1G71800	CSTF64	cleavage stimulating factor 64	Component of RNA 3'-processing complex. Silence FLC by 3' processing FLC antisense transcripts	Vernalization
	c153674_g1	SS	1.637974	-0.10965	SS	1.63797	-0.109652	U	AT1G76710	SDG26	SET domain group 26	SET domain protein	0
	c124221_g1	SS	-0.10601	-0.18074	SS	-0.106	-0.180735	B	AT2G19520	FVE, ACG1, MS14, NFC4, NFC04, ATMS14	Transducin family protein	MS1-homologue	Autonomous
	c168574_g1	SS	-0.39078	-0.47929	SS	-0.3908	-0.479286	K	AT2G23380	CLF, ICU1, SDG1, SET1	SET domain-containing protein	SET domain protein component of polycomb repressive complex 2	0
	c148297_g2	SS	0.406138	0.2191	SS	0.40614	0.219097	A	AT2G43410	FPA	RNA binding	RNA-binding protein	Autonomous
	c148405_g2	SS	-0.217	-0.18787	SS	-0.217	-0.187871	A	AT3G04610	FLK	RNA-binding KH domain-containing protein	RNA binding protein	Autonomous
	c158357_g1	SS	0.303608	0.02359	SS	0.30361	0.023593	H	AT3G10390	FLD	Flavin containing amine oxidoreductase family protein	Hisonc deacetylation associated protein	Autonomous
	c169102_g1	SS	0.108639	-0.47797	SS	0.10864	-0.477968	S	AT3G12810	PIE1, SRCAP, chr13	SNF2 domain-containing protein Required for FLC transcriptional activation	Similar to ATP-dependent, chromatin-remodeling proteins of the ISWI and SWI2/SNF2 family	0
	c156551_g2	SS	-0.03807	0.12402	SS	-0.0381	0.124023	Z	AT3G33520	ATARP6, ARP6, SUF3, ESD1	actin-related protein 6. Required for FLC transcriptional activation		0
GA	c167135_g2	SS	0.171313	0.01404	SS	0.17131	0.014045	K	AT4G02560	LD	Homeodomain-like superfamily protein	Homeodomain protein	Autonomous
	c145290_g1	SS	0.89123	0.33319	SS	0.89123	0.333189	K	AT4G32980	ATH1	homeobox gene 1	MAINTAINS flc repression after cold treatment	0
	c165806_g1	SS	0.122553	0.14254	SS	0.12255	0.14254	K	AT5G04240	ELF6	Zinc finger (C2H2 type) family protein	Jumonji protein involved in histone demethylation	-
	c150513_g1	SS	1.115927	0.53188	SS	1.11593	0.531876	Q	AT1G30040	ATGA2OX2, GA2OX2	gibberellin 2-oxidase		
	c172017_g2	SS	0.308914	-0.05013	SS	0.30891	-0.05013	E	AT1G78440	ATGA2OX1, GA2OX1	Arabidopsis thaliana gibberellin 2-oxidase 1		
Meristem responses	c154977_g1	SS	-0.28534	0.09658	SS	-0.2853	0.09658	K	AT3G11440	ATMYB65, MYB65	myb domain protein 65		
	c172069_g2	SS	0.131323	0.46357	SS	0.13132	0.463565	T	AT3G63010	ATGID18, GID18	alpha		
	c156312_g1	SS	1.686285	0.89556	SS	1.68629	0.895559	E	AT4G21200	ATGA2OX8, GA2OX8	gibberellin 2-oxidase 8		
	c171620_g2	SS	0.296131	-0.41824	SS	0.29613	-0.41824	K	AT1G24260	SEP3, AGL9	K-box region and MADS-box transcription factor family protein	Flower development	MADS-box transcription factor
	c169698_g7	SS	-0.25177	0.37779	SS	-0.2518	0.377791	K	AT2G27990	BLH8, PNF	BEL1-like homeodomain 8	Inflorescence meristem development	BEL1-like homeodomain transcription factor

Supplementary Table 3. 7 Cranberry Unigenes with expression pattern DS in apical buds from vegetative and SS in fruiting uprights (BD = transition from floral induction stages 'before' and 'during'; DA= transition from floralinduction stages 'before' and 'during'). Fold change ≥ 2 .

Pathway	unigene	VEGETATIVE UPRIGHTS			FRUITING UPRIGHTS			COG category	BLAST RESULTS				
		V_pattern	V_BD_fc hange	V_DA_fc hange	F_patte rn	F_BD_fc hange	F_DA_fc hange		Gene Model	Symbol	Description	Protein function	
Photoperiod	c148048_g1	D S	-1.69213	1.3457	S S	-1.69213	1.3457	K	AT1G01060	LHY, LHY1	Late elongated hypocotyl Homeodomain-like superfamily protein	Myb transcription factor	photoperiod, circadian clock
	c111153_g1	D S	-1.38258	0.91757	S S	-1.38258	0.91757	K	AT1G69570	CDF5 (CYCLING DOF FACTOR 5)	Dof-type zinc finger DNA-binding family protein	DOF transcription factor	Photoperiod
	c162013_g2	D S	-1.18725	0.40972	S S	-1.18725	0.40972	S	AT2G25930	ELF3, PYK20	hydroxyproline-rich glycoprotein family protein	unknown (nuclear localized)	photoperiod, circadian clock
	c166008_g1	D S	-0.4945	0.27705	S S	-0.4945	0.27705	O	AT2G32950	COP1, ATCOP1, DET340, FUS1, EMB1	COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1) Transducin	E3 ubiquitin ligase	Photoperiod
	c92267_g1	D S	-0.73681	0.16983	S S	-0.73681	0.16983	K	AT5G57660	ATCOL5, COL5	CONSTANS-like 5	Zinc finger transcription factor	Photoperiod
Autonomous/Vernalization	c253001_g1	D S	-1.04913	-0.43018	S S	-1.04913	-0.43018	U	AT1G77300	EFS, SDG8, CCR1, ASH2, LAZ2	histone methyltransferases(H3-K4 specific);histone methyltransferases(H3-K36 specific) . Required for FLC transcriptional activation		SET domain protein involved in histone modification probably functionally related to Paf1C (polymerase-associated factor 1 complex). Flowering inhibitor
	c171372_g1	D S	-0.67957	-0.32266	S S	-0.67957	-0.32266	BK	AT2G31650	ATX1, SDG27	ATX1 (ARABIDOPSIS TRITHORAX 1) homologue of trithorax --> expressed in gynoecium primordium. Required for FLC transcriptional activation	-	
	c157938_g2	D S	-0.87941	-0.19935	S S	-0.87941	-0.19935	S	AT3G48430	REF6	relative of early flowering 6	-	Jumonji protein that is involved in histone
	c159739_g1	D S	-0.59713	-0.22238	S S	-0.59713	-0.22238	A	AT5G13480	FY	Transducin	Autonomous	Polyadenylation factor
GA	c216281_g1	D S	-0.51983	0.3805	S S	-0.51983	0.3805	K	AT1G14920	GAI, GIBBERELIC ACID INSENSITIVE, R	DELLA GRAS family transcription factor family		
Meristem responses	c200713_g1	D S	-0.24597	0.12055	S S	-0.24597	0.12055	K	AT2G22540	SVP, AGL22	K-box region and MADS-box transcription factor family protein	Floral repressor	MADS-box transcription factor
	c153633_g1	D S	-1.53099	-0.77972	S S	-1.53099	-0.77972	S	AT5G03840	TFL1, TFL-1	PEBP (phosphatidylethanolamine-binding protein) family protein	Inflorescence architecture	Phosphatidylethanolamine-binding protein
Woody sps	c158898_g1	D S	-0.93003	-0.80048	S S	-0.93003	-0.80048	J	flowering gene gi 337733642		gb AEI72270.1 HM596721	eukaryotic translation initiation factor protein [Citrus trifoliata]	

Supplementary Table 3. 8 Cranberry Unigenes with expression pattern SD in apical buds from vegetative and SS in buds from fruiting uprights (BD = transition from floral induction stages 'before' and 'during'; DA= transition from floralinduction stages 'before' and 'during'). Fold change ≥ 2 .

Pathway	unigene	VEGETATIVE UPRIGHTS			FRUITING UPRIGHTS			COG category	BLAST RESULTS				
		V_pattern	V_BD_fc hange	V_DA_fc hange	F_patte rn	F_BD_fc hange	F_DA_fc hange		Gene Model	Symbol	Description	Protein function	
Photoperiod	c143616_g1	S D	0.045798	-0.39632	S S	0.045798	-0.39632	K	AT2G38880	HAP3A, NF-YB1 . Regulates Co, GI, FT	nuclear factor Y, subunit	CCAAT-box binding	Photoperiod

Supplementary Table 3. 9 Cranberry Unigenes with expression pattern IS in apical buds from vegetative and SS in buds from fruiting uprights (BD = transition from floral induction stages 'before' and 'during'; DA= transition from floralinduction stages 'before' and 'during'). Fold change ≥ 2 .

Pathway	unigene	VEGETATIVE UPRIGHTS			FRUITING UPRIGHTS			COG category	BLAST RESULTS				
		V_pattern	V_BD_fc hange	V_DA_fc hange	F_patte rn	F_BD_fc hange	F_DA_fc hange		Gene Model	Symbol	Description	Protein function	
Photoperiod	c139760_g1	IS	0.855091	0.26162	S S	0.855091	0.26162	LT	AT1G04400	CRY2, FHA, AT-PHH1, PHH1, ATCRY2	cryptochrome 2	Blue light photoreceptor	Light perception
	c149845_g1	IS	1.902098	-0.15255	S S	1.902098	-0.15255	S	AT2G28550	RAP2.7 TOE1 (TARGET OF EAT 1)	related to AP2.7	AP2-domain transcription factor	Photoperiod
Aging	c149845_g1	IS	1.902098	-0.15255	S S	1.902098	-0.15255	S	AT2G28550	RAP2.7 . TOE1	related to AP2.7	AP2-domain transcription fa	Photoperiod
Autonomous/Vernalization	c158075_g1	IS	0.201896	-0.11953	S S	0.201896	-0.11953	O	AT1G14400	UBC1, ATUBC1	ubiquitin carrier protein 1		E2 enzyme involved in monoubiquitination of histone 2B
	c158343_g1	IS	0.347098	0.07944	S S	0.347098	0.07944	O	AT1G55250	HUB2	histone mono-ubiquitination 2		E3 enzyme involved in monoubiquitination of histone 2B
	c168986_g3	IS	0.384533	-0.20516	S S	0.384533	-0.20516	P	AT2G06210	ELF8, VIP6	binding	-	Component of Paf1c. Flowering inhibitor
	c158963_g2	IS	0.230204	-0.05024	S S	0.230204	-0.05024	O	AT3G49600	UBP26, SUP32, ATUBP26	ubiquitin-specific protease 26		Protease that removes ubiquitin from histone 2B
GA	c122927_g1	IS	1.298987	-0.23324	S S	1.298987	-0.23324	E	AT4G25420	GA5, GA200X1, AT2301, ATGA200X1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein		

Supplementary Table 3. 10 Cranberry Unigenes with expression pattern SS in apical buds from vegetative uprights and DS in buds from fruiting uprights (BD = transition from floral induction stages 'before' and 'during'; DA= transition from floralinduction stages 'before' and 'during'). Fold change ≥ 2 .

Pathway	unigene	VEGETATIVE UPRIGHTS			FRUITING UPRIGHTS			COG category	BLAST RESULTS				
		V_pattern	V_BD_fchan ge	V_DA_fchan nge	F_patter n	F_BD_fchang e	F_DA_fchang e		Gene Model	Symbol	Description	Protein function	
Photoperiod	c159673_g1	SS	-1.07802	1.359954	D S	-1.07802	1.359954	K	AT1G68840	RAV2, RAP2.8, TEM2, EDF2, AtRAV2	related to ABI3, Tempranillo 2. Floral repressor	AP2-domain transcription factor	Floral repressor

Supplementary Table 3. 11 Cranberry Unigenes with expression pattern SS in apical buds from vegetative uprights and IS in buds from fruiting uprights (BD = transition from floral induction stages 'before' and 'during'; DA= transition from floralinduction stages 'before' and 'during'). Fold change ≥ 2 .

Pathway	unigene	VEGETATIVE UPRIGHTS			FRUITING UPRIGHTS			COG category	BLAST RESULTS				
		V_pattern	V_BD_fchange	V_DA_fchange	F_pattern	F_BD_fchange	F_DA_fchange		Gene Model	Symbol	Description	Protein function	
Photoperiod	c152356_g1	SS	0.00044451	-0.0816375	IS	0.000444507	-0.081637524	K	AT3G54990	SMZ	Integrase-type DNA-binding superfamily protein	AP2-domain transcription factor	Photoperiod
	c168943_g3	SS	0.338947	0.123273	IS	0.338947004	0.123272989	O	AT5G46210	CUL4, ATCUL4	cullin4	Component of ubiquitin ligases	Light signaling
Aging	c152356_g1	SS	0.00044451	-0.0816375	IS	0.000444507	-0.081637524	K	AT3G54990	SMZ	Integrase-type DNA-binding superfamily protein	Encodes a AP2 domain transcription factor that can repress flowering. SMZ and its paralogous gene, SNARCHZAPFEN (SNZ), share a signature with partial complementarity to the miR172 microRNA, whose precursor is induced upon flowering.	Photoperiod
Autonomous/Vernalization	c200602_g1	SS	-0.0859427	-0.1891814	IS	-0.085942729	-0.189181422	T	AT4G39400	BRI1, CBB2, DWF2, BIN1, ATBRI1	Leucine-rich receptor-like protein kinase family protein	Brassinosteroid receptor. + regulates transition to flowering. Repress FLC	
	c167548_g1	SS	0.08901638	0.1875975	IS	0.08901638	0.187597494	K	AT5G23150	HUA2 , enhancer of AG-4 2.	Tudor	Involved in mRNA processing	

Supplementary Table 3. 12 Cranberry Unigenes with expression pattern unknown in apical buds from vegetative and fruiting uprights (BD = transition from floral induction stages 'before' and 'during'; DA= transition from floralinduction stages 'before' and 'during'). Fold change ≥ 2 .

Pathway	unigene	VEGETATIVE UPRIGHTS			FRUITING UPRIGHTS			COG category	BLAST RESULTS				
		V_pattern	V_BD_fchange	V_DA_fchange	F_pattern	F_BD_fchange	F_DA_fchange		Gene Model	Symbol	Description	Protein function	
Photoperiod	c157685_g1	NS	NA	1.069405	NN	NA	1.069404967	K	AT1G51140	ABA-RESPONSIVE KINASE SUBSTRATE 1, AKS1	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	photoperiodism, flowering, regulation of stomatal movement, regulation of transcription, DNA-templated, transcription, DNA-templated	
Autonomous/Vernalization	c167711_g4	NS	NA	0.076493	NN	NA	0.076493	K	AT4G16845	VRN2	VEFS-Box of polycomb protein	POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) component; homologue of Su(z)12	
Meristem responses	c151529_g1	NS	NA	0.904285	NN	NA	0.904285	K	AT5G60910	AGL8, FUL	AGAMOUS-like 8	Floral integrator and flower meristem identity	MADS-box transcription factor

Chapter 4.

A vigor study based on three self- and cross-pollinated cranberry cultivars

4.1 Abstract

Cranberry (*Vaccinium macrocarpon* Ait.) is known to produce fruits via self- or cross-pollinations, but it has been observed that seed number is reduced in selfed fruit. However, the effect of the proportion of self- and cross-pollinated seeds in fruits produced in the field has not been reported. On the other hand, lower vigor in seedlings from self-pollinations has been mentioned, but there have not been studies reporting these results. In the present study, we analyzed the vigor of seedlings coming from self- and cross-pollinated seeds of fruits growing in field conditions of HyRed, Ben Lear and Stevens. In addition, relationship between fruit traits and number of seeds selfed or outcrossed were evaluated. The results show that vigor, given by height of the seedling and leaf number was reduced in seedlings from self-pollinations compared to cross-pollinated seedlings and varied depending on the cultivar. Fruit size was positively related with the number of seeds, and the presence of cross-pollinated seeds. Size, volume and density of the fruits are positively related with the number of seeds. Our results suggest that fruit production in cranberry could benefit more for cross-pollinations, than self-pollinations. Self-pollinations are most likely to occur in higher proportions in beds where only one genotype is present than in a heterogeneous bed, as shown by principal component analysis.

4.2 Introduction

The benefits of cranberries to human health have been known for centuries beginning with the Native Americans who first used the cranberry fruit for medicinal purposes. Despite its qualities, cranberry was not domesticated and commercialized until the late 1800s in Cape Cod, Massachusetts when wild cranberry plants were collected and planted as a crop. Twenty years later, cranberry cultivation expanded to Wisconsin (Caruso *et al.*, 2000), where its popularity grew making the state currently the main producer worldwide (USDA, 2014)

In the 1920s traditional and controlled plant breeding was applied for the first time in cranberry to produce cultivars with improved agricultural traits. As a result of these first breeding efforts, the USDA released seven cranberry cultivars in the 1950s and 1960s, including Stevens, the most widely grown cultivar today, with increased fruit production and improved fruit quality traits (McCown and Zeldin, 2003; Roper, 2001; Vorsa and Johnson-Cicalese, 2012). In the late 1990s and early 2000s, 50 years later, a second generation of cranberry cultivars was released by the University of Wisconsin-Madison and Rutgers University and other independent breeders. The released cultivars possess improved yield and quality traits coupled with reduced alternate bearing tendencies (Vorsa and Johnson-Cicalese, 2012).

As a crop, cranberry plants grow in specially designed large, sunken plantings (0.5 to 5 hectares), called beds. Plants are propagated clonally within each cranberry bed, but numerous beds of multiple cultivars can be arranged in extensive field plantings. Thus, each bed is mostly

composed of one clonal genotype, but consecutive beds could have the same cultivar or different cultivars (i.e., genotypes) depending on the farm. Although the protandric characteristic of cranberry, where the androecium (anthers and pollen) matures earlier than the gynoecium (carpel and ovules), inhibits self-pollination due to the clonal nature of cranberry plantings, individual flowers tend to be pollinated from other flowers on the same upright or in the same clonal bed. Therefore, fruits and seeds from one bed are most likely the result of self-pollinations (pollen from uprights of the same cultivar) or the result of cross-pollinations when pollen is transported by insects from other cultivars planted in neighbor beds. Previous studies have characterized the effect of self- and cross-pollinations on the count of seeds per berry and the berry set capability of the upright in a diverse number of cultivars (MacKenzie, 1994; Sarracino and Vorsa, 1991); unfortunately, studies characterizing the extent of self-pollination in a cranberry bed have not been conducted and the effect that seed number has on the size and shape of cranberry fruit has not thoroughly been explored.

Because the clonally propagated cranberry in beds can remain productive for decades, the genetic homogeneity of a bed can gradually decrease due to the chance establishment of selfed or outcrossed seedlings from unharvested fruits (Fajardo, Morales, Zhu, Steffan, Harbut, Bassil, and Zalapa, 2012). Investigations on inbreeding depression comparing the vigor of self- vs. cross-pollinated seedlings have not been conducted; and therefore, uncertainty remains about whether the “off-genotypes” established in these commercial beds commonly arise from self-fertilized seedlings or cross-pollinated seedlings. In addition, since inbreeding depression has not been well characterized in cranberry, increasing understanding about its prevalence could have implications about breeder and selection strategies for cranberry germplasm improvement.

Therefore, this chapter aims to determine: 1) the effect of seed number on cranberry fruit size and shape, 2) the proportion of self- vs. cross-pollinated seeds in commercial cranberry beds and within individual fruits in those beds, 3) the effect of self-pollination vs. cross-pollination on the total number of seeds within a fruit, 4) the number of self- and cross-pollinated seeds within a fruit known to contain at least one cross-pollinated seed, 5) the effects of self-pollination on cranberry seedling vigor, and 6) the relationship between fruit and seedling vigor in cranberry. These studies utilized ripe fruits collected from a commercial cranberry farm. Data were collected on the size, shape, and seed number of each fruit, and seeds were germinated from a subset of these fruit for the seedling vigor (measured in terms of height and leaf number over a month) analysis. DNA fingerprinting analysis was used to determine whether the individual seedlings arose from self- or cross-pollination events. Relationships between seedling vigor and pollination type were found across the three cultivars, and significant effects of seed number on various cranberry fruit traits were observed. In addition, greenhouse experiments designed to induce growth of fruiting uprights and self- and cross-pollinations are described.

4.3 Materials and methods

4.3.1 Fruit collection from the field.

During the fruit harvesting season, in October 2014, 160 fully mature fruits were collected during flood harvest from three commercial cultivars: Ben Lear, Stevens, and HyRed, each planted in 1.5 hectare beds at a commercial marsh in Necedah, WI. After collection, fruits were

stored at 4°C until their evaluation and seed planting. Data of diameter, length, weight and number of seeds per fruit were recorded. The volume of the fruits was calculated as the volume of an ellipsoid: $Volume = 2\pi[b^2a - \frac{1}{3}b^2a]$ where a is half the length of the fruit and b is half the width of the fruit, and density, calculated as $mass / volume$ (Supplementary Table 4.1).

4.3.2 Outline of experiments using field collected fruits

The following experiments, illustrated also in Figure 1, were carried out with data obtained from fruits and seedlings produced in field conditions.

4.3.2.1 Effect of seed number of cranberry fruit size and shape. Using data for all 160 fruits, Pearson correlations were performed to determine the relationships between the number of seeds and fruit traits such as: volume, density, length, width, and weight. Additionally, linear mixed models (LMM) were fit to determine the effect of seed number on the fruit traits previously mentioned. A total of five models were analyzed. For each model, seed number was used as a fixed effect term and fruit cultivar was used as a random effect (random intercept). The significance of the fruit cultivar random effect was determined using likelihood ratio tests implemented in the *lmerTest* R package. Significance of the fixed effects was determined using restricted maximum likelihood (REML) t-tests (Bates *et al.*, 2015).

Planting seeds from the fruit collected in the field and growing seedlings.

In order to carry out the study of the proportion of self- vs. cross-pollinated seeds in commercial cranberry beds, and the fruit effects on seedling vigor in cranberry and to evaluate vigor of self- and cross-pollinated seeds and seedlings, seeds were selected, planted and genotype as described below:

A total of 58 fruits were randomly selected (~20 per cultivar: HyRed, Stevens and Ben Lear) from the 160 field collected fruits in total. All the seeds were extracted from these fruits and stored separately at 4° C for 4 months to ensure that all chilling requirements were met. After stratification, approximately 10 seeds from each fruit were randomly selected and planted.

The seeds from each cultivar were planted in four 10 x 20 inch germination trays containing packed and moist peat moss. 50 seeds were planted in each tray in 5 rows and 10 columns, where each row corresponded to the seeds of one berry. Each seed was tracked so that it could be traced back to the fruit it was originally extracted from. The trays were then placed in a growth chamber in a completely randomized design, and kept under 16/8 hours photoperiod, light intensity at approximately 400 $\mu\text{mol}/\text{m}^2/\text{s}$, and a constant temperature around 22°C.

Each seedling was genotyped to determine origin (self or cross-pollinations) as described by (Schlautman *et al.*, 2015a). Briefly, after collecting ~6 leaves per seedling, leaves were freeze-dried and DNA extracted with Macherey–Nagel (MN) Plant II kit (Düren, Germany) following manufacturer's instructions. 12 SSR markers selected from the high density cranberry map

(Schlautman *et al.*, 2015a) were used to genotyped the cranberry seedlings (Supplementary Table 4.2). The markers were arrayed in 4x multiplex PCR reactions performed as described by (Schlautman *et al.*, 2015b). The PCR products from the multiplex reactions were pooled for fragment analysis using an ABI 3730 fluorescent sequencer (Applied Biosystems, Foster city, CA, USA). Allele scoring for the SSR loci was performed through GeneMarker software v.1.91 (SoftGenetics LLC, State College, PA, USA).

Seedlings were determined to be selfed or outcrossed by using two methods. First, selfs were identified as those seedlings which did not contain any alleles not inherited from the assumed mother. Conversely, outcrosses were identified as those seedlings which contained one or more alleles that could not have been inherited from the seedling's assumed mother. Secondly, to confirm self vs. outcross determination, principal component analysis PCA was performed using the *adeigenet* package (Jombart, 2008) in R platform v3.1.2 to ensure that identified outcrossed seedlings did not "group" with the clusters of selfed seedlings. Finally, since some cranberry cultivars such as Ben Lear were selected more than 100 years ago, and intracultivar genetic heterogeneity has previously been observed for multiple cranberry cultivars (Fajardo *et al.*, 2012), we tested to see if some of the seeds may have arisen from fruits which were from some unknown cultivar rather than the assumed cultivar propagated in the bed. We determined a seed to have a mother other than the assumed cultivar when it did not group with the correct cultivar in the PCA, when it did not contain at least one allele from its assumed mother at every locus, or when one of its sibling seeds from the same fruit did not meet the above criteria.

4.3.2.2 The prevalence of self vs. cross-pollinated seeds in commercial cranberry beds. Using the genotype information of each seedling (self vs. cross-pollination), whose maternal cultivar and fruit of origin were tracked, we tested whether the number of self-pollinated seed was equal to the number of cross-pollinated seeds in a bed. Chi-square tests were performed with a null hypothesis (H_0): proportion of self-pollinated seeds = 0.5, proportion of cross-pollinated seeds = 0.5. Specifically, we performed $\chi^2 = \sum \frac{(\text{observed \# of selfs} - \text{total seeds} \times 0.5)^2}{(\text{total seeds} \times 0.5)^2}$ with degrees of freedom equivalent to $k-1=1$ where k is equal to the number of possible responses. In this case the possible responses were self-pollinated or cross-pollinated seedling, then k was 2.

4.3.2.3 Effect of self-pollination vs. cross-pollination on the total number of seeds within a fruit. In order to determine if the number of seeds within a fruit is related to the percentage of cross-pollinated seeds in the fruit, a LMM was fit using *lme4* to explain the number of seeds within a fruit using the proportion of outcrossed seeds in the fruit (as determined previously by genotyping seedlings from those fruits) as a fixed term and using cultivar as a random intercept. Significance of the variance components for the random effects terms included in the model were determined by likelihood ratio tests performed using *lmerTest*. Significance of the fixed effect coefficients was determined using REML t-tests.

4.3.2.4 Number of self- versus cross-pollinated seeds within a fruit known to contain at least one cross-pollinated seed. Based on preliminary observations of pollen from the three cultivars growing on culture medium, it was confirmed that the pollen is released in the form of tetrads, and all four pollen grains from each tetrad germinated, as it was previously reviewed by Vorsa

and Johnson-Cicalese, (2012). Based on the former, it was hypothesized that when one cross-pollinated seed was present in a fruit, at least one additional cross-pollinated seed was also likely to be present. To test this hypothesis, a one sample t-test was performed with $H_0: \mu \leq 1$ (when fruits have cross-pollinated seeds, fruits can have at least one cross-pollinated seed; and $H_1: \mu > 1$ (when fruits have cross-pollinated seeds, there should be more than one cross-pollinated seed). Specifically: $t = (\text{sample mean} - 1)/SE$ where $SE = s/\sqrt{n}$ and degrees of freedom = $n - 1$. Where s is the standard deviation of the sample mean and n is the number of fruits.

4.3.2.5 Effect of inbreeding on cranberry seedling vigor. Length of the epicotyl of all seedlings was measured at 2, 4, and 6 weeks after planting. In addition to seedling length, the number of leaves per seedling was also counted on these three dates. These measurements were used as measures of vigor in this study.

Descriptive statistics of seedling length and seedling leaf number explained by the cultivar of seedling's mother (whether the seedling was self- or cross-pollinated), and measurement date were explored using regression trees constructed with the *partykit* R package (Hothorn *et al.*, 2006). Breaks/nodes in the regression tree were interpreted as potential interactions, and means of the individuals in each node at the branch tips was calculated. In addition, we used package *lme4* (Bates *et al.*, 2015) to perform linear mixed effects analysis (LMM) of the relationship between seedling length or seedling leaf number and the mother cultivar and seedling type (self vs. outcross). As fixed effects, we entered the seedling type nested within cultivar into the model. As random effects, we had intercepts for genotype (i.e., each seedling), for fruit (i.e., one

of the 58 fruits from which seedlings were extracted), and data collection date (2, 4, and 6 weeks after planting). Significance of fixed effect terms was tested using restricted maximum likelihood (REML) t-tests using Satterthwaite approximations to degrees of freedom. Significance of random terms was determined using likelihood ratio tests with a chi-square distribution implemented in the *lmerTest* R package.

4.3.2.6 Quantifying maternal (fruit) effects on seedling vigor in cranberry: Because significant variance components for fruit effect (maternal influence) in seedling vigor were observed for both the seedling length and seedling leaf number models, best linear unbiased predictors (BLUPs) from the LMMs were predicted for each fruit from each model. Additional LMMs were fit using the fruit BLUPs as response variables explained by all possible fruit trait measurements (used in Experiment 1) including seed number, percentage of outcrossed seed, width, length, weight, volume, density and their interactions. Significance of the effects was determined as in previous LMMs.

.

4.3.3 Greenhouse experiments.

4.3.3.1 Collection, storage, and sorting of uprights. Dormant uprights were collected from three beds from each of the following cultivars: Stevens, Ben Lear, HyRed, and GH1. The collection of the plant material was performed in the third week of May once the ice covering the beds was completely melted and the uprights were accessible to harvest. The apical buds from the uprights collected were in the stage of tight bud as described by (Workmaster *et al.*, 1997).

Healthy uprights were collected at randomly in different zones of the bed, wrapped in moist paper towels and placed on labeled bags and stored at 4°C until removed for greenhouse plantings. Since reproductive and vegetative buds have been found to differ in size, uprights with potential to be reproductive (flower production) were sorted and categorized by the width of the apical bud (Workmaster *et al.*, 1997). Uprights with apical rounded buds with a width equal or larger than 1.2 mm were considered reproductive. Only reproductive uprights with 15 to 20 cm long were separated and all the leaves below the first 5 cm from the apical bud of those uprights were removed and discarded. Subsequently, the uprights were rinsed with distilled water, prior to be stored at 4°C. About 250 dormant uprights (with reproductive buds) per bed per cultivar were stored.

4.3.3.2 Growing uprights under greenhouse conditions. Immediately after removing the uprights from storage, the uprights were placed in moistened, packed medium in groups of ten in pots. The lower half of the uprights was immersed in the medium, which consists in 80% peat moss, 10% autoclaved sand, 10% metro mix. Immediately after that, the plants were transferred for rooting to a plastic fog tent in the greenhouse under continuous ultrasonic generated fog, sufficient to keep the uprights with a relative humidity (RH) between 97-99%. The fog tent had a porous bottom and vented top to keep the temperature inside the tent within 3°C above greenhouse temperature.

The greenhouse conditions were set to keep constant temperature around 22°C. The 16/8 hours photoperiod was controlled by using supplemental high intensity discharge lighting at approximately 400 $\mu\text{mol}/\text{m}^2/\text{s}$. After rooting, the uprights (from here on called plants) were kept in the tent for one week more and the fog levels were reduced progressively until reaching the greenhouse RH. The plants were then transferred to the open greenhouse and fertilized once a week with a dilute soluble fertilizer (1g/L Miracid 30-10-10, Scotts Company Marysville, Ohio) until flowering (Zeldin, 2013 personal communication). Once rooted, 4-6 leaves per plant per cultivar were collected and placed separately in coded paper bags, freeze-dried and analyzed using SSR markers (Fajardo *et al.*, 2012) to verify the cultivar identity of each upright.

4.3.3.3 Pollination and production of selfed and crossed progenies under greenhouse conditions. A pollen diversity experiment was performed in order to study the vigor of the progeny of three cultivars (Ben Lear, Stevens and HyRed) by self-pollinations or cross-pollinations. From the 250 uprights per cultivar collected and stored, four sets of 20 uprights per bed per cultivar were planted consecutively. The first two plantings were done to obtain pollen from each cultivar to obtain the bulk of pollen needed to perform self- and cross-pollinations. The last two plantings were done to grow uprights with flowers able to be pollinated.

Due to the low flower production of the uprights, there were only enough flowers to obtain pollen from all the cultivars. From the last plantings done for the pollinations, Ben Lear and Stevens uprights produced flowers; therefore self- and cross-pollinations were carried out only for these cultivars. To generate the self-pollinated plants, Ben Lear flowers were pollinated using

pollen from the same cultivar (Ben Lear). Similar procedure was used for Stevens. In order to produce cross-pollinated populations, flowers from BenLear were pollinated with a pool of pollen from the other cultivars: Stevens, and HyRed. In the case of Stevens, the pool of pollen came from Ben Lear and HyRed. Manual pollinations were performed daily once flowering commenced and the flowers were ready to be pollinated. A flower was considered ready for pollination when the style had elongated past the androecium and the stigma was receptive, and the ones use for cross pollinations were emasculated. Flowers were re-pollinated until a visible clump of pollen was observed on the stigmas. Once the bottom three flowers on an upright were pollinated, any remaining flowers above that were removed. Fully mature fruits were harvested after 3 months from the pollination time and storage in the dark at 4°C for 3 months more to break seed dormancy.

4.4 Results

4.4.1 Fruit collection from the field.

4.4.1.1 Effect of seed number of cranberry fruit size and shape. Pearson correlations comparing the relationships between fruit traits (weight, length, volume, and density), and seed number, revealed large, positive correlations between fruit length, width, volume, and weight (Table 4.1). The correlation between volume and the length and width traits was expected since volume is a function of these two traits. Seed number was positively correlated with all the fruit traits except density (Table 4.1). Density was negatively correlated with length, width, and

volume (denominator of the density equation) and was positively correlated with mass (i.e. numerator of the density equation).

Data of fruit traits collected from 160 fruits was analyzed to determine if these traits are related to number of seeds within the fruit. Individual LMMs of cranberry fruit volume, density, weight, length, and width explained by seed number were performed. REML t-tests showed that seed number was not significant in predicting cranberry fruit density; however increase in seed number was significant in predicting increases in fruit volume, weight, length, and width ($p=0.006$, $p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively). All the fruit traits in the three cultivars increased when the number of seeds in the fruit increased (Figure 4.2).

Planting seeds from the fruit collected in the field and growing seedlings.

At total of 140 seedlings in 19 fruits of Ben Lear, 165 seedlings from 20 berries of HyRed and 174 seedlings from 19 fruits of Stevens, were grown and genotyped with 12 microsatellite markers. An average of 7, 8, and 9 seedlings per fruit in Ben Lear, Hyred and Stevens, respectively, were genotyped. All these 479 seedlings, whose seeds came from 58 randomly selected fruits, were determined to be either self- or cross-pollinated based on the presence or absence of maternal alleles.

In addition, PCA was performed using the allele scores of each seedling at the 12 loci. Based on the PCA, there were three distinct clusters representing the three known parental cultivars (Ben

Lear, Stevens, and HyRed). Within each cluster, two or more additional groups were distinguished and represented seedlings resulted from either self- or cross-pollinations (Figure 4.3). We also observed that one cluster of outcrossed Ben Lear seedlings arising from 4 different fruits clustered closer to Stevens than to Ben Lear (Figure 4.3). When further exploring the genotypes of these misplaced Ben Lear seedlings, it was observed that many of the 33 seedlings from these fruits had loci which did not have a Ben Lear allele.

4.4.1.2 The prevalence of self vs. cross-pollinated seeds in commercial cranberry beds. Chi-square tests of the number of seeds resulting from self-pollination versus cross-pollination revealed that there were significantly more self-pollinated seedlings than out-crossed seedlings in the HyRed and Stevens (Table 4.2). However, there were not significant differences between the number of self-pollinated and cross-pollinated seedlings in the Ben Lear beds (Table 4.2). The 33 seedlings which did not appear to meet the assumption that Ben Lear was their mother were excluded from further statistical analyses.

4.4.1.3 Effect of self-pollination vs. cross-pollination on the total number of seeds within a fruit. Based on the LMM model and REML t-tests ($p=0.01$), the estimated number of seeds per fruit, when all the seeds in the fruit were self-pollinated, was 11.7 (intercept of the model). Furthermore, and according to REML t-tests, increases in the proportion of cross-pollinated seeds within a fruit significantly increased the number of seeds in the fruit ($p=0.07$). Specifically, a fruit with all cross-pollinated seeds was estimated to have 2.96 more seeds than a fruit with all self-pollinated seeds.

4.4.1.4 Number of self- versus cross-pollinated seeds within a fruit known to contain at least one cross-pollinated seed. One sample t-tests were performed to determine whether the average number of cross-pollinated seeds within a fruit known to contain at least one outcrossed seed, was greater than or equal to one, since cranberry pollen is released in the form of tetrads. A total of 26 fruits were analyzed, from which 12 fruits were from Ben Lear, 11 fruits for Stevens, and 3 fruits for HyRed. We found that the average number of outcrossed seeds per fruit was more than one for all cultivars combined, for the cultivar Ben Lear on its own and for the cultivar Steven's on its own (Table 4.3). For HyRed, the mean of outcrossed seeds per fruit, that contained at least one outcrossed seed, was 2.7 seeds; however, the one-sample t-test was not significant most likely due to the small sample size of three fruits (Table 4.3).

4.4.1.5 Effect of inbreeding on cranberry seedling vigor.

The regression tree of seedling length predicted by collection date, mother cultivar, and self-/cross-pollinated origin of the seed (Figure 4.4), revealed that the seedlings had significantly different lengths ($p < 0.001$) depending on the week the data was collected (2, 4 and 6 weeks after planting). Overall, seedlings from Ben Lear as a mother, regardless if they were derived from self-cross pollinated seeds, were shorter than seedlings from Stevens and HyRed (p values < 0.001).

Significant differences ($p\text{-value} < 0.001$) in seedling length were even noticed by the first weeks of data collection (2 and 4 weeks after planting). By the second, and fourth week of planting regardless their origin (from self- or cross-pollinations), Ben Lear seedlings were significantly shorter compared to HyRed and Stevens. The length of the seedlings was similar for the HyRed and Stevens by the second week of planting ($p\text{ value} < 0.001$). When seedlings reached the 4 weeks of planting, significant differences in length were observed for the first time between self- and cross-pollinated seedlings in both, HyRed and Stevens ($p\text{ value} = 0.02$).

After 6 weeks of planting, seedlings had significant differences in height, with p -values lower than 0.001. Thus, Ben Lear seedlings were shorter than Stevens and HyRed. Ben Lear selfs seedlings were markedly shorter than the outcrossed seedlings from the same cultivar, HyRed and Stevens. Cross-pollinated seedlings from HyRed and Stevens were significantly taller than selfs from those same cultivars. Within self-seedlings, the tree reveals that Stevens seedlings are taller than HyRed ($p\text{-value} = 0.006$)

The regression tree of seedling leaf number predicted by collection date, mother cultivar, and self/outcross revealed that the seedlings had significantly different leaf numbers depending on the collection data ($p\text{-value} < 0.001$), regardless of the seedlings mother or origin as a self/outcross. In general, cross-pollinated seedlings had more leaves compared to self-pollinated seedlings (Figure 4.5).

Similar as seedling height, by the second and fourth week of planting, differences in the number of leaves were observed among cultivars ($p\text{-value} < 0.001$), but not within seedlings (self and cross-pollinated) of HyRed and Ben Lear. However, Ben Lear seedlings consistently had fewer leaves compared to the other cultivars. In contrast, HyRed seedlings had more leaves than the other cultivars during the second and fourth week after planting.

By the sixth week after planting, HyRed and Stevens seedlings had more leaves than Ben Lear seedlings ($p\text{-value} < 0.001$). Within Ben Lear, cross-pollinated seedlings had more leaves than self-pollinated seedlings ($p\text{-value} = 0.015$). There were not significant differences between HyRed and Stevens seedlings, therefore the regression tree kept them in the same node. As observed in Ben Lear, cross-pollinated seedlings from both, HyRed and Stevens, had more leaves than self-pollinated seedlings from these two cultivars ($p\text{-value} < 0.001$).

Linear Mixed Model (LMM) was performed in order to determine if the seedling length and leaf number are influenced by the effect of the mother cultivar and the seedling type (self vs. cross). For doing that, the mother cultivar as well as the seedling type were kept as fixed effect and nested within seedling mother on seedling length and seedling leaf counts. The results of the analysis are presented in Table 4.4.

HyRed and Stevens seedlings, self- and cross-pollinated, were significantly different in height compared to Ben Lear. Cross-pollinated seedling of HyRed and Stevens were 72 mm and 70 mm, and 55 mm and 58 for self-pollinated seedlings, respectively; contrasting with the height of crossed, and self-pollinated seedlings in Ben Lear, which were 48 mm and 39 mm, respectively. In addition, self-pollinated seeds within each mother cultivar were significantly shorter than seedlings from cross-pollinated, especially for HyRed and Stevens. Such differences were also found in Ben Lear (Table 4.4).

LMM model also revealed that Stevens seedlings had significantly more leaves than HyRed and Ben Lear. Moreover, cross-pollinated seedlings for this cultivar had more leaves than self-pollinated seedlings. Significant differences were also found in leaf number of cross- vs self-pollinated seedlings of Ben Lear.

4.4.1.6 Quantifying maternal (fruit) effects on seedling vigor in cranberry. Best linear unbiased predictors (BLUPs) from the LMMs explaining seedling length and seedling leaf number for each of the 58 fruits were predicted and used the response variables in additional linear models attempting to determine the components of the significant fruit effect. However, none of the fruit traits (length, width, volume or density) and/or their interactions were significant predictors in the models using the BLUPs as response variables.

4.4.2 Greenhouse experiments.

All four plantings of uprights with reproductive buds resulted in the growth of new fruiting uprights. However, most of them failed to grow well developed flowers, and the floral bud development was arrested, as shown in Figure 4.6. From the first two plantings, destined to pollen collection to perform the pollen diversity experiments, a total of 4 uprights of Ben Lear produced 6 flowers, 11 uprights of Stevens produced a total of 30 flowers, and none of HyRed uprights developed flowers, even though floral buds were observed on the uprights.

A preliminary upright collection from the three cultivars was done the previous year in November (2013), and planted a month before the plantings for the experiments started. This preliminary collection and planting was done in order to set up greenhouse conditions for the experiments. From this planting, 13, 10, and 11 uprights of Ben Lear, Stevens, and HyRed, respectively successfully grew at least one flower and pollen was collected from there. Due to the fact that from the first two plantings, uprights of HyRed did not developed viable flowers, pollen collected from the preliminary planting just described was used for carrying out the pollinations.

Prior to pollinations, a sample of pollen from the three cultivars was placed on culture medium, and percentage of grains of pollen germinated was recorded to assure viability. After 8 hours of incubation at 25°C, the majority, if not all, pollen grains germinated (developed pollinic tube), which guaranteed their use for the pollination experiments.

In Stevens, from the two plantings for pollination purposes, only 17 uprights from 150 planted grew viable flowers. Flowers from seven of those uprights were cross-pollinated; meanwhile flowers from the remaining 10 uprights were self-pollinated. From the uprights cross-pollinated, 10 berries became growth arrested and stony; the four remaining successfully matured. 16 berries from self-pollinations became stony and three matured.

In the case of Ben Lear, out of 130 uprights planted, only 15 produced flowers. 6 uprights were cross-pollinated and the other 9 uprights were self-pollinated. From the cross-pollinations, 5 developing fruits became stony and 8 matured. From the self-pollinations, 8 berries were arrested and 4 berries matured. Fingerprinting analysis of the uprights planted, showed that all HyRed, and Stevens uprights collected from the field were true to genotype. Other case was Ben Lear, from the three beds sampled, one bed displayed an arrangement of alleles different from Ben Lear. Surprisingly, most of the uprights producing berries (9 in total) were harvested from that particular bed. Therefore, from uprights true to cultivar, only 2 berries resulted from cross-pollinations and none from self-pollinations.

Due to the low number of mature berries to compare between self and cross pollinations in Stevens and the lack of self-pollinated berries from Ben Lear, no further analysis was performed.

4.5 Discussion

4.5.1 Effects of seed number on fruit quality, and the effects of self- versus cross-pollination on seed number

Seed number influences the size and shape of cranberry fruits. Seed number was positively correlated with fruit weight, volume, length, and width; conversely, seed number is negatively correlated with fruit density. Significant seed number effects were observed in the linear mixed models explaining cranberry fruit weight, length, volume, and density. Although predictions of fruit size and shape differed for three cultivars in this study (i.e., HyRed, BenLear, and Stevens), increases in seed number tended to increase the length, width, weight, and volume of cranberry fruit for the three cultivars (Figure 4.2). Dogterom, *et al.*, (2000) studied pollen loads and source in fruit production of two blueberry cultivars and found similar relationships between fruit traits, time to fruit and number of seeds, especially when cross-pollinations occurred. The observed positive relationship between seed number and fruit size and shape suggests that commercial cranberry growers can influence the size and shape of fruits in their beds by adopting practices which increase pollination and seed set; studies have previously shown that significant differences in pollen load servability exist between cranberry insect pollinators (Cane and Schiffhauer, 2003). Furthermore, although yield data was not collected during this particular study, past cranberries studies have found that fruit weight was associated to cranberry yield for Stevens and Ben Lear (DeVetter et al., 2015); and therefore, increasing seed set could be an important means of increasing cranberry yield.

Significantly more self-pollinated seeds than cross-pollinated seeds are present in fruits within commercial cranberry beds. Cranberry is capable of self-fertilization (Vorsa and Johnson-cicalese, 2012), therefore, it was hypothesized that the majority of seeds present within individual fruits and within an entire cranberry bed would arise mainly from self- rather than from cross-pollination events since beds are clonally propagated with a single cultivar. Chi squared analysis revealed that the majority of seedlings from HyRed and Stevens were self-pollinated, whereas for Ben Lear self- and cross-pollinated seedlings were equally proportioned in the bed (Table 4.2). The disproportionate number of self- and cross-pollinated seeds within commercial cranberry beds, while not surprising, suggests that the potential effect of the proportion of self-pollinated seeds on the total number of seeds in a fruit is worth studying, especially since seed number was found to have a positive relationship with fruit size and shape (Figure 4.2). It is likely that the intra-cultivar genetic heterogeneity of Ben Lear seen in the PCA analysis (Figure 4.3) resulted in a higher number of outcrossed seedlings than for the other two cultivars because multiple genotypes could have occurred in Ben Lear bed and pollen from those genotypes were able to pollinate any flower from other of the co-existing genotypes in the bed.

Cross-pollination increases the total number of seeds within a fruit. Our analysis of the relationship between total seeds in a fruit and the origin of those seeds (self- vs. cross-pollinated) showed that fruits had less seeds when all seeds were self-pollinated, than fruits with cross-pollinated seeds. Similar results have been obtained previously by studies of self- and cross-pollinations in a diallel experiment with eight cultivars (Sarracino and Vorsa, 1991), and with two early selections (MacKenzie, 1994). Studies in blueberry (*Vaccinium corymbosum*) also revealed that cross-pollinations favored the development of higher number of seeds (Dogterom *et*

al., 2000; Vander Kloet, 1984). The lower number of seeds in fruits self-pollinated could suggest that some form of inbreeding depression due to self-pollination may have led to the abortion of embryos in the fruit. Shriveled seeds were observed in the fruits in the present study as it has also been observed in cranberry by Sarracino and Vorsa (1991) and in blueberry by Hokanson and Hancock (2000). However, these aborted seed have not been taken into account for the total seeds number per fruit. It would be interesting to include the number of shriveled seeds in fruits with self- and crossed- pollinated seeds to determine if an early acting inbreeding depression could affect cranberry as it was shown for three species of blueberry by Hokanson and Hancock (2000). But most importantly, the fact that cross-pollination increases the total number of seeds within a fruit suggests that cranberry growers could potentially achieve the increases in fruit size and total yield in their cranberry beds by adopting practices which promote cross-pollination on the farms. Such practices could include a variety of strategies including planting adjacent beds in different cultivars, planting a mix of cultivars in a single bed, or choosing the active pollinators which move between multiple beds in order to increase the spread of foreign pollen.

For example the larger predicted size of the Ben Lear fruits in this study (Figure 4.2) could be attributed to the larger proportion of cross-pollinations with a more diverse pollen given by the heterogeneity of the bed shown in the chi-square results (Table 4.2). Such diversity in pollen could favor the fertilization of most, if not all, the eggs in the fruit, as it was hypothesized to occur in blueberry (Dogterom *et al.*, 2000). Vander Kloet and Tosh (1984) studied the effect of the number of pollen donors in seed production and weight in highbush blueberry. Their study showed that when flowers were pollinated with pollen of three donors, the fruit set, seed number

and weight were higher than when pollen of a single donor was used. On the contrary, HyRed, which was mainly self-pollinated in this study, had expectedly smaller berry sizes.

The mean number of cross-pollinated seeds in fruit known to contain at least one cross-pollinated seed is more than 1. Because pollen is released in the form of tetrads, and all four pollen grains the tetrad have the potential to germinate, as previously reviewed by Vorsa and Johnson-Cicalese, (2012), we hypothesized that when a single tetrad from a foreign parent landed on a cranberry flower, it was likely that more than one of those pollen grains would fertilize an embryo within that flower. In the analysis of fruits with at least one cross-pollinated seed, we found that the mean number of cross-pollinated seeds was greater than one, which supported our hypothesis (Table 4.3). However, in HyRed, the small size sample did not allow significance testing due to very low number of fruits that contained cross-pollinated seeds. In contrast, Ben Lear had the highest mean number of outcrossed seeds per fruit likely due to the high genetic heterogeneity found in the sampled bed. With more than one genotype represented in the Ben Lear bed, it is likely that the pollen from those genotypes competed with Ben Lear pollen to fertilize eggs, and therefore more cross-pollinated seeds were observed. Pollen germination capacity is out of the scope of this experiment, but it would be interesting to test whether or not our results are related to a genetic capacity of pollen grains to fertilize eggs. The implication of this particular study is that since cross-pollination increases seed set, a single foreign pollen tetrad can increase the presence of cross-pollinated seeds by four rather than only one; and therefore, have a fairly large effect on overall seed set and potentially fruit size and yield.

4.5.2 Effects of inbreeding and fruit effects on seedling vigor.

Inbreeding reduces seedling vigor in cranberry. Differences in vigor of seedlings from cross and self-pollinations in three cultivars were observed at early stages of plant growth, up to 6 weeks of growth. Seedlings from self-pollinations were shorter and a reduced number of leaves compared to seedlings from cross-pollinations. Additionally height and number of leaves differs based on the cultivar. When the vigor of seedlings was analyzed, we found that both length and leaf number were higher in HyRed and Stevens, which are more recent cultivars, than Ben Lear. Moreover, HyRed seedlings tended to have a similar height and leaf number to Stevens, its paternal cultivar than Ben Lear, its maternal cultivar. Additionally, these results showed that differences in vigor are noticeable even at fairly early stages of plant growth. Initially, by the second week of planting, no differences in vigor between self- and cross-pollinated seedlings were observed, which could be due to the use of nutrients provided by the cotyledons, and therefore provided a uniform start to the seedlings. However, by the fourth week, differences in vigor began to be revealed, and two weeks later the vigor of the seedlings, expressed in terms of leaf number and plant height was evident in cross-pollinated than in self-pollinated plants.

From the LMM model analysis of the seedling length and leaf number, we found significantly shorter seedling lengths and lower seedling leaf counts for self-pollinated versus cross-pollinated seedlings within the three mother cultivars, as well as the regression tree partitions, provided evidence of inbreeding depression in cranberry. Moreover, the reduction of

vigor as evidenced by the shorter length of the seedlings and number of leaves in self-pollinated plants was different depending on the cultivar.

The differences in vigor between self- and crossed-pollinated seedlings were more accentuated in newer cultivars than in Ben Lear. Overall, Ben Lear is shorter compared to the other cultivars, but when comparing estimates from the LMM analysis (Table 4.4), self-pollinated plants of Ben Lear were 8.62 mm shorter than their cross-pollinated counterpart. While for HyRed, self-pollinated seedlings were 17.11 mm shorter than cross-pollinated seedlings, and for Stevens, the difference in height was 11.71 mm. Our results demonstrated that cranberry benefits from the ability to outcross to produce more vigorous plants. Additionally, our results supports that cranberry can tolerate certain levels of inbreeding depression since more advanced self-pollinated seeds are available (Roper and Vorsa 1997). It would be interestingly to evaluate the fruit production capability of these self-pollinated seedlings compared to their cross-pollinated counterparts as well as to test the vigor of more advanced selfing lines. Finally, it would be interesting to continue inbreeding in this population to obtain inbred lines which could be used in the first analysis of heterosis in cranberry.

Although fruit effects on seedling vigor existed, our study failed to determine which fruit qualities contributed to the observed effect. Finally, significant variance components for the fruit effect on seedling vigor were observed in LMMs. Therefore, we were interested in learning if the fruit traits evaluated here would be responsible for contribution (maternal effect) on the seedling vigor (seedling length and leaf number). To answer this question, we estimated BLUPs from the LMMs. However, when using the obtained BLUPs as response variables in LMM

models which included the combinations fruit measurements from experiment 4.4.1.1 (seed number, weight, width, length, volume, density) and their interactions, no significant effects were detected. Among the possible reasons that could have contributed to the inability to detect significant effects are: lack of enough fruits to have enough power to predict the true fruit effect BLUPs and use that effect as a response variable in subsequent models; failure to collect data for the true components/predictors of the observed fruit effects (measure of carbohydrate content rather than morphological traits); failure to measure seed vigor or seedling vigor at an earlier enough date so that all vigor was associated with the fruit effect and not confounded by the seedlings own vigor (i.e. ability to undergo normal plant processes such as photosynthesis) due to its unique genetic make up and status as a self- or cross-pollinated seedling.

4.5.3 Greenhouse experiments

Cranberry is relatively easy to propagate due to the natural stoloniferous growth of its runners. The establishment of in vitro cultures of this species with the goals of micropropagation or transformation is relatively easy and cranberries require minimal salts and growth regulators (McCown and Zeldin 2005). Likewise, cranberry plants are routinely propagated in nurseries with well drained soils and N-P-K fertilizations.

One of the goals of the present study was to establish greenhouse conditions that permit not only the vegetative growth of cranberry, but also the growth of reproductive and dormant buds into fruiting uprights able to grow well-developed flowers available for breeding purposes. While the majority of the reproductive buds from the three cultivars studied (BenLear, Stevens, and

HyRed) grew new uprights, then failed to produce flowers. All the uprights developed from the apical buds were fruiting uprights since it was evident that they had arrested floral buds that did not progress through further developmental stages (Figure 4.6).

We were curious about the rationale as to floral bud abortion, and while it has been observed in Japanese pears growing in different areas of Brazil thus, abortion was attributed to an inadequate chilling treatment during dormancy (Petri *et al.*, 2002). Unfortunately, the likelihood of inadequate chilling is minimal as all buds were collected after the ice melted from the plantations (beds) and before the buds started to grow. Potential desiccation during uprights collection or excess of temperature might have been responsible.

In fact, environmental conditions, including high temperatures have been found to be factors to induce floral bud abortion (in our case arrested, since floral buds do not detached from the upright). Based on this, we could speculate that uprights growing under greenhouse conditions could have been exposed to high temperatures inside the greenhouse, which would cause most of the flower buds to become arrested. In cowpea (Ahmed and Hall, 1993) and *Arabidopsis* (Zhang *et al.*, 2013), it has been found that stress by high temperatures (30°C) can cause abortion of floral buds. In *Arabidopsis*, this is due by the upregulation of genes related to stress and cell death (Zhang *et al.*, 2013). Additionally, differences in the ability of the uprights to sustain flower development between uprights harvested and grown in the previous year and the uprights that were grown for carrying out the experiments (and in which flower development was poor), could be due to internal physiological differences between developed uprights from the two sets

of buds harvested. This could have been responsible for the differences in the ability of the uprights to sustain the development of the flowers and fruits. Additional, experiments are needed in cranberry to investigate the growth of floral buds under greenhouse conditions.

Unfortunately, the study of vigor based on the pollen diversity could not be carried out due to the low number of uprights that were able to develop into full flowers for pollen collection and also for pollination treatments. Therefore, the number of berries produced was very limited, impeding further analysis and comparison.

These results suggest re-evaluating current commercial cranberry cultivation practices and the implications of self- and cross-pollinations in the fruit quality. Cranberry cultivation is traditionally done using established beds of one cultivar per bed, therefore most of the fruits produced are the result of self-pollinations. Whether or not a single genotype cultivation practice can negatively affect fruit production is still unknown. However, our results demonstrate that cross-pollination increases total seed numbers, and thus could favor the production of larger fruits. Further experiments including a higher number of experimental units (fruits and seedlings) would help to clarify this question.

4.6 References

- Ahmed, F. E., and Hall, A. (1993). Heat injury during early floral bud development in Cowpea. *Crop Science Society of America*, 33(4), 764–767.
doi:10.2135/cropsci1993.0011183X003300040026x

- Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*; Vol 1, Issue 1 (2015) . Retrieved from <http://www.jstatsoft.org/index.php/jss/article/view/v067i01>
- Cane, J. H., and Schiffhauer, D. (2003). Dose-response relationships between pollination and fruiting refine pollinator comparisons for cranberry (*Vaccinium macrocarpon* [Ericaceae]). *American Journal of Botany*, 90(10), 1425–1432. doi:10.3732/ajb.90.10.1425
- Caruso, F. L., Bristow, P. R., and Oudemans, P. V. (2000). Cranberries: The Most Intriguing Native North American Fruit. *APSnet Feature Articles*. doi:10.1094/APSnetFeature-2000-1100
- DeVetter, L., Colquhoun, J., Zalapa, J., and Harbut, R. (2015). Yield estimation in commercial cranberry systems using physiological, environmental, and genetic variables. *Scientia Horticulturae*, 190, 83–93. doi:10.1016/j.scienta.2015.04.016
- Dogterom, M. H., Winston, M. L., and Mukai, A. (2000). Effect of pollen load size and source (self, outcross) on seed and fruit production in highbush blueberry cv. “Bluecrop” (*Vaccinium corymbosum*; Ericaceae). *American Journal of Botany*, 87(11), 1584–1591. Retrieved from <http://www.amjbot.org/content/87/11/1584.abstract>
- Fajardo, D., Morales, J., Zhu, H., Steffan, S., Harbut, R., Bassil, N., and Zalapa, J. (2012). Discrimination of American Cranberry Cultivars and Assessment of Clonal Heterogeneity Using Microsatellite Markers. *Plant Molecular Biology Reporter*, 31(2), 264–271. doi:10.1007/s11105-012-0497-4
- Hokanson, K., and Hancock, J. (2000). Early-acting inbreeding depression in three species of *Vaccinium* (Ericaceae). *Sexual Plant Reproduction*, 13, 145–150. doi:10.1007/s004970000046
- Hothorn, T., Hornik, K., and Zeileis, A. (2006). Unbiased Recursive Partitioning: A Conditional Inference Framework. *Journal of Computational and Graphical Statistics*, 15(3), 651–674. doi:10.1198/106186006X133933
- Jombart, T. (2008). adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics*, 24 (11), 1403–1405. doi:10.1093/bioinformatics/btn129
- MacKenzie, K. E. (1994). Pollination requirements in cranberry. *Journal of Small Fruit and Viticulture*, 2(3), 33–44. doi:10.1300/J492v06n02_04
- McCown, B. H., and Zeldin, E. L. (2003). “HyRed”, an Early, High Fruit Color Cranberry Hybrid. *HortScience*, 38(2), 304–305. Retrieved from <http://hortsci.ashspublications.org/content/38/2/304.short>
- McCown, B. H., and Zeldin, E. L. (2005). *Vaccinium spp.* Cranberry. In R. E. Litz (Ed.), *Biotechnology of Fruit and Nut crops* (pp. 247–261). Cambridge, MA: CABI publishing.
- Petri, J. L., Leite, G. B., and Yasunobu, Y. (2002). Studies on the causes of floral bud abortion of Japanese pear (*Pyrus pyrifolia*) in Southern Brazil. *Acta Horticulturae*, 584, 375–380. Retrieved from http://www.actahort.org/books/587/587_50.htm

- Roper, T. (2001). “Steven” cranberry. *Journal American Pomological Society*, 55(2), 66–67.
- Roper, T., and Vorsa, N. (1997). Roper and Vorsa 1997 cranberry botany and horticulture.pdf. In J. Janick (Ed.), *Horticultural reviews* (pp. 215–249). New York: John Wiley and Sons, Inc.
- Sarracino, J. M., and Vorsa, N. (1991). Self and cross fertility in cranberry. *Euphytica*, 58(2), 129–136. doi:10.1007/BF00022813
- Schlautman, B., Covarrubias-Pazarán, G., Díaz-García, L. a., Johnson-Cicalese, J., Iorizzo, M., Rodríguez-Bonilla, L., ... Zalapa, J. (2015a). Development of a high-density cranberry SSR linkage map for comparative genetic analysis and trait detection. *Molecular Breeding*, 35(8), 177. doi:10.1007/s11032-015-0367-5
- Schlautman, B., Fajardo, D., Bougie, T., Wiesman, E., Polashock, J., Vorsa, N., ... Zalapa, J. (2015b). Development and Validation of 697 Novel Polymorphic Genomic and EST-SSR Markers in the American Cranberry (*Vaccinium macrocarpon* Ait.). *Molecules*, 20(2), 2001–2013. doi:10.3390/molecules20022001
- USDA NASS. (2014). Statistics of fruits, tree nuts, and horticultural specialties. In R. Holcomb, C. Foster, P. Hilliard, T. Ray, and S. Pendarvis (Eds.), *Agricultural Statistics* (pp. v1–v61). Washington, D.C: <http://www.nass.usda.gov/>.
- Vander Kloet, S. P., and Tosh, D. (1984). Effects of Pollen Donors on Seed Production, Seed Weight, Germination and Seedling Vigor in *Vaccinium corymbosum* L. *American Midland Naturalist*, 112(2), 392–396. doi:10.2307/2425444
- Vorsa, N., and Johnson-cicalese, J. (2012). American Cranberry. In M. L. Badenes and D. H. Byrne (Eds.), *Fruit Breeding* (pp. 191–223). Boston, MA: Springer US. doi:10.1007/978-1-4419-0763-9
- Workmaster, B., Palta, J., and Roper, T. (1997). Terminology for Cranberry Bud Development and Growth. *Cranberries*, 61, 11–14.
- Zhang, J., Li, Q.-F., Huang, W.-W., Xu, X.-Y., Zhang, X.-L., Hui, M.-X., ... Zhang, L.-G. (2013). A Vacuolar Processing Enzyme RsVPE1 Gene of Radish Is Involved in Floral Bud Abortion under Heat Stress. *International Journal of Molecular Sciences*, 14(7), 13346–59. doi:10.3390/ijms140713346

Table 4. 1. Pearson correlations between length, width, volume, density, weight, and seed number of 160 fruits from HyRed, Ben Lear, and Stevens different cranberry cultivars. Values closer to 1 denote strong correlations

	Length	Width	Volume	Density	Weight	Seeds
Length						
Width	0.665095					
Volume	0.814435	0.944148				
Density	-0.30498	-0.34321	-0.42021			
Weight	0.762888	0.884731	0.853431	0.005565		
Seeds	0.322961	0.519853	0.480695	-0.03515	0.527297	

Table 4.2 Number of seedlings, pollination type, and p-value from chi-square test to evaluate proportions of self- and cross-pollinated seedlings in beds from three commercial cultivars of cranberry. (n= 479 seedlings)

Cultivar	Total Seedlings	Selfed Seedlings	Outcrossed Seedlings
HyRed	165	157	8***
Stevens	174	146	28***
BenLear	107	53	54
BenLear off-type	33	-	-

*** significant at $p < 0.001$

Table 4.3 Mean number of outcrossed seeds within a fruit containing at least one outcrossed seed in three cultivars of cranberry. And one-sample t-test statistics from the hypothesis that the: $\mu \leq 1$ seed and $H_1: \mu > 1$ seed where μ is the mean number of outcrossed seeds within a fruit containing at least one outcrossed seed (n=26 fruits).

Cultivar	degrees of freedom	mean # outcross seeds per fruit
BenLear	8	5.63***
Stevens	10	2.52*
HyRed	2	2.67
All	22	3.75***

* significant at $p < 0.05$; ** significant at $p < 0.01$;

*** significant at $p < 0.001$

Table 4.4. Estimates and standard error from linear mixed models predicting the length (mm) and leaf number (count) of seedlings. Fixed effects in the LMM included cultivar (the seedling's known mother) and seedling type (self- or cross-pollinated). Random effect terms with random intercepts included date of measurement (3 levels), seedling (446 levels), and fruit from which the seed was taken (54 levels). P-values are from restricted maximum likelihood (REML) t-tests using Satterthwaite approximations to degrees of freedom.

Trait	Cultivar	Seedling type	
		Cross $\pm$ SE	Self $\pm$ SE
Length (mm)	BenLear	48.14 $\pm$ 19.89 ^S	39.52 $\pm$ 19.88 *
	HyRed	72.52 $\pm$ 20.48 ***	55.41 $\pm$ 19.76 **
	Stevens	70.54 $\pm$ 19.96 ***	58.83 $\pm$ 19.77 ***
Leaf Number	BenLear	9.87 $\pm$ 3.45 ^S	8.81 $\pm$ 3.45 *
	HyRed	12.61 $\pm$ 3.51 **	11.59 $\pm$ 3.44 n
	Stevens	12.39 $\pm$ 3.46 ***	10.7 $\pm$ 3.44 ***

n: $p = 0.17$; S: significant at $p < 0.10$; * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$. SE = standard error of the estimates.

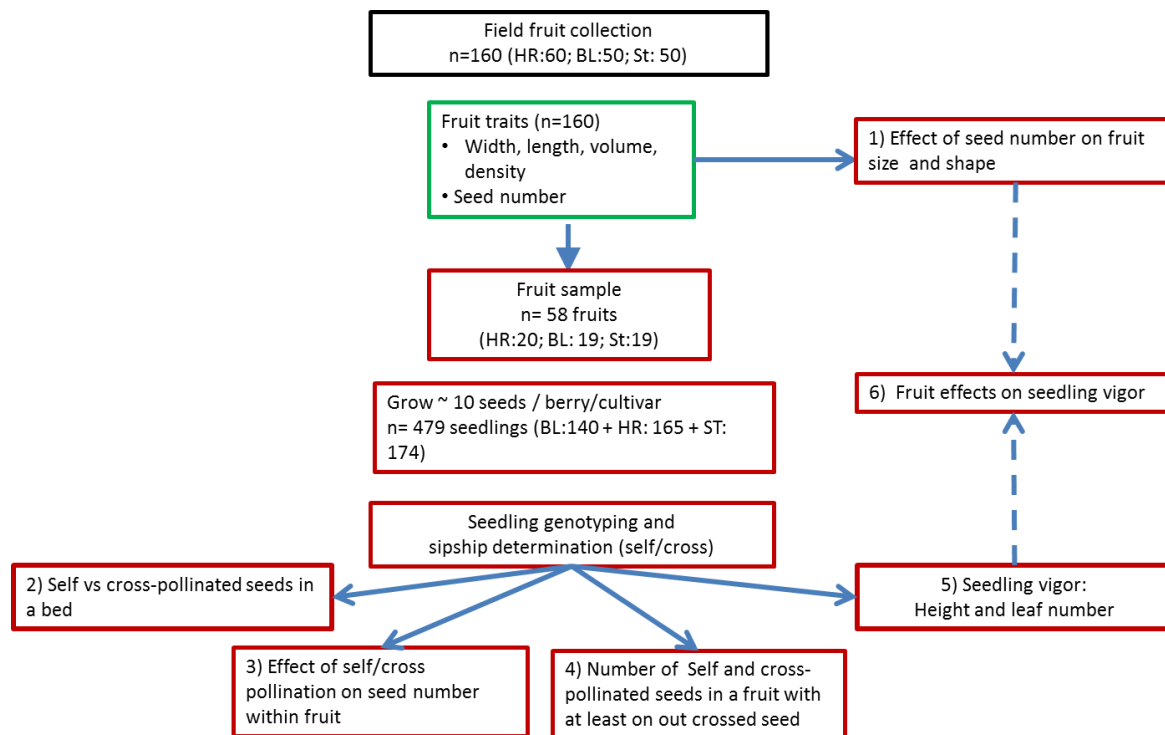


Figure 1. Flow chart of the list of experiments performed from fruits and seedlings from three cultivars of cranberry. Number of units are different based on the experiment performed and are shown in boxes.

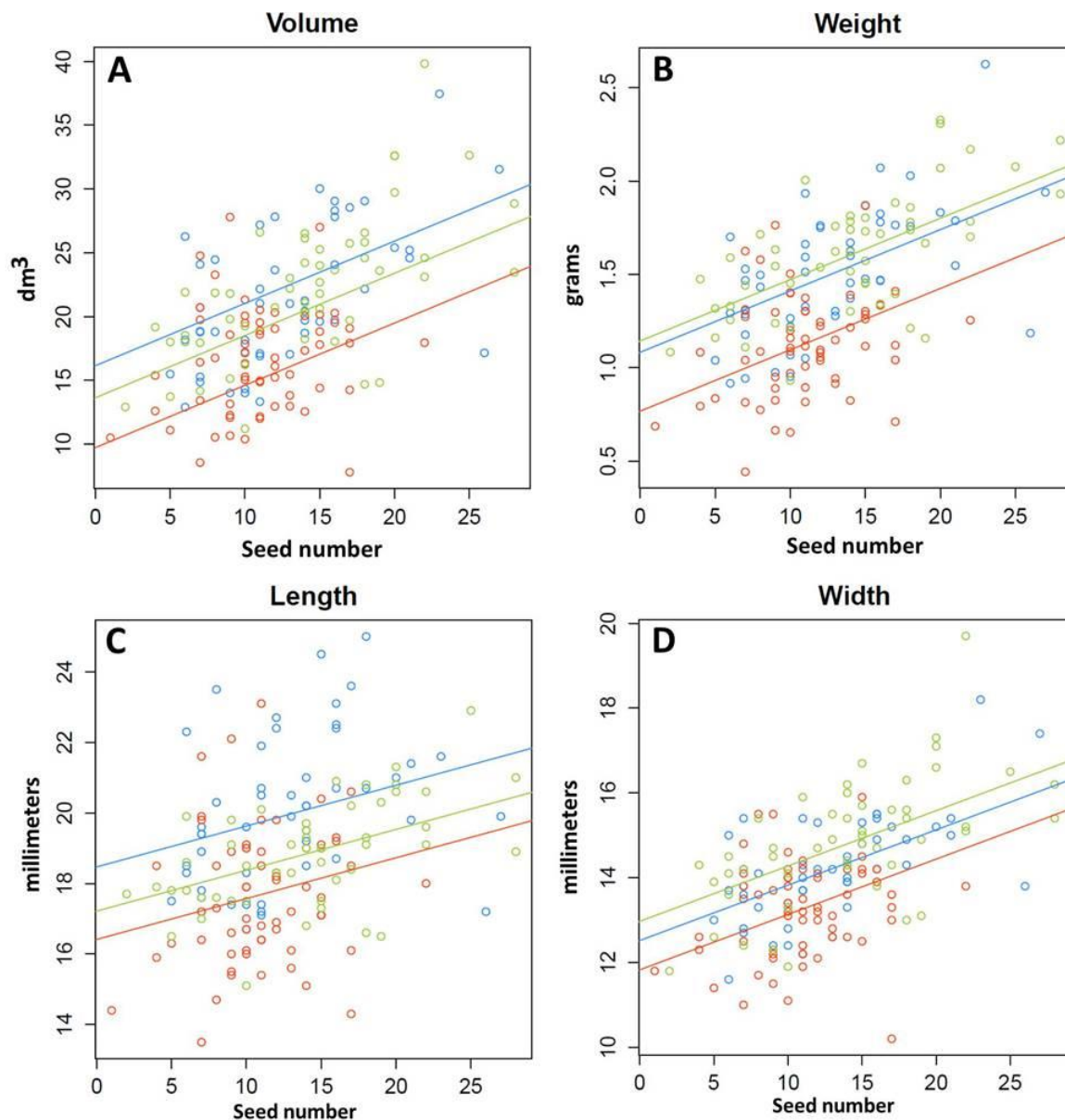


Figure 4.2 Relationship between cranberry fruit traits and seed number. Linear mixed models to understand the relationship of the seed number and the fruit traits were done. Fruit traits such as volume, weight, length and width of the fruits were plotted as a function of the seed number. Red line = HyRed, Green = Stevens, blue = Ben Lear. There was a positive relationship between fruit traits and seed number, the higher the number of seeds per cultivar, the bigger the fruit.

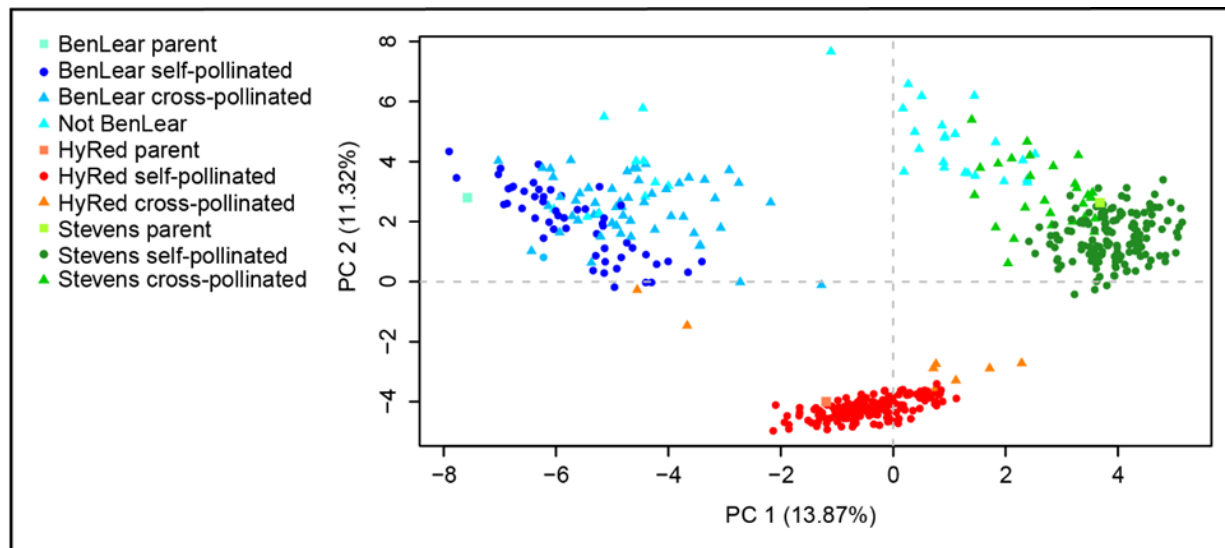


Figure 4.3 Principal components analyses (PCA) of 479 cranberry seedlings (140 seedlings of BenLear, 165 seedlings of HyRed, and 174 seedlings of Stevens) based on 12 microsatellite markers. Seedlings are colored according to their assumed parent cultivars HyRed (red), Ben Lear (blue), and Stevens (green). Within each cluster, squares represent the cultivar parent used as a reference for scoring alleles, circles represent self-pollinated seedlings, and triangles represent cross-pollinated seedling. An additional category was included for Ben Lear, called not Ben Lear. Not Ben Lear cluster is closer to Stevens than others from Ben Lear main cluster

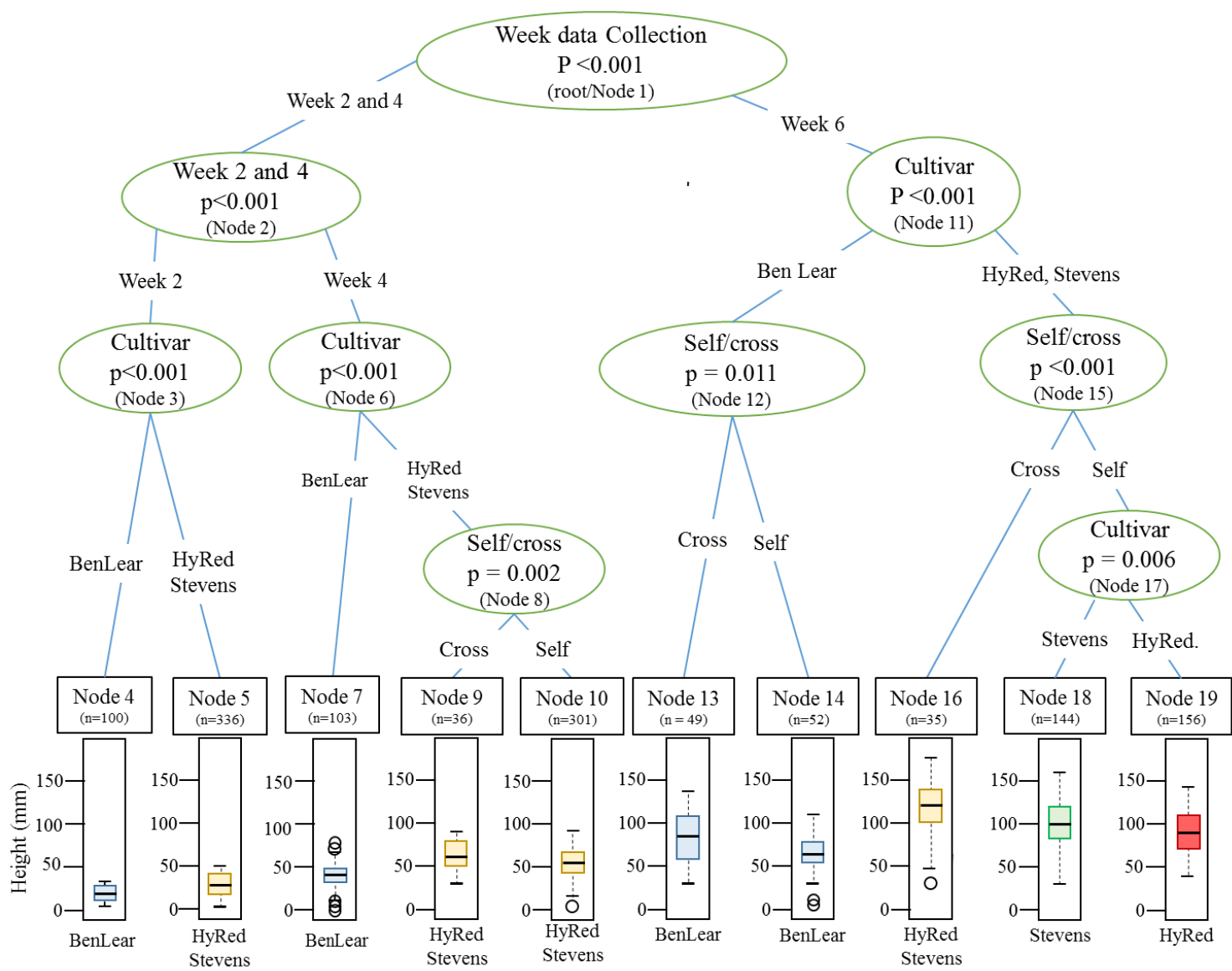


Figure 4.4 Regression tree for cranberry seedling length. 19 nodes represent the significant data partitions according to the binary classification. n= number of seedlings that conform the more basal nodes. Blue boxes represent Ben Lear seedlings, red to HyRed, green to Stevens, yellow to the node combining HyRed and Stevens data. In general, length of the seedlings is explained by the week of data collection. Seedlings from fruits collected in Ben Lear bed and whose genotype was not Ben Lear, were removed from the analysis. Ben Lear seedlings were significantly shorter than HyRed and Stevens. Based on the origin (self/cross), self-pollinated seedlings were significantly shorter compared to cross-pollinated seedlings. By the second week after seeds were planted, no differences in height were observed between self and cross-pollinated, only differences between Ben Lear and the other cultivars (HyRed and Stevens) were observed. By the 6th week, significant differences in height from self and crossed pollinated seedlings within cultivars were observed.

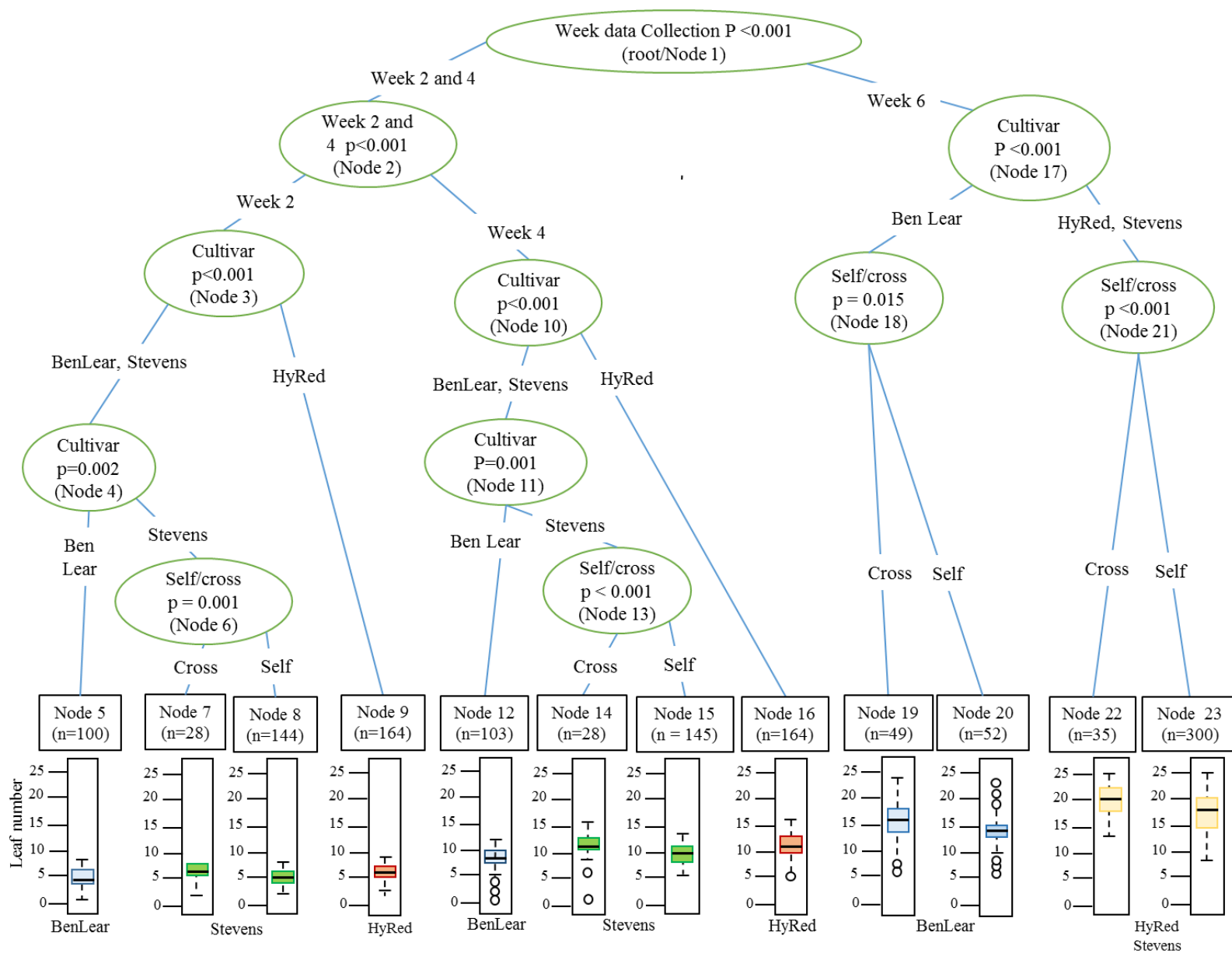


Figure 4.5. Regression tree for cranberry seedling leaf number. 23 nodes represent the significant data partitions according to the binary classification. n= number of seedlings that conform the more basal nodes. Blue boxes represent Ben Lear seedlings, red to HyRed, green to Stevens, yellow to the node combining HyRed and Stevens data. In general, and similar to height, number of leaves per seedling is explained by the week of data collection (weeks after planting). During the second and fourth weeks after planting, only Stevens showed differences in leaf number, whereas by the 6th week, crossed-pollinated seedlings had more leaves than seedlings coming from self-pollinations.

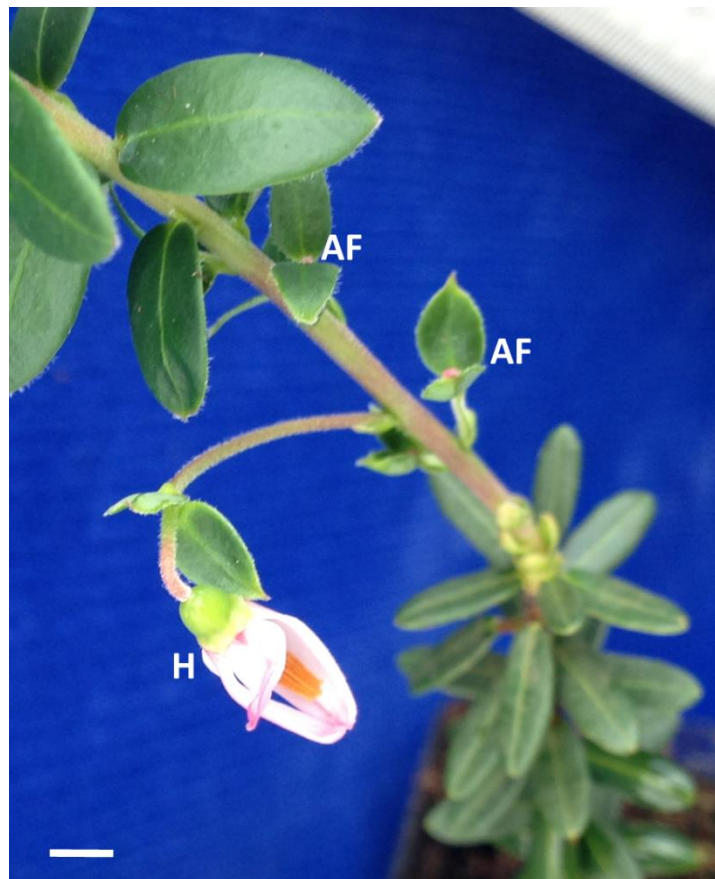


Figure 4.6 Cranberry arrested flower buds. Most of the uprights from reproductive buds of HyRed, Ben Lear, and Stevens collected from field did not successfully develop flowers for pollen collection and pollination. This figure shows a Stevens upright, where floral buds in position 1 and 3 failed to develop, and become arrested (AF). Floral bud in position 2 grew as a normal flower, here in hook stage (H). Scale bar = 1 cm

Supplementary Table 4.1 Fruit traits collected from fruits flood harvested from HyRed, Ben Lear and Stevens cultivars.

Cultivar	Berry	Length	Width	Volume	Density	Weight	Seeds	Fruit Used*	Cultivar	Berry	Length	Width	Volume	Density	Weight	Seeds	Fruit Used*	Cultivar	Berry	Length	Width	Volume	Density	Weight	Seeds	Fruit Used*
BenLear	1	24.5	15.3	3002.946	0.000623	1.87	15	1	HyRed	1	18.9	14.4	2052.038	0.000544	1.116	11		Stevens	1	19	15.1	2268.329	0.000694	1.573	15	1
BenLear	2	21.6	18.2	3746.236	0.000701	2.626	23		HyRed	2	19	14.2	2005.991	0.000693	1.39	14		Stevens	2	19.3	16.2	2652.076	0.000656	1.74	14	2
BenLear	3	20.5	14	2103.82	0.000757	1.593	11	2	HyRed	3	17.2	13.5	1641.325	0.000634	1.041	7		Stevens	3	19.5	15.4	2421.445	0.000737	1.784	14	3
BenLear	4	27.7	21.6	6766.84	0.000306	2.072	21		HyRed	4	18.9	13.7	1857.383	0.000698	1.297	9		Stevens	4	20.1	15.9	2660.657	0.000754	2.005	11	4
BenLear	5	21	15.2	2540.417	0.000721	1.832	20		HyRed	5	16.4	12.5	1341.722	0.000607	0.815	7		Stevens	5	20.8	17.3	3259.524	0.000714	2.327	20	
BenLear	6	23.1	15.5	2905.855	0.000613	1.78	16	3	HyRed	6	19.8	13.8	1974.34	0.000665	1.312	7		Stevens	6	19.9	14.5	2190.724	0.000726	1.59	6	
BenLear	7	22.4	14.2	2364.957	0.000745	1.762	12	4	HyRed	7	17.9	13.8	1784.883	0.00065	1.16	10		Stevens	7	17.6	15.4	2185.51	0.000785	1.715	8	
BenLear	8	22.3	15	2627.157	0.000648	1.701	6		HyRed	8	18.5	13.6	1791.629	0.000626	1.121	17		Stevens	8	20.3	14.9	2359.757	0.000706	1.667	19	
BenLear	9	17.1	14.8	1961.183	0.000753	1.476	15	5	HyRed	9	19.1	14.6	2131.757	0.000706	1.504	10		Stevens	9	19.1	15.2	2310.57	0.000737	1.702	22	
BenLear	10	19.4	13.6	1878.79	0.000627	1.177	7		HyRed	10	19.3	13.9	1952.475	0.000684	1.335	16		Stevens	10	19.1	16.3	2657.096	0.0007	1.859	18	
BenLear	11	22.7	15.3	2782.322	0.000629	1.751	12	6	HyRed	11	19	14.2	2005.991	0.000699	1.403	10		Stevens	11	19.1	14.9	2220.264	0.000731	1.623	13	5
BenLear	12	19.4	15.4	2409.028	0.000635	1.529	7		HyRed	12	17.1	14.1	1780.053	0.000732	1.303	15		Stevens	12	21.3	17.1	3261.148	0.000708	2.308	20	
BenLear	13	19.6	14.2	2069.338	0.000631	1.306	7		HyRed	13	18.3	14.1	1904.969	0.000654	1.245	12		Stevens	13	20.8	15.4	2582.875	0.000689	1.78	18	6
BenLear	14	20.2	13.9	2043.523	0.000671	1.372	14	7	HyRed	14	18.5	14.2	1953.201	0.000668	1.304	11		Stevens	14	18.9	15.4	2346.939	0.000823	1.931	28	
BenLear	15	21.9	15.4	2719.469	0.000711	1.934	11		HyRed	15	16.7	14	1713.844	0.000701	1.201	10		Stevens	15	20.9	14.7	2364.719	0.000727	1.718	16	7
BenLear	16	21.4	15	2521.128	0.000614	1.548	21		HyRed	16	16.4	13.2	1496.202	0.000772	1.155	11		Stevens	16	17.8	14.1	1852.921	0.000718	1.33	6	
BenLear	17	19.9	17.4	3154.643	0.000615	1.94	27		HyRed	17	16.8	13	1486.602	0.000678	1.008	11		Stevens	17	17.5	15	2061.67	0.000707	1.457	15	8
BenLear	18	18.3	13.4	1720.518	0.000735	1.265	10		HyRed	18	16.9	12.1	1295.556	0.000815	1.056	12		Stevens	18	16.8	14.4	1824.034	0.000714	1.302	14	9
BenLear	19	17.2	13.8	1715.083	0.000692	1.186	26		HyRed	19	17.3	13.6	1675.416	0.000649	1.087	8		Stevens	19	18.1	13.8	1804.826	0.000744	1.343	16	10
BenLear	20	20.7	14.3	2216.364	0.00075	1.662	11		HyRed	20	15.4	11.5	1066.387	0.000775	0.826	9		Stevens	20	19.1	12.3	1513.011	0.000819	1.239	9	
BenLear	21	19.8	15.4	2458.698	0.000727	1.788	21		HyRed	1	22.1	15.5	2780.061	0.000635	1.764	9		Stevens	1	19	14.3	2034.344	0.000746	1.517	14	
BenLear	22	21	13.9	2124.455	0.000766	1.627	14		HyRed	2	20.4	15.9	2700.369	0.000692	1.868	15		Stevens	2	17.3	16.7	2526.258	0.000714	1.804	15	
BenLear	23	17.5	13	1548.543	0.000672	1.04	5		HyRed	3	18.5	15.5	2327.2	0.000679	1.579	8		Stevens	3	19.8	14.5	2179.716	0.000749	1.633	9	
BenLear	24	17.4	13.7	1709.972	0.000885	1.513	11		HyRed	4	16	13.4	1504.278	0.000735	1.106	10		Stevens	4	20.2	15.6	2573.945	0.000732	1.884	17	
BenLear	25	19.9	14.2	2101.011	0.000621	1.304	13	8	HyRed	5	19.2	14.2	2027.106	0.000659	1.335	16	1	Stevens	5	17.8	13.9	1800.728	0.000732	1.318	5	
BenLear	26	20.2	13.3	1870.912	0.000778	1.455	14	9	HyRed	6	19.8	13.5	1889.432	0.000727	1.374	11	2	Stevens	6	21	16.2	2885.679	0.000769	2.219	28	
BenLear	27	18.5	14.5	2036.603	0.000786	1.6	14	10	HyRed	7	18	13.8	1794.855	0.000699	1.255	22	3	Stevens	7	19.6	19.7	3982.788	0.000545	2.17	22	
BenLear	28	19.2	14	1970.407	0.000848	1.67	14		HyRed	8	19	14	1949.882	0.000718	1.4	10	4	Stevens	8	22.9	16.5	3264.39	0.000636	2.077	25	
BenLear	29	22.4	15.4	2781.558	0.000656	1.825	16		HyRed	9	17.9	13.6	1733.522	0.000702	1.216	14		Stevens	9	19.5	16	2613.805	0.000694	1.814	14	
BenLear	30	17.2	13.7	1690.317	0.000786	1.328	11		HyRed	10	19.1	14.2	2016.549	0.000625	1.261	15	5	Stevens	10	17.5	14.7	1980.028	0.00078	1.545	9	
BenLear	31	17.1	12.2	1332.645	0.000789	1.051	11		HyRed	11	18.2	13	1610.485	0.00076	1.224	12	6	Stevens	11	17.5	13.3	1620.839	0.00075	1.216	10	
BenLear	32	17.4	12.4	1400.849	0.000763	1.069	10		HyRed	12	17.6	12.5	1439.897	0.000775	1.116	15	7	Stevens	12	18.5	14.1	1925.788	0.000755	1.453	10	
BenLear	33	18.3	11.6	1289.335	0.000711	0.917	6		HyRed	13	14.4	11.8	1049.845	0.000654	0.687	1		Stevens	13	20.6	16.6	2972.227	0.000696	2.07	20	
BenLear	34	18.9	13.8	1884.597	0.000779	1.468	7		HyRed	14	16.1	13	1424.66	0.000731	1.042	17		Stevens	14	17.9	14.3	1916.566	0.00077	1.475	4	
BenLear	35	19.6	13.3	1815.34	0.000673	1.222	10		HyRed	15	16.1	12.8	1381.161	0.000683	0.943	13		Stevens	15	19.3	15.6	2459.264	0.000707	1.738	18	
BenLear	36	18.7	14.2	1974.317	0.000745	1.47	16		HyRed	16	16.6	12.3	1314.973	0.000723	0.951	9		Stevens	16	18.6	13.6	1801.314	0.000698	1.257	6	
BenLear	37	20.5	12.6	1704.094	0.00075	1.278	13		HyRed	17	20.6	13.3	1907.959	0.000739	1.41	17	8	Stevens	17	18.4	14.3	1970.101	0.000709	1.396	17	
BenLear	38	25	14.9	2906.104	0.000698	2.029	18		HyRed	18	17.2	13.1	1545.502	0.000743	1.149	13	9	Stevens	18	18.6	15.7	2400.551	0.000604	1.451	15	
BenLear	39	22.5	15.5	2830.379	0.000732	2.071	16		HyRed	19	16.4	11.9	1216.008	0.000736	0.895	11	10	Stevens	19	18.3	14.7	2070.544	0.000743	1.538	12	
BenLear	40	20.7	14.9	2406.254	0.000609	1.466	16		HyRed	20	18.5	12.6	1537.841	0.000704	1.083	4		Stevens	20	16.5	12.6	1371.588	0.000847	1.161	5	
BenLear	41	23.6	15.2	2854.945	0.000618	1.765	17		HyRed	1	17.1	14.5	1882.482	0.000683	1.286	15		Stevens	1	17	14.2	1794.834	0.000803	1.441	7	1
BenLear	42	18.5	13.7	1818.074	0.000711	1.293	6		HyRed	2	19.9	14.1	2071.524	0.000615	1.273	7		Stevens	2	20.6	15.1	2459.347	0.000725	1.784	22	2
BenLear	43	23.5	14.1	2446.272	0.000612	1.497	8		HyRed	3	21.6	14.8	2477.284	0.000656	1.625	7		Stevens	3	18.3	15.5	2302.041	0.000764	1.759	13	3
BenLear	44	17.8	14.2	1879.297	0.000738	1.386	37		HyRed	4	16.7	13.2	1523.572	0.00071	1.082	12		Stevens	4	19	14.8	2179.092	0.000794	1.731	15	4
BenLear	45	17.6	12.7	1486.342	0.000634	0.943	7		HyRed	5	16	12.1	1226.562	0.000726	0.89	9	1	Stevens	5	19.7	14.3	2109.293	0.000769	1.622	14	5
BenLear	46	17.4	12.4	1400.849	0.000696	0.975	9		HyRed	6	16.3	11.4	1109.164	0.000754	0.836	5		Stevens	6	15.1	11.9	1119.617	0.000832	0.932	10	6
BenLear	47	16.7	12.8	1432.633	0.000665	0.953	10		HyRed	7	15.4	12.2	1200.16	0.000681	0.817	11	2	Stevens	7	17.7	11.8	1290.434	0.00084	1.084	2	
BenLear	48	20.3	13.3	1880.174	0.000762	1.433	8		HyRed	8	17.9	13.2	1633.05	0.000666	1.088	10	3	Stevens	8	16.5	13.1	1482.604	0.000781	1.158	19	7
BenLear	49	20.7	14.3	2216.364	0.000793	1.758	18		HyRed	9	17	13.1	1527.531	0.000636	0.971	10	4	Stevens	9	16.6	13	1468.904	0.000825	1.212	18	8
BenLear	50	17.8	12.8	1526.998	0.000843	1.287	7		HyRed	10	19.8	14	2031.982	0.000539	1.096	12		Stevens	10	17.6	12.4	1416.95	0.000783	1.11	7	9
									HyRed	11	18.1	13.3	1676.411	0.00062												

Supplementary Tabel 4.2 SSRs markers selected and used to determine genotypes of the seedlings

Multiplex Comb	Primer ID	Primer Origin	NCBI ID	Linkage Group	Position
Combo 3 NED	SCF213102	Schlautman et al. (2015)	KP278953	2	37.117
	SCF92414	Schlautman et al. (2015)	KP278793	6	34.936
	409500_K63	Schlautman et al. (2015)	KP279177	10	53.026
Combo 9 FAM	SCF140628	Schlautman et al. (2015)	KP278876	4	33.902
	SCF59248	Schlautman et al. (2015)	KP27874	1	52.752
	SCF107715	Schlautman et al. (2015)	KP278816	10	39.343
	SCF73288	Schlautman et al. (2015)	KP278758	2	65.011
Combo 14 HEX	SCF113389	Schlautman et al. (2015)	KP278831	4	31.538
	314797_K70	Schlautman et al. (2015)	KP279218	8	48.577
	vm27120	Zhu et al. (2012)	JF834265	6	41.895
	308539_K70	Schlautman et al. (2015)	KP27929	11	54.516

Supplementary Tabel 4.2 SSRs markers selected and used to determine genotypes of the seedlings

Multiplex Comb	Primer ID	Primer Origin	NCBI ID	Linkage Group	Position
Combo 3 NED	SCF213102	Schlautman et al. (2015)	KP278953	2	37.117
	SCF92414	Schlautman et al. (2015)	KP278793	6	34.936
	409500_K63	Schlautman et al. (2015)	KP279177	10	53.026
Combo 9 FAM	SCF140628	Schlautman et al. (2015)	KP278876	4	33.902
	SCF59248	Schlautman et al. (2015)	KP27874	1	52.752
	SCF107715	Schlautman et al. (2015)	KP278816	10	39.343
	SCF73288	Schlautman et al. (2015)	KP278758	2	65.011
Combo 14 HEX	SCF113389	Schlautman et al. (2015)	KP278831	4	31.538
	314797_K70	Schlautman et al. (2015)	KP279218	8	48.577
	vm27120	Zhu et al. (2012)	JF834265	6	41.895
	308539_K70	Schlautman et al. (2015)	KP27929	11	54.516

Chapter 5

General conclusions and future perspectives

Cranberry fruit production depends of several environmental and physiological factors. The breeding process of this fruit crop is still in early stages of development, as very few cultivars that are grown in commercial beds are the product of two cycles of controlled plant breeding. Those new cultivars have been selected for their increase in vigor, fruit yield and for some, the capability of overcome alternate bearing, which guarantees stable fruit production. However, relatively little is known about the physiology and molecular aspects that govern fruit production in cranberry. Thus, it is imperative to learn more about the vigor of plants produced by self-pollinations and cross-pollinations in order to establish more inbred lines useful in the study of important agricultural traits, and to develop molecular tools that guarantee the production of more cultivars with improved characteristics.

Based on the previous premises, the results from the research performed in the present dissertation will contribute to our understanding about flowering induction and the effect on vigor of self and cross-pollinated plants. The histological analysis performed on apical buds of the alternate bearing cultivar (Ben Lear) and a non-alternate bearing cultivar (HyRed), showed that floral initials differentiation occurred at different times in alternate and non-alternate bearing cultivars during the growing season. Moreover, in HyRed, floral initials are visible 2 weeks earlier in apical buds from vegetative uprights than fruiting uprights. Due to the earliness in formation, floral initials from apical buds of vegetative uprights differentiate into floral meristems early. Other aspect covered in this study was to relate the observation of floral initials with a phenological stage. In general, floral initials were visible when fruiting uprights were in berry growth stages (Stages V and VI). Finally, we learned that bud size is related to the presence of floral initials and once the floral initials are formed the weekly rate of growth of the

bud in Ben Lear increased 300 times compared with buds with no floral initials. While in HyRed buds with floral initials grew 68 times more.

Further histological studies of reproductive apical buds of HyRed from vegetative and fruiting uprights after fruit harvesting season are recommended. Those studies will give us more insights about the differentiation of the floral meristems from buds of vegetative and fruiting uprights. Additionally, extending this study to other cultivars of commercial importance, alternate and non-alternate bearing cultivars, will help to elucidate if the patterns of floral initials formation observed here is a generalized process across cultivars.

The histological study was also carried out to determine the time that floral initials were observed for the first time, and from there, select the samples that will represent stages named as before, during and after floral induction in vegetative and reproductive buds of Ben Lear. Transcriptome analysis in these three floral induction stages was performed in order to determine possible gene candidates that are involved in flowering induction in cranberry.

Genes known to be involved in the five flowering pathways so far known, were BLAST against the transcripts. The results showed that genes involved in Gibberellic acid pathway were upregulated in reproductive buds before and during floral induction stages, and were kept with constant expression throughout the floral induction stages in vegetative buds. These results, suggest that gibberellic acid pathway could be involved in flowering process in cranberry,

contrasting what it has been found in field trials. Whether or not this pathway is truly responsible of flowering in cranberry is still debatable. Studies of the differential expression of genes related to the GA biosynthesis genes and *LFY* in reproductive buds compared to vegetative buds via RT-PCR and *in situ* hybridization, will help to clarify the role of GA in flowering. Additionally field experiments evaluating the effect of GA and anti-GA. treatments in the differentiation of floral meristems as well as the internal concentration of GA and the expression of flowering genes presumably under the control of GA, such as *LFY*, will be decisive in clarifying the participation of this hormone in flowering in cranberry.

The transcriptome generated in this dissertation, not only was useful in evaluating the expression of 148 genes related to flowering in apical buds of Ben Lear, but will be an important tool to find molecular markers, such as SNPs in breeding populations with important agronomical traits.

From PCA analysis of the proportion of self-pollinated seedlings vs cross-pollinated seedlings in Ben Lear, HyRed and Stevens in fruits harvested from field, it was observed that the older the cultivar is, the more heterogeneous the cultivar is; that is, the more cross-pollinated seedlings were observed.

Vigor in seedlings from self- and cross-pollinations from Ben Lear, HyRed, and Stevens was studied in terms of height and number of leaves, and showed that differences in vigor are

evident even at 4 to 6 weeks after seeds were planted. Across cultivars, cross-pollinated seedlings had increased vigor, compared to self-pollinated seedlings.

When comparing height of cross-pollinated seedlings, Stevens and Ben Lear, the parental cultivars of HyRed, showed the highest and lowest height, respectively. However, the loss of vigor in self-pollinated seedlings was less accentuated in Ben Lear than in HyRed and Stevens. It has been observed in field conditions that HyRed uprights are shorter than Stevens; however the fruit production is higher in HyRed. It would be interesting to continue evaluating and comparing the vigor of the seedlings in these cultivars, and further study the yield, in order to determine how the plant vigor (height and leaf number) could affect fruit production in terms of fruit number and fruit traits. On the other hand, it would be important to study self and cross pollinated plants of newly released cultivars. The last research about this topic was published in 1991, two decades earlier than the new cultivars were released.

In addition fruit traits (length, width, volume and density) were evaluated and from there, it was observed that the higher the number of seeds in the fruit, the bigger the fruit it is. However, no relationship between fruit traits and the vigor of the seedling was found. Evaluation of the same traits but including more fruits, would give more power to BLUP analysis, and inclusion of other fruit traits, such as the thickness of the “flesh” and area of the fruit locules would help to understand the effect of the fruit traits, if any, with the seedling vigor. In addition, our results showed that fruit size benefit from cross-pollinations, therefore similar studies in other cultivars will reinforce these findings and could establish a new view of how cranberry

commercial plantations could benefit from heterogeneous genotypes growing together rather than uniform and clonal propagated cultivars in individual beds.