Characterization of the primocane flowering trait in the red raspberry (*Rubus idaeus* L.) and the effect of low temperature

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A mi hermana Estela,
mi estrella en el camino

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Contents

Chapter 1

General Introduction

The red raspberry (*Rubus idaeus* L.) has different flowering habits, and two groups of cultivars can be identified. The primocane cultivars, that bloom on lignified canes in the early summer and on the new growing shoots in late summer and autumn. The floricane cultivars, bloom only on lignified canes and produce fruit once, during the early summer (Heide and Sønsteby, 2011).

Currently, primocane cultivars are of greater economic importance because this character extends the harvest; therefore, these cultivars can be more productive than floricane, also are better adapted to warm climate regions (Pritts, 2008). Thus, several breeding programs are focused on obtaining new primocane cultivars, the primocane fruiting trait is a priority (Hall *et al*., 2009). However, flowering in seasonal shoots is a poorly studied trait, and breeders often find it difficult to evaluate.

Several studies have been to understand the physiological basis of the different flowering habits of raspberry by identifying the environmental factors that lead to floral induction. Temperature and photoperiod are the primary environmental factors that influence the induction process (Heide and Sønsteby, 2011). The expression of the primocane fruiting trait is strongly influenced by the environment, and it has been observed that this character is expressed in different degree (Gambardella *et al*., 2016). Some cultivars, have been observed with an intermediate behaviour; studies have described this behavior in the cultivars ‘Glen Clova’ (Dale and Daubeny, 1987) and ‘Glen Moy’ (Carew *et al.*, 2000; Ourecky, 1976). Thus, this cultivar classified as

floricane can flower at the tip of the shoot by the end of the growing period, before the winter rest, when the genotype is exposed to different environmental conditions. Among the first primocane cultivars obtained through genetic improvement is ‘Heritage’ (Daubeny *et al*., 1992), North American cultivar released in 1969, this cultivar is the most important in Chile, where he arrived at the beginning of the '80s (Bañados, 2002). In Chile exist about 12,000 ha cultivated with raspberry, distributed between 34.70° and 37.47° South latitudes, where climate is cold in winter and hot in summer. Despite the importance of this crop, in the country there is an almost mono-varietal situation, being ‘Heritage’ the main variety grown. However, it has been observed that, in temperate zones with maritime influence, the flowering in the shoot of the season can be minimal, even non-existent (Gambardella *et al*., 2016).

In primocane cultivars floral induction, differentiation and floral development occur early in the season and are favoured at temperatures higher than 20°C (Sønsteby and Heide, 2009; Lockshin and Elfving, 1981; Vasilakakis *et al*., 1980); thus, flowering occurs in the summer and fruits are produced in the early autumn. By contrast, in floricane cultivars, buds of the shoot of the season are induced to flower under short-day conditions and when temperatures are below 13°C and therefore bloom the next spring. Nevertheless, when days are long, and temperatures are low below 13 °C, floral induction may also occur (Sønsteby and Heide, 2008). In this case, the effect of temperature is greater than that of photoperiod, and these cultivars are not strictly dependent on day length.

Although low temperatures (< 18°C) are a prerequisite for floral induction in floricane cultivars, also a positive effect of cold (2°C) has been observed in some primocane cultivars, such as ‘Heritage’ (Takeda, 1993) and two genotypes of primocane

blackberry (*Rubus spp*.), studied by López-Medina and Moore (1999). In both cases was observed an increase of flowering in plants subjected to cold. Some primocane cultivars show a vernalisation-type because the flowering was stimulated at low temperature (Sønsteby and Heide, 2009; Carew *et al*., 2001).

The vernalisation process was defined by Chouard (1960) as "the acquisition or acceleration of the ability to flower by chilling treatment". This process corresponds to an adaptation mechanism to temperate climates in some species, which require a cold period during plant development before being receptive to another signal for floral induction (Koskela *et al*., 2017; Rantanen *et al*., 2015). This It could be of the primary differences between the two groups of raspberry cultivars, in relation to the requirement of cold, as a stimulus for floral induction. So, the cold requirements for flower induction could be an important factor for the differences in the expression of primocane fruiting trait for each cultivar.

At the molecular level, the induction and flowering development have been widely studied in model species such as *Arabidopsis thaliana*, *Hordeum vulgare* and *Triticum aestivum*, which have shown that the flowering process is activated by both internal and external plant stimuli. Gibberellin and the plant's age are internal control factors, while photoperiod and vernalisation are environmental stimuli that trigger the flowering process (Blázquez and Weigel, 2000). Molecularly this process involves a complex network of highly specific genes, associated with different metabolic pathways (Tofiño *et al*., 2013). The genes SUPPRESSOR OF CONSTANS 1 (SOC1), APETALA 1 (AP1) and LEAFY (LFY), are among the most critical genetic processes that must be activated to identify the floral meristem (Vincent-Prue *et al*., 2010, Bewley *et al*., 2000).

The leaves receive the photoperiod signal, where light stimulates genic expression of FLOWERING LOCUS T (FT), a signal that is transferred to the meristematic apex stimulating the gene SOC1, which is the main flowering gene (Vincent-Prue *et al*., 2010). In Arabidopsis, flowering is induced by cold treatments by epigenetic silencing of FLOWERING LOCUS C (FLC) (Horvath, 2009). In this species, prolonged cold treatments can reverse late flowering (Bewley *et al*., 2000). This response to vernalisation has revealed specific repressor genes for each species, which can be inactivated with periods of cold exposure, thus inducing flowering (Koskela *et al*., 2017).

Although no detailed descriptions are available of the genes involved in raspberries, reviewing what happens in other species of *Rosaceae* families with similar reproductive behaviours is of interest. In *Fragaria vesca*, flowering is induced on short days with low temperatures during the autumn, and after a period of winter recess, the flowers emerge in the spring (Koskela *et al*., 2012). Cultivars with this trait are called short-day cultivars, and raspberries exhibit similar behaviour in floricane cultivars. Some strawberry cultivars, called neutral-day cultivars, feature continuous flowering, where the repressor gene, TERMINAL FLOWER 1, is inactive (Iwata *et al*., 2012). This gene, which is regulated by temperature and photoperiod, is the major flowering repressor in *Rosaceae*, similar to the FLC, VRN2 and *Bv*FT1 genes in Arabidopsis, wheat and beets, respectively. In *Fragaria vesca*, *Fv*TFL1 is repressed at low temperatures on short days. However, on long days with temperatures below 13°C, *Fv*TFL1 expression is also suppressed, regardless of photoperiod (Kurokura *et al*., 2013), while at temperatures above 20°C flowering is inhibited because *Fv*TFL1 mRNA is increased (Rantanen *et al*., 2015).

This work postulates that the difference in the flowering habit of the red raspberry is given by different requirements of low temperatures, so that the plant is competent to receive the signals to trigger the floral induction, reflected in the repression of TFL1 gene with low temperatures.

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Hypothesis

Genotypes that do not present flowering in the primocane, require low temperatures for the stimulation of the floral induction, through the process known as vernalisation.

General objective

To study the primocane flowering trait by, the characterization of different genotypes of red raspberry, to determine the effect of the low temperatures in genotypes floricane and primocane, in the expression of the character.

Specific objectives

- Characterize the degree of primocane flowering trait, in five raspberry genotypes with different flowering habits.

- Determiner the influence of low temperatures on floral induction in the primocane.

- Evaluate the difference of expression of the main gene repressor of flowering, in a floricane and primocane cultivars.

Chapter 2

Phenotyping primocane fruiting trait in raspberry (*Rubus idaeus*)

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PHENOTYPING PRIMOCANE FRUITING TRAIT IN RASPBERRY (*Rubus idaeus*)

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Keywords: Primocane, floricane, inheritance, remontancy, chilling.

Abstract

In raspberry (*Rubus idaeus*), two groups of varieties are known: the floricane, not remontant, which has a biennial shoot life cycle and fructifies in the spring only on the shoot lignified in the previous season, and the primocane, remontant, in which fruiting occurs twice, in the spring on the shoot lignified in the previous season and late in the summer or in autumn on the shoot of the season. Primocane fruiting can thus extend harvest periods, resulting in higher yields, and therefore it is an objective of current raspberry breeding programs for new varieties. However, phenotypic identification of this trait is not easy because there is a strong interaction genotype/environment, influenced mainly by temperature and photoperiod. In addition, there is a gradual expression of the character.

With the objective of generating tools to make a more efficient selection of this character in segregating populations, a description of the degree of primocane fruiting was performed in five raspberry genotypes, ranging from high primocane fruiting to no primocane fruiting. The genotypes studied in order of increasing primocane fruiting were: 'Meeker', 'Tulameen', 'Heritage', 'Autumn Bliss', and UC103

(advanced selection). Plant height was measured, together with number of nodes, number and development of lateral shoots, and number of fruits per lateral shoot. Days from planting to first flower were also measured. With these values, a 1-5 scale was developed, that identifies the different degrees of primocane fruiting. Additionally, in the same genotypes we studied the effects of a chill treatment (2 ºC for 30 days) applied to plants in the early stages of development. This treatment was effective only in Heritage. This characterization will allow progress in determining the type of inheritance of this character.

Keywords: Primocane, floricane, inheritance, remontancy, chilling.

Introduction

The great dynamism showed by the raspberry crop in recent years has intensified research efforts aimed at developing techniques that allow off-season production and the expansion of the area under cultivation to warmer regions (Heide and Sønsteby, 2011, Oliveira et al., 1996). Studies about the flowering period have been based mainly on the environmental characteristics that affect this character; however, it has been observed that it is not only affected by the environment, but that there is also great diversity among cultivars and a strong genotype/environment interaction. This often results in a complex behavior, which is unpredictable and difficult to manage. As is generally known, red raspberry varieties are divided into two groups. Floricane: are non-remontant varieties with a biennial life cycle of the shoot, in which in the first year only a shoot with vegetative growth is produced, and in late summer and early autumn buds enter dormancy. Therefore, flowering and

fruiting occur during the second year, when the shoot, or cane, already lignified restarts its development after the winter, and flowering and fruiting take place during the spring. Primocane: are remontant varieties, which are characterized by their ability to flower both in the shoot of the season and in the cane or lignified shoot from the previous season. Thus, two crops are produced: in autumn and spring. Spring production comes from the lignified shoots that were developed in the previous season, while the autumn fruit comes from the shoots of the same season. Hence, remontancy can be defined as the ability of a plant to flower in late summer or in autumn on shoots that develop in the same growing season (Heide and Sønsteby, 2011, Keep, 1961).

Currently, there are several breeding programs for this species, in which the remontancy trait is a priority (Hall *et al*., 2009). One of the main advantages of remontant varieties is that they allow extending the harvest period and therefore can be more productive than non-remontant ones. Moreover, remontant varieties are well suited for hot climate regions, with limited accumulation of chilling hours, which would affect the development of dormant buds in non-remontant varieties (Pritts, 2008). In Chile there are about 12,000 ha cultivated with raspberry, distributed between 34.70° and 37.47° South latitudes, in foothill environments, where climate is cold in winter and hot in summer. Despite the importance of this crop, in the country there is an almost mono-varietal situation, being 'Heritage' the main variety grown. This variety is very hardy and well adapted to growing conditions in the area. It is also considered as remontant, as it fructifies both in spring and autumn. However, it is an ancient variety with very small fruit, and as the crop is cultivated northward with warmer climates, its remontant capacity decreases.

The above mentioned encouraged the development of a breeding program at Pontificia Universidad Católica de Chile, aimed mainly at replacing the Heritage variety with a remontant genotype, more productive in different environments and with larger fruit. This program, based on intervarietal crosses and selection, is being carried out in the town of Santo Domingo (33.6° South latitude), located in an area with a temperate climate and maritime influence. Approximately 30 crosses are performed with 5,000 seedlings being evaluated annually. In these trial plots, a high degree of diversity was verified for the remontancy character, with widely varying responses in flowering habits.

Differences observed in this character are mainly due to different temperature and photoperiod requirements for the processes of floral induction and differentiation. In non- remontant varieties, flower buds are induced under short days and when temperatures are below 13 °C (Williams, 1960). Nevertheless, it has been observed that the effect of the photoperiod disappears when temperatures are lower during the summer. Sønsteby and Heide (2008) found that at temperatures below 12 °C, under long-day conditions, buds can also be induced to flower; conversely, under temperatures of over 18 °C and long-day conditions, induction is not produced.

Another feature of non-remontant varieties is that shoots originating from the root undergo a period of "juvenility", since they need to achieve a certain development. It has been noticed that before reaching 15-20 nodes at least, they are not able to respond to low temperatures and short days so that flower induction occurs (Sønsteby and Heide, 2008, Williams, 1960). In these varieties, growth continues until the end of the season, when growth slows down until it stops. At the same time, the highest apical and lateral buds are induced to flower and then go dormant before

flowering takes place (Sønsteby and Heide, 2011). Additionally, for dormancy to come to an end, a period of low temperatures during several weeks is required (chilling).

On the contrary, in remontant varieties, floral induction and differentiation occurs in the shoot of the season regardless of photoperiod and with high temperatures (28 °C, and even 30 °C for some varieties). Sønsteby and Heide (2009) studied the effects of temperature and photoperiod on a remontant variety, 'Polka' to determine its behavior under protected cultivation, for the production of off-season fruit in Norway. Results indicated that Polka did not need low temperatures for the process of flower induction and formation. The plant flowered at high temperatures (30 °C) and days to anthesis were reduced with the increase in these temperatures.

Moreover, in remontant varieties, no juvenility factor is observed as the response to conditions of floral induction occurs with a stage of development of 5 nodes (Sønsteby and Heide, 2009). Shoot development throughout the season does not stop, and floral induction takes place during the summer, long before days are shortened. Thus, floral development of the apex and higher axillary buds does not stop during the same season, resulting in flowering, fruit set, and fall fruit production.

To these two types of raspberry varieties, some authors add a third one. This would be an intermediate, so-called "tip flowering" type (Neri *et al*., 2012, Dale, 2008, Carew *et al*., 2000, Ourecky, 1976). It is characterized by production of few flowers and fruits on the shoot tip at the end of the first growing season, while the rest of the buds will become flowers and fruits in the second season. This type of behavior can be observed in non-remontant and remontant varieties, when subjected to certain growing conditions. An example of this was described by Dale and Daubeny (1987)

in the non-remontant variety ‘Glen Clova’, which showed flower bud sprouting at the end of the season. These authors named it as "hidden remontant", as it developed flowers on the tip of the primocane at the end of the season.

However, this third type is not sufficient to describe what was observed in the segregating populations from intervarietal crosses evaluated as part of the breeding program of Pontificia Universidad Católica de Chile. In these populations, more gradual differences were observed in flower distribution along the shoot, and the period of beginning of flowering. It is likely that this greater variation in the character is due to the fact that under temperate climate conditions, an increased expression of the differences between genotypes is possible, which are masked when genotypes are cultivated in climates with higher thermal oscillation between day and night.

Several authors have proved that the type of flowering depends on cultivar and location, indicating that factors such as temperature and light strongly influence the time when flowering occurs. Influence of other abiotic factors, such as water and nutrient availability, has also been observed (Privé *et al*., 1993, Hoover *et al*., 1989). At the time of the selection in segregating populations derived from intervarietal crosses, breeders face the difficulty of having no objective parameters to properly assess the character. In general, plants are classified only according to the two types already described, without considering that there are intermediate behaviors, with a gradual expression of the character. That is, those plants that on the shoot of the season have flowers and fruits, regardless of the quantity and location along the shoot, are classified as remontant. On the contrary, those plants that on the shoot of the season are not able to produce any type of flower are considered non-remontant.

The strong environmental influence further interferes in the evaluation of the seedlings. Therefore, a more accurate analysis is necessary, together with the development of tools that help to better understand the responses of each plant, to maximize production results.

A common practice among growers is to expose plants to cold temperatures in their early stages of development to increase flowering in the shoot of the season in the case of the Heritage variety. Apparently, this treatment would have a positive effect, especially when the plantation is established in warmer areas, but it has not been studied whether it is effective and if it would have an effect on other varieties.

The goal of this study was to establish a scale of degrees of remontancy by analyzing the expression of the character in selected raspberry cultivars. The selection included cultivars ranging from zero flowering to abundant flowering in the shoot of the season. In these same genotypes, an evaluation was made of the effect of low temperature treatments –applied in the early stages of development– on the flowering habit.

Material and Methods

Five raspberry cultivars with varying degrees of remontancy were chosen, according to descriptions in literature and previous observations in the same field where the trial took place. The cultivars were: ‘Meeker’, ‘Tulameen’, ‘Heritage’, and ‘Autumn Bliss’ (Jennings, 1988). Selection UC103, from the raspberry breeding program of Universidad Católica, was also included. This selection is remontat and characterized by a very abundant flowering along the shoot of the season.

The trial was established in the town of Santo Domingo, which has a Mediterranean climate with maritime influence, and average temperatures of 20 °C in summer and 7 °C in winter. Plants were established in a substrate cultivation system with 6.5 l pots and a mixture of 30% peat and 70% coconut fiber. Standard fertilization was applied weekly through irrigation, according to local production system.

To evaluate the effect of low temperatures on the five genotypes, chill treatments were applied, which consisted in keeping the plants in cold dark chambers at 2 °C, for 30 days continuously. This was done when the plants reached an average height of 6 cm. Upon completion of the treatment, plants were established in the field, together with the control plants that did not receive the cold treatment, in both cases, the plants had an average height of 6 cm.

Records of growth rate, by measuring the total height in cm, were kept for each plant throughout the season. Number of nodes, lateral shoots, and fruits per lateral shoot were also counted. Evaluations were carried out weekly from planting, in early November, until the end of the season, in April.

The experimental design was completely randomized, with an experimental unit of three plants, and four replications. The Statistix-9 program was used for the analysis.

Results and discussion

The analysis of the growth curve of the genotypes studied (Fig. 1) shows that varieties ‘Autumn Bliss’, UC103, ‘Tulameen’, and ‘Heritage’ have a similar curve, a simple sigmoid curve, with an accelerated growth period and a halt therein approximately in week 15-16 from planting, although there are significant differences in the final height they reach (Table 1). ‘Meeker’, instead, showed a longer growth

period, which only slowed down at the end of the season, coinciding with a sharp drop in temperatures. The differences in height of the shortest varieties would be caused by a lower average internode length.

According to some authors, the halt in growth and flower initiation result from the same mechanism of internal induction. The continued growth of shoots would require high temperatures and long photoperiods (Sønsteby and Heide, 2008). In this case, the above would be verified in the Meeker variety, in which growth was not halted and there was no flowering. However, in the 'Tulameen' variety, the growth curve did halt early, but there was no flowering. This could demonstrate that environmental factors interact differently depending on the genotype, both in remontant and non-remontant varieties.

Table 1 also shows the number of buds that developed side shoots with flowers, from the apex to the base of the main shoot. It is noted that among the 5 genotypes, only three had flower development. As expected, selection UC103 was the one with the greater number of differentiated buds (18.1), followed by 'Autumn Bliss' (10.7), and 'Heritage' (3.5), with these differences being significant. 'Tulameen' and 'Meeker' did not develop flowers. It is worth noting the low percentage of buds that flowered in 'Heritage', since this variety is normally classified as remontant. Nevertheless, under these conditions of mild temperatures, an erratic behavior, close to tip remontancy, is observed (Sønsteby and Heide, 2008, Dale, 2008 Carew *et al*., 2000, Ourecky, 1976).

Since the goal of this study was to establish parameters that can be used to differentiate genotypes in segregating populations, where many individuals are usually evaluated, results obtained were transformed to percentage of buds in the

main shoot that reached flowering, and on this basis a scale of 1 to 5 was designed. Fig. 2 shows a diagram of how flowers are distributed in each of these types of individuals, according to the following "degrees of remontancy": (1) non-remontant, (2) poorly remontant, (3) moderately remontant, (4) highly remontant, and (5) fully remontant, corresponding to 0%, 1-20%, 21-40%, 40-60%, and greater than 60% respectively. In the five genotypes studied, was not observed type 5, but in the previous season, the UC103 selection could have classified in this type of flowering habit.

Regarding the effect of the chill treatment, Table 2 presents a comparison of the parameters evaluated in the same genotypes under both conditions, with and without a chill treatment. Results show that only in the ‘Heritage’ variety there were statistically significant differences in all parameters, that is, height, number of nodes, and degree of differentiation. This variety, which according to our classification was at degree 2 (poorly remontant), with the chill treatment could be classified at degree 3. We can assume that the chilling induced a higher vigor to the growing shoots as it is when vernalisation is applied to seeds and the higher number of nodes which increased the fruiting capacity (the remontancy). In the other varieties, no effect was observed of the cold treatment on the number of buds and so that did not change the number of buds which reached the flowering. However, in the Meeker variety, an effect on plant height and number of nodes was observed, while ‘Tulameen’ only showed a difference in the number of nodes. Thus, it would be expected a higher production in the next spring in both floricane varieties. A microscopic bud analysis could reveal an effect at the differentiation level.

Conclusions

The results obtained in this study showed that there is a gradual expression of the character, because significant differences were found between genotypes studied in relation to percentage of flowering in the shoot of the season. Based on these data, a scale of remontancy was defined, which can be used for the classification of individuals from segregating populations. This classification could help to develop frequency histograms that allow understanding the type of inheritance of this character.

On the other hand, these results show that Heritage is a poorly remontant variety and is responsive to cold treatments applied in the initial stages of plant development. Apparently, varieties with a high degree of remontancy do not respond to these treatments.

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Tables

Table 1. Phenotyping of the five genotypes evaluated

Variety	Average height (cm)	No. of nodes	Height/No. of nodes ratio	No. of differentiated nodes
UC103	118.4 b	35.3 bc	3.4 b	18.1 a
Autumn Bliss	124.9 b	35.7 b	3.5 ab	10.7 b
Heritage	85.3 c	31.3 c	2.7 c	3.5 c
Meeker	164.4 a	45.2 a	3.6 a	0
Tulameen	85.0 c	32.3 bc	2.6 c	0

Table 2. Effect of low temperatures in seedlings on the number of differentiated nodes

Parameter	Treatment	UC103	Autumn Bliss	Heritage	Meeker	Tulameen
Average height (cm)	Not cold	118.4 a	124.9 a	85.3 b	164.4 b	85. 0 a
	Cold	118.9 a	118.0 a	120.5 a	205.7 a	102.1 a
No. of nodes	Not cold	35.3 a	35.7 a	31.3 b	45.2 b	32.3 b
	Cold	36.4 a	31.7 a	42.1 a	53.8 a	38.8 a
No. of side shoots	Not cold	18.3 a	10.7 a	3.5 b	0.0	0.0
	Cold	18.7 a	12.4 a	11.9 a	0.0	0.0

Note: The statistical analysis was performed within the same variety to determine the effect of cold. See vertical comparison for each parameter.

Figures

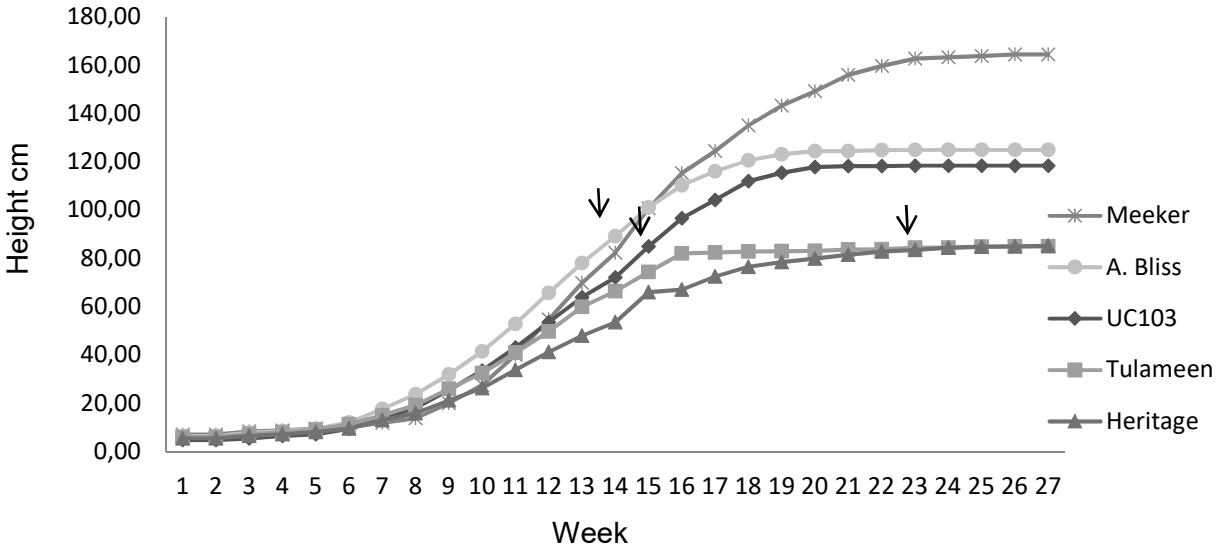

Figure 1. Growth curve of the five genotypes studied. (Arrows indicate the beginning of flowering).

Figure 2. Remontancy scale according to percentage of flowering.

Chapter 3

Effect of low temperature in the first development stage for five red raspberry genotypes

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Effect of low temperature in the first development stage for five red raspberry genotypes

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Abstract

In raspberry, the expression of the primocane fruiting trait is influenced by the environment. Although there are several factors that influence the expression of this character, it is well known that low temperatures that occur during the growth season of the primocane, are important and affect the flowering. In this study, plants in their early stages of development were exposed to low temperatures (2°C) for one month, in a dark cold chamber. The following genotypes primocane and floricane were used: 'UC103', 'Autumn Bliss', 'Heritage', 'Meeker' and 'Tulameen'. Flowering and growth were recorded until the end of the season and the morphology of the meristem was characterized in this moment. Interaction between cold and genotype was detected in all parameters studied. In 'Heritage', a slight primocane, growth and flowering were favored by exposure to cold. Thereby, low temperature affects flowering, but this effect depends on primocane fruiting degree of each genotype, slight primocane

the cold favored flowering and growth. However, strongly primocane the cold had no effect on flowering and growth.

Keywords: flowering, induction, primocane fruiting, vernalisation

Introduction

The red raspberry (*Rubus idaeus* L.) has different flowering habits, and two groups of cultivars can be identified: primocane and floricane. The primocane cultivars can bloom both on lignified canes in the early summer and on the new growing shoots in late summer and autumn. In contrast, floricane cultivars bloom only on lignified canes and produce fruit once during the early summer. Thus, commercially, the primocane cultivars are called annual raspberry, whereas the floricane cultivars are called biennal raspberry (Heide and Sønsteby, 2011).

Currently, primocane cultivars are of greater economic importance because this character extends the harvest; therefore, these cultivars can be more productive than floricane. Primocane cultivars are also better adapted to warm climate regions (Pritts, 2008). Thus, several breeding programs are focused on obtaining new cultivars of the primocane type.

The expression of the primocane fruiting trait or autumn fruiting is strongly influenced by the environment and depending on the geographic location of cultivation this character is expressed in different degree. The percentage of the buds that differentiate into flowers on the growing shoots determines the degree of primocane fruiting (Gambardella *et al*., 2016). Some cultivars have been observed with an intermediate behavior; thus, some cultivars classified as floricane can flower at the

tip of the shoot by the end of the growing period, before the winter rest, when the genotype is exposed to different environmental conditions. Studies have described this behavior in the cultivars ‘Glen Clova’ (Dale and Daubeny, 1987) and ‘Glen Moy’ (Carew *et al.*, 2000, Ourecky, 1976).

The aim of several studies has been to understand the physiological basis of the different flowering habits of raspberry by identifying the environmental factors that lead to floral induction. Temperature and photoperiod are the primary environmental factors that influence the induction process (Heide and Sønsteby, 2011). However, a genetic component with great diversity among cultivars and a strong genotype x environment interaction has been discovered. The process of floral induction/differentiation is flexible in raspberry, resulting in complex, scarcely predictable and difficult to handle behavior (Neri *et al.*, 2012). This flexibility leads to a different response of the flowering habit that is dependent on the environmental conditions of cultivation; therefore, raspberry cultivars cannot be definitively classified as primocane or floricane (Carew *et al.*, 2000), based on exposure to only one environment.

In primocane cultivars floral induction, differentiation and floral development occur early in the season and are favored at temperatures higher than 20°C (Sønsteby and Heide, 2009, Lockshin and Elfving, 1981, Vasilakakis *et al.*, 1980); thus, flowering occurs in the summer and fruits are produced in the early autumn. By contrast, in floricane cultivars, buds of the shoot of the season are induced to flower under short-day conditions and when temperatures are below 13°C and therefore bloom the next spring. Nevertheless, when days are long, and temperatures are low below 13 °C, floral induction may also occur (Sønsteby and Heide, 2008). In this

case, the effect of temperature is greater than that of photoperiod, and these cultivars are not strictly dependent on day length.

Although low temperatures (< 18°C) are a prerequisite for floral induction in floricane cultivars, also a positive effect of cold (2°C) has been observed in some primocane cultivars, such as 'Heritage' (Takeda, 1993) and two genotypes of primocane blackberry (*Rubus* spp.), studied by López-Medina and Moore (1999). In both cases was observed an increase of flowering in plants subjected to cold. In other primocane cultivars, such as 'Polka', flowering and growth improves their response with exposure to high temperatures (Sønsteby and Heide, 2009). However, some primocane cultivars show a vernalisation-type, because the flowering was stimulated at low temperature, (Sønsteby and Heide, 2009, Carew *et al*., 2001).

The vernalisation process defined by Chouard (1960) as "the acquisition or acceleration of the ability to flower by chilling treatment" it could be of the primary differences between the two groups of raspberry cultivars, in relation to the requirement of cold, as a stimulus for floral induction. So, the cold requirements for flower induction could be an important factor for the differences in the expression of primocane fruiting trait for each cultivar. Thus, the purpose of this study was to evaluate the response of flowering and growth in plants subjected to cold treatment at early stages of development using five raspberry genotypes with different degrees of primocane fruiting.

Materials and Methods

Five raspberry genotypes were selected according to the degree of primocane fruiting trait, based previous observations in the field. The primocane cultivars were

‘Heritage’ ‘Autumn Bliss’, and UC103 selection from the breeding program of Universidad Católica de Chile, and the floricane cultivars were ‘Meeker’ and ‘Tulameen’. In our experiment the primocane fruiting trait was evaluated according to the classification of "degrees of primocane fruiting" described by Gambardella *et al.* (2016).

Plants were obtained from roots through etiolated shoots and were established in 6.5-liter pots with a substrate mixture of 30% peat and 70% coconut fiber in a greenhouse at temperatures that ranged from 25 to 27 °C, and without artificial light. The plants were obtained during September and, on October 01. 2014, 12 plants of each genotype were subjected to a cold treatment. Plants were placed in a cold chamber at 2°C in the dark for 30 consecutive days with a total of 720 artificial chilling hours. The control plants were obtained during October, without the cold treatment, under the same propagation condition of plant for cold treatment. The objective was to obtain plants that were at the same stage of development, with an average of four expanded leaves at the establishment of the trial on November 06. 2014.

After treatment in the cold chamber, the trial was established in pots in the locality of Santo Domingo 33°38′ S, 71°39’ W, which has a Mediterranean climate with maritime influence, with an average summer temperature of 20 °C and an average winter temperature of 7 °C. The maximum, minimum and mean temperatures during the test period are presented in Table 1. In relation to the photoperiod, the light hours during the trial evaluation period ranged from 10-15 hours (Fig. 1). Fertilization was applied weekly through irrigation at doses used in commercial production systems.

Days to the first visible floral button, number of floral lateral shoots, number of flowers per plant, height and number of nodes were recorded weekly. These evaluations

were conducted from the establishment of the trial in November 2014 to the end of the season in April 2015. Moreover, after the plants entered winter rest on April 30. 2015, the state of differentiation of buds was determined with a stereomicroscope (63x magnification). Classification of buds as vegetative or reproductive was performed according to the scale published by Neri *et al.* (2012).

The experimental design consisted of completely randomized blocks with four replications, with an experimental unit of three plants. All evaluated parameters, except the meristems, were analyzed using a factorial analysis, with genotype and cold treatment as the factors. For the comparison of averages among genotypes according to treatments, the Tukey test was performed, with a level of significance of p=0.05. The Statistix-9 program (Thomas and Maurice, 2009) was used for statistical analyses.

Results

The primocane cultivars showed different degrees of primocane fruiting, according to the number of floral lateral shoots developed from the apex to the base of the shoot, in the case of control plants (Table 2). 'UC103' selection had the highest number of floral lateral shoots (18.1), followed by 'Autumn Bliss' (10.7) and 'Heritage' (3.5). These differences were significant ($p<0.05$). The two floricane cultivars 'Tulameen' and 'Meeker' did not develop flowers in local climatic conditions. The studied genotypes had degrees of primocane fruiting between 4 and 1 (Table 2).

The factorial analysis showed that both, cold and genotype factors, induced statistically significant differences ($p<0.05$) in each parameter studied. The interaction of these factors also showed statistically significant differences ($p<0.05$)

for each parameter, except in the number of days to the first visible floral button p=0.0977 (Table 3).

For ‘UC103’ selection and ‘Autumn Bliss’, the cold treatment did not significantly affect the number of floral lateral shoots and number of flowers. In contrast, for ‘Heritage’, the values of both parameters were significantly higher in cold-treated plants than in those without cold storage.

Although a genotype x cold interaction was not detected for the number of days to the first visible floral button, an effect of the cold was observed in ‘Heritage’. The treated plants of this cultivar had significantly earlier onset of flowering than the control plants (Table 3).

The meristematic characterization of buds according to genotype and cold treatment is illustrated in Figure 2. For the three primocane cultivars, a differentiation in the basipetal direction was observed. In ‘UC103’ selection, in plants with and without cold, a similar number of nodes and few differentiated buds were observed at the entry of winter rest. ‘Autumn Bliss’ developed fewer floral lateral shoots than ‘UC103’ but a larger proportion of differentiated buds with and without cold. In this genotype, plant architecture was similar under different conditions. In ‘Heritage’, plants without cold had fewer nodes, little development of floral lateral shoots, and fewer differentiated buds than plants that received cold treatment.

‘Meeker’ (Fig. 2) had several differentiated buds along the shoot before entering dormancy, for both cases, with and without treatment whereas ‘Tulameen’ showed similar numbers of differentiated buds and vegetative buds. For ‘Tulameen’, the apex was vegetative for both cases.

In relation with the growth and development, the growth pattern of plants was adjusted to a normal sigmoid curve, with some differences observed among cultivars. As shown in Figure 3, for ‘UC103’ and ‘Autumn Bliss’, no difference was detected in total shoot height, and the growth rates were similar with and without cold treatment. In ‘Heritage’, cold-treated plants reached a larger height, whereas control plants stopped growth earlier. Floricane cultivars showed differences between treated and untreated plants, with taller cold-treated plants. Comparatively, ‘Meeker’ achieved a larger height than that of ‘Tulameen’, cultivar that had a growth rate like that of the three primocane genotypes.

Table 4 shows results for shoot height and number of nodes according to genotype and cold treatment. For both parameters, the interaction was significant. Cold-treated plants of Meeker and Heritage had higher growth rates and therefore were significantly taller by the end of the season than not treated plants. A similar response was observed for the number of nodes in both genotypes. ‘Tulameen’ plants did not show a difference in height, but a significant increase in the number of nodes was observed.

Discussion

Low temperature removes the effects of dormancy but may also have a vernalisation effect on floral development in some species. The response to chilling treatments could depend physiological stage it has been applied, knowing that cold is necessary for breaking dormancy, as well as for vernalisation process (Metzger, 1996). The developmental stage when plants are exposed to low temperatures and duration of

the exposure will determine whether the result is a stimulus for satisfying the chilling requirement for breaking dormancy or a vernalisation effect on flower development. In these studies, the objective was evaluated the low temperatures as a vernalisation effect. For this, plants in their early stages with four development nodes were exposed to low temperatures. Interaction between cold and genotype was found for most of the parameters analysed, related to both flowering and growth. The significant interaction found between low temperature and genotypes was consistent with work by López-Medina and Moore (1999) on blackberry (*Rubus* spp.), in which cold improved flowering and favored growth in the shoot of the season in 2 of the 3 primocane genotypes studied. This led them to conclusion that the response to low temperatures is genotype dependent.

Genotype 'UC103', classified as strongly primocane, showed the greatest development of floral lateral shoots compared with the two other primocane cultivars studied (Table 3). However, the cold had no effect on any flowering or growth parameters, being this cultivar insensible to the cold. Sønsteby and Heide (2009) observed a similar response in 'Polka', which did not require low temperatures for flower formation; by contrast, the response improved at high temperatures. In 'UC103', the proportion of floral lateral shoots and differentiated buds was not modified by cold application, and in both treated and control, plants entered winter rest with a small proportion of differentiated buds (between 9% and 16%), because many of them had previously developed. The early floral differentiation is consistent with that observed by Neri *et al.* (2012) in primocane genotypes, such as 'Dolomia', 'Erika' and 'L03', on similar dates. The absence of a response to cold suggests that flowering of the primocane fruiting in this genotype is not determined by exposure to

cold. Therefore, flowering may be induced early in the season, and with high temperatures plants can develop abundant flowering before winter rest. The response observed in the 'UC103' is of great interest, because this genotype could be used to either expand the cultivation in higher temperature growing areas or reduce the effect of high temperatures due to the climate changes that are occurring in many areas of cultivation.

With a similar response to 'UC103', the effect of cold in 'Autumn Bliss' was not significant for any of the parameters evaluated, related to both flowering and growth. Nevertheless, the total number of flowers was approximately 10% higher in plants subjected to cold treatment than in those without this treatment. Plants treated with artificial cold produced the same number of floral lateral shoots but these shoots had more flowers on average. Carew *et al.* (2001) studied the effect of temperatures between 6 and 7°C on new shoots from roots from 2 to 10 weeks in 'Autumn Bliss'. The authors observed a shorter period to anthesis in plants from roots treated with cold, associating this result with a vernalisation response. In our studies, plants treated with artificial cold initiated flowering 9 days before control plants; however, the difference was not significant, and plant height was not affected. In combination with the bud analysis, in this study 'Autumn Bliss' is not induced of vernalisation process and is also not favored by the cold.

'Heritage' showed a response to the artificial cold for all parameter evaluated, and poor development of floral lateral shoots was observed in plants not exposed to cold. The response is consistent with other studies performed using this genotype, which show that although cold is not mandatory for the beginning of flowering, such treatment accelerates the process and shortens the time to anthesis (Vasilikakis *et*

al., 1980; Takeda, 1993). Once induced, high temperatures are better for flower differentiation, as reported by Lockshin and Elfving (1981). These authors grew plants of 'Heritage' under 16-hour photoperiod and 29/24 °C and 25/20 °C day/night temperatures. They found that plants in the high temperature treatment flower two weeks earlier and produce more flowers. Because of the diversity of responses of this genotype to temperature, further studies are required.

Results of meristem dissection showed a differentiation in the basipetal direction for the three primocane genotypes, as described for other cultivars of this type (Heide and Sønsteby, 2011). The results indicated that floral differentiation of control plants of 'Heritage' was later in relation with that in the two other primocane genotypes. In treated plants low temperatures promoted floral induction, because was observed more buds differentiate so, when are favorable the temperatures for flower development, the production of flower will be higher. The above results confirmed that a period of low temperatures was a determining factor in the process of floral induction in this genotype, unlike the process that occurred in the two other primocane genotypes. Despite this promote of flowering, the proportion of differentiated buds was lower than that reported by Neri *et al.* (2012) in 'Dolomia', 'Erika' and 'L03', that did not undergo artificial cold treatment but were cultivated in an alpine climate with low night temperatures during the spring/early summer period. Of note, in this study 'Heritage' showed intermediate behavior between strongly primocane and floricane cultivars, like the behavior observed by Neri *et al.* (2012) in the 'Lagorai' cultivar.

Under conditions of this study, control plants of the 'Heritage' cultivar, usually classified as primocane, presented a low proportion of floral lateral shoots. This

behavior has been referred to as "primocane fuiting tip" by several authors, although described in floricane cultivars (Sønsteby and Heide, 2008, Carew *et al.*, 2000, Ourecky, 1976). The response observed in control plants was consistent with the response to cold treatment, because at the locality in which the study was conducted, temperatures are moderate (average minimum temperature of 7°C and average maximum temperature of 20°C) with low thermal oscillation. The lack of cold in this cultivar is known to have little effect on flowering, however, in conditions of cold temperatures or greater daily oscillation, this cultivar would be classified as highly primocane.

For the growth response of shoots, only the ‘Heritage’ cold-exposed individuals had an increase in growth rate, like the response observed by López-Medina and Moore (1999) in 2 of 3 blackberry primocane genotypes. Takeda (1993) observed a different effect in ‘Heritage’ plants and found a reduction of growth in response to low temperatures. These antecedents suggest that when plants are exposed to low temperatures is a key factor for the effect on growth, which may be fostered when this exposure occurs in early stages of development. In full growth cold could be a factor inducing dormancy, resulting in a decrease of activity. Takeda (1993) also noted that cold-exposed adult plants had reduced height and number of nodes, suggesting there may be an optimum cold exposure depending on the genotype. Further research in this area is required.

Plants of ‘Meeker’ and ‘Tulameen’, treated and not treated with artificial cold, did not flower on the growing shoot. However, ‘Meeker’ presented more differentiated buds before the entry into winter rest than the ‘Tulameen’, in which the vegetative apex continued to be observed. This characterization indicated that ‘Meeker’ was most

likely less floricane than ‘Tulameen’ and could be potentially primocane under conditions of greater exposure to cold during the growth period, like of the behavior of tip flowering observed in others floricane cultivars (Sønsteby and Heide, 2008, Carew *et al.*, 2000, Dale and Daubeny, 1987, Ourecky, 1976).

In ‘Tulameen’, the undifferentiated apex at the beginning of the winter rest is consistent with the description by Heide and Sønsteby (2011). They noted that floricane cultivars have a differentiation that begins from bud five to ten below the apex, and therefore, the process advances in two directions, basipetal and acropetal. For the growth response, ‘Meeker’ plants subjected to cold-treatment were taller and had more nodes, whereas ‘Tulameen’ plants only showed more nodes. ‘Tulameen’, despite a growth rhythm that was like that of primocane cultivars, the growth was stopped early in the season, and a cessation of growth did not was accompanied by floral differentiation.

Conclusions

This study demonstrated that cold applied in early stages of plant development had a favorable effect on flowering of ‘Heritage’, because of the reduction in days to anthesis, the increase in the number of floral lateral shoots, and the increase in the number of flowers per plant, in addition to an increase in growth rate and larger height and number of nodes in the shoot in ‘Heritage’ and ‘Meeker’. The cold was applied to young plants, therefore, this effect of cold could be attributed to vernalisation.

Low temperatures stimulated flowering primarily in genotypes with an intermediate behavior, such as ‘Heritage’, suggesting that a different vernalisation requirement

between primocane and floricanes cultivars may be involved. These requirements are likely quantitative and would generate gradualness in the expression of primocane fruiting in accordance with the environmental conditions of the locality in which the crop is grown. ‘UC103’ and ‘Autumn Bliss’ require little or no cold to flower and are insensitive to this factor in the flowering process, whereas in ‘Heritage’, the exposure to cold increases degree of primocane fruiting. It is possible that the amount of cold applied to ‘Meeker’ and ‘Tulameen’ was insufficient to observe a difference in the sprouting of flower buds, which should be considered in future studies. A quantitative expression of the primocane fruiting trait in raspberry cultivars can be hypothesized, with higher sensitivity to low temperature in weak primocane types or with an intermediate behavior.

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Tables

Table 1. Temperatures recorded during the red raspberry trial evaluation period in Santo Domingo, Chile

Temperature	Nov	Dec	Jan	Feb	Mar	Apr
Min.	3.9	4.0	6.0	4.0	4.1	3.8
Max.	26.9	27.2	27.1	29.4	30.4	30.9
Mean	14.2	15.9	17.8	16.0	16.8	14.6

* Evaluation period: Nov. 2014 to Apr. 2015.

Table 2. Number of floral lateral shoots developed and evaluation of "degree of primocane fruiting" (1-5) according to the scale of Gambardella *et al*. (2016)

Cultivar	No. of differentiated nodes	Degree of Primocane fruiting
UC103	18.1 a	4, highly primocane
Autumn Bliss	10.7 b	3, moderately primocane
Heritage	3.5 c	2, slightly primocane
Meeker	.	1, floricane
Tulameen	.	1, floricane

* All data correspond to averages of four replications, each represented by three plants.
' Different letters within the column indicate significant differences ($p<0.05$) according to the Tukey test.

Table 3. Effect of cold on flowering of the three primocane genotypes

Cultivar	Treatment	No. of floral lateral shoots	No. of total flowers	No. of days to the first visible floral button
UC 103	No cold storage	18.3 a	96.4 a	99.8 bc
	Cold storage	18.7 a	92.8 a	94.8 ab
Autumn Bliss	No cold storage	10.7 b	46.3 b	97.3 abc
	Cold storage	12.4 b	72.5 ab	88.8 a
Heritage	No cold storage	3.4 c	8.3 c	148.0 d
	Cold storage	11.8 b	51.7 b	110.8 c

* All data correspond to averages of four replications, each represented by three plants.
'Different letters within the columns indicate significant differences ($p<0.05$) according to the Tukey test.
** Data adapted from Gambardella *et al.* (2016).

Table 4. Effect of cold treatment on height and number of nodes in the five genotypes

Cultivar	Treatment	Height (cm)	No. of nodes
UC 103	No cold storage	119.7 c	35.3 de
	Cold storage	118.9 c	36.4 cde
Autumn Bliss	No cold storage	124.9 c	35.7 de
	Cold storage	118.0 c	31.7 e
Heritage	No cold storage	85.2 d	31.3 e
	Cold storage	120.5 c	42.1 bc
Meeker	No cold storage	164.4 b	45.2 b
	Cold storage	205.7 a	53.8 a
Tulameen	No cold storage	85.0 d	32.3 e
	Cold storage	102.1 cd	38.8 bcd

* All data correspond to averages of four replications, each represented by three plants.
' Different letters within the columns indicate significant differences ($p<0.05$) according to the Tukey test.
** Data adapted from Gambardella *et al.* (2016).

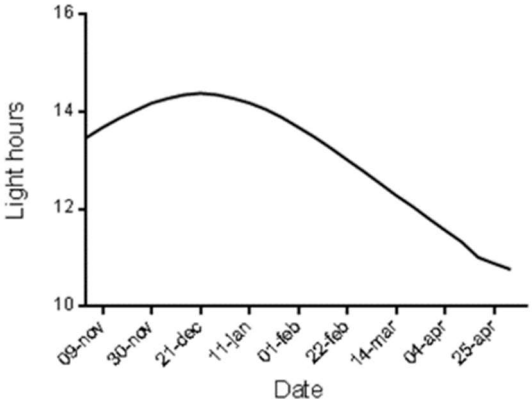

Figure 1. Light hours in Santo Domingo, during evaluation period of trial.

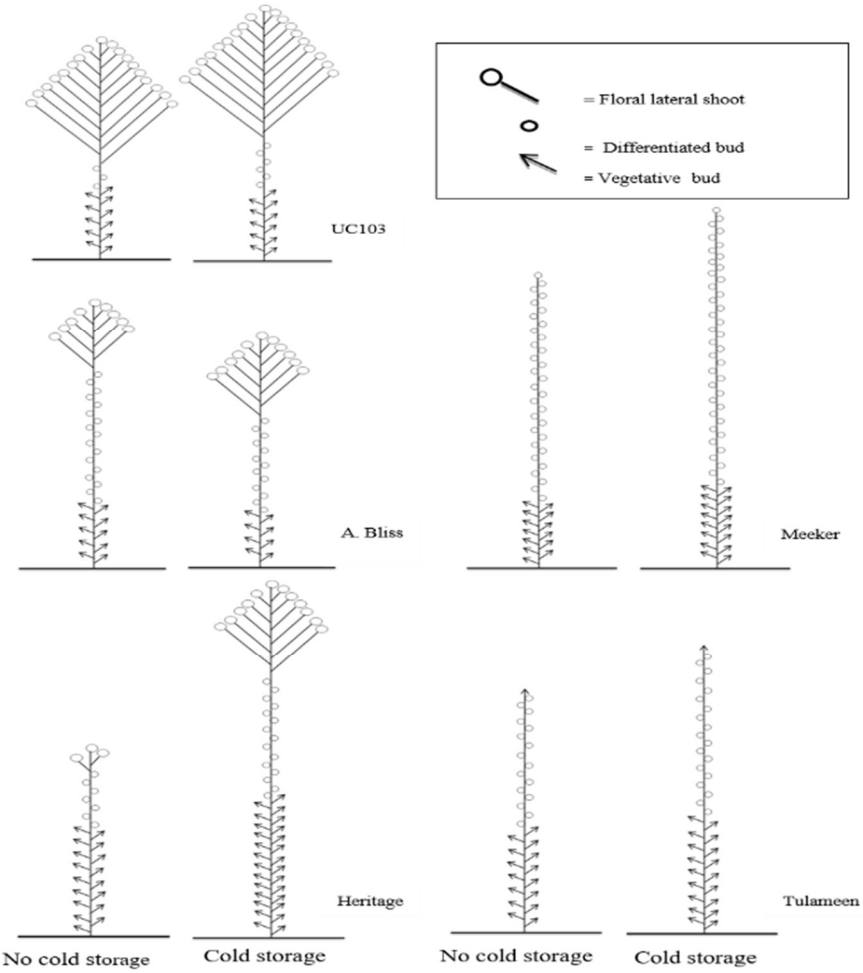

Figure 2. Plant architecture and state of floral differentiation at the entry into winter rest, according to genotype and cold treatment.

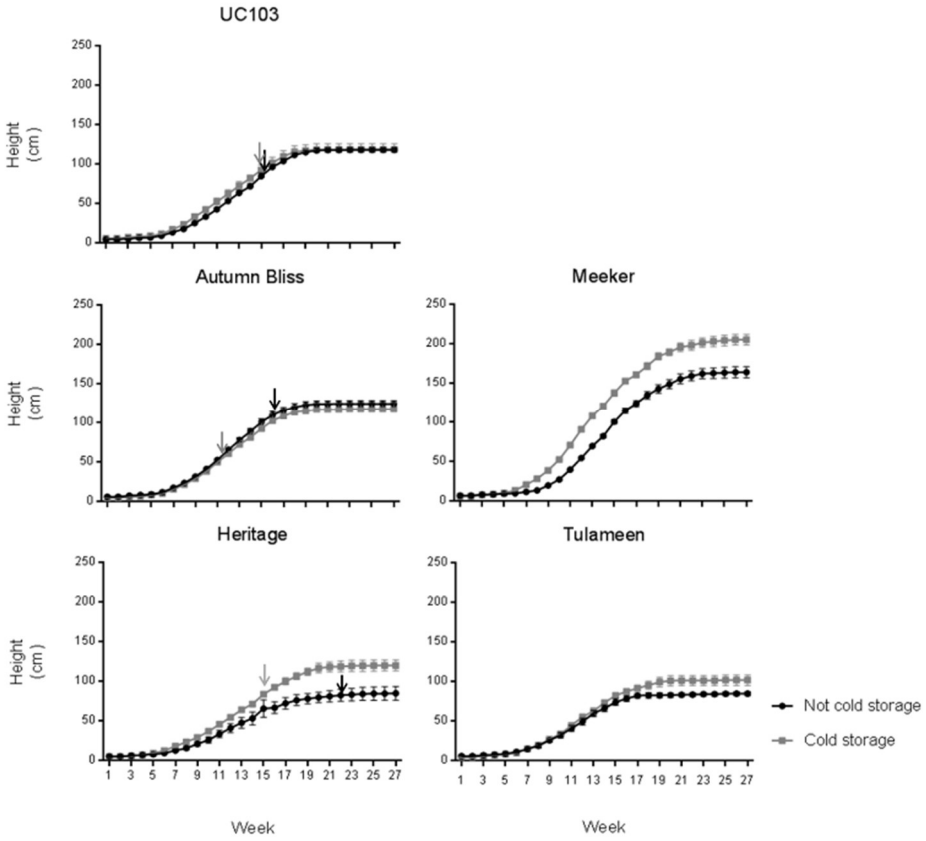

Figure 3. Plant height of cultivars with and without cold storage: primocane and floricane. Arrows indicate the time when the first flower buds were observed at the apex.

Chapter 4

Low night temperature during the growing period enhances primocane fruiting trait in red raspberry (*Rubus idaeus* L.)

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Low night temperatures during the growing period enhance primocane fruiting trait in red raspberries (*Rubus idaeus*).

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ABSTRACT

BACKGROUND:

In red raspberries, two fruiting traits are distinguished, namely, primocane and floricane. Primocane cultivars represent greater economic relevance since they adapt to warm zones and have elongated production periods. However, this classification can be complicated since fruiting is affected by environmental and genetic factors. 'Heritage', the main cultivar used in Chile, presents a relevant case as flowering is inhibited in seasonal shoots grown in temperate zones with low thermal oscillation.

OBJECTIVE:

This study evaluated the effect of cold temperatures during shoot growth on fruiting parameters in different genotypes.

METHODS:

'Autumn Bliss', 'Heritage', 'Meeker' and 'Tulameen' cultivars were tested at low night temperatures. We evaluated the growing characteristics per cultivar, days at first

floral bud, number of floral laterals and meristematic bud differentiation. We also analysed the variations associated with expression of the gene, *Ri*TFL1, on flowering inhibition.

RESULTS:

The results revealed the effect of cold on flowering, growth and *Ri*TFL1 expression, mainly in the 'Heritage' and 'Meeker' cultivars. Shoot flowering was enhanced in the 'Heritage' cultivars, while 'Meeker' exhibited an intermediate behaviour, since seasonal shoots managed to flower with the 60-night cold treatment. In both cultivars, the growth was lower, and *Ri*TFL1 was repressed in treated plants.

CONCLUSIONS:

Genotypes with intermediate behaviour require low temperatures for floral induction, which could be associated with *Ri*TFL1 gene repression in low-temperature treatments.

Keywords: Induction, vernalisation, primocane, cold.

1. INTRODUCTION

In red raspberries (*Rubus idaeus* L.), two cultivar groups can be distinguished by their fruiting traits. Floricanes only flower in second-year lignified stems and therefore present summer production. Primocanes can flower in either lignified or seasonal stems, resulting in both summer and autumn production (Heide and Sønsteby, 2011).

Primocane cultivars have increasing economic importance because they flower and produce fruit twice per season, enabling extended harvest periods, increased profits

and greater adaptation to temperate climates (Pritts, 2008). Primocane cultivars have also allowed off-season fruit production and expanded cultivation areas (Heide and Sønsteby, 2011, Oliveira *et al*., 1996). However, flowering in seasonal shoots is a poorly studied trait, and breeders often find it difficult to evaluate. A segregated population has shown graduality in expressing this trait, and some genotypes may feature reduced fructification in the head of the shoot, while others can bear fruit in the upper section of the seasonal shoot, thus indicating high primocane-fruiting genotypes (Gambardella *et al*., 2016).

'Heritage' was one of the first cultivars defined as primocane, which was obtained via genetic improvement and released in North America in 1969 (Daubeny *et al*., 1992). 'Heritage' has been widely cultivated in several countries because of its high rusticity, and it is the most important cultivar in Chile to date (Leiva *et al*., 2017). Despite its primocane fruiting trait, 'Heritage' has shown higher fruiting prevalence in seasonal stems and in climates with marked day-night temperature oscillations. However, when taken to temperate zones with maritime influence, fruiting in seasonal stems can be minimal and even nonexistent (Gambardella *et al*., 2016).

Several studies have been conducted to evaluate the physiological aspects that determine different raspberry fruiting traits and the influence of the environmental factors involved. Primocane and floricane fruiting, although genotypic traits (Keep, 1988, Keep, 1961), have shown marked environmental influences mainly determined by the interaction of temperature and photoperiod (Heide and Sønsteby, 2011, Sønsteby and Heide, 2008, Carew *et al*., 2000).

Floricane cultivars need short photoperiods and low temperatures to induce flowering. However, trials conducted with this cultivar subjected to different

treatments showed that temperatures above 18°C inhibited floral induction despite being under short photoperiod conditions. However, at temperatures below 15°C, floral induction occurs regardless of the photoperiod (Sønsteby and Heide, 2008, Dale and Daubeny, 2008).

In primocane cultivars, temperature and photoperiod are less influential for floral induction, which can occur at higher temperatures and longer photoperiods. However, flowering can be stimulated in some cultivars by subjecting the plants to low temperatures during the growth period (Sønsteby and Heide, 2009, Carew *et al*., 2001).

Vernalisation is the process by which low temperatures favour inception of the reproductive stage (Chouard, 1960). This process corresponds to an adaptation mechanism to temperate climates in some species, which require a cold period during plant development before being receptive to another signal for floral induction (Koskela *et al*., 2017, Rantanen *et al*., 2015).

In 'Heritage' cultivars, the need for vernalisation in seasonal shoots may explain the differences in fruiting traits in different climatic environments. Under temperate climate conditions of maritime influence (average temperatures of 20°C in the summer and 7°C in the winter), flowering is scarce and is concentrated in the upper part of the shoot, while in areas with marked thermal amplitude (premontane zones), flowering extends from the apex downwards due to high bud prevalence. Therefore, ‘Heritage’ has been classified as slightly primocane-fruiting, while other recently obtained cultivars classified as highly primocane-fruiting are insensitive to this type of stimulus (Gambardella *et al*., 2016).

Induction and flowering development have been widely studied in model species such as *Arabidopsis thaliana*, *Hordeum vulgare* and *Triticum aestivum*, which have shown that the flowering process is activated by both internal and external plant stimuli. Gibberellin and the plant's age are internal control factors, while photoperiod and vernalisation are environmental stimuli that trigger the flowering process (Blásquez and Weigel, 2000).

Molecularly, this process involves a complex network of highly specific genes, associated with different metabolic pathways (Tofiño *et al*., 2013). The genes, SUPPRESSOR OF CONSTANS 1 (SOC1), APETALA 1 (AP1) and LEAFY (LFY), are among the most critical genetic processes that must be activated to identify the floral meristem (Vincent-Prue *et al*., 2010, Bewley *et al*., 2000).

The leaves receive the photoperiod signal, where light stimulates genic expression of FLOWERING LOCUS T (FT), a signal that is transferred to the meristematic apex stimulating the gene, SOC1, which is the main flowering gene (Vincent-Prue *et al*., 2010). In *Arabidopsis*, flowering is induced by cold treatments by epigenetic silencing of FLOWERING LOCUS C (FLC) (Horvath, 2009). In this species, prolonged cold treatments can reverse late flowering (Bewley *et al*., 2000). This response to vernalisation has revealed specific repressor genes for each species, which can be inactivated with periods of cold exposure, thus inducing flowering (Koskela *et al*., 2017).

Although no detailed descriptions are available of the genes involved in raspberries, reviewing what happens in other species of *Rosaceae* families with similar reproductive behaviours is of interest. In *Fragaria vesca,* flowering is induced on short days with low temperatures during the autumn, and after a period of winter

recess, the flowers emerge in the spring (Koskela *et al*., 2012). Cultivars with this trait are called short-day cultivars, and raspberries exhibit similar behaviour in floricane cultivars. Some strawberry cultivars, called neutral-day cultivars, feature continuous flowering, where the repressor gene, TERMINAL FLOWER 1, is inactive (Iwata *et al*., 2012). This gene, which is regulated by temperature and photoperiod, is the major flowering repressor in *Rosaceae*, similar to the FLC, VRN2 and *Bv*FT1 genes in *Arabidopsis*, wheat and beets, respectively. In *Fragaria vesca*, *Fv*TFL1 is repressed at low temperatures on short days. However, on long days with temperatures below 13°C, *Fv*TFL1 expression is also suppressed, regardless of photoperiod (Kurokura et al., 2013), while at temperatures above 20°C, flowering is inhibited because *Fv*TFL1 mRNA is increased (Rantanen et al., 2015).

This study evaluated the effect of cold treatments at night on floral induction and bud differentiation in four raspberry cultivars. Additionally, *Ri*TFL1 gene expression/repression was evaluated in the plants subjected to the cold to determine whether *Ri*TFL1 is involved in vernalisation and whether the differences in expression are related to the degree of primocane-fruiting in raspberry genotypes. In the trials, we used the moderate primocane-fruiting cultivar, ‘Autumn Bliss’, the weak primocane-fruiting cultivar, ‘Heritage’, and the floricane cultivars, ‘Meeker’ and ‘Tulameen’.

2. MATERIALS AND METHODS

2.1 Plant materials and experimental design.

Plants propagated by etiolate buds of the cultivars ‘Autumn Bliss’, ‘Heritage’, ‘Meeker’ and ‘Tulameen’ were placed in 6.5-litre pots, using a substrate of coconut

fibre and peat (70:30), fertilised with 1 gr/Basacote plant (6 months) slow release-fertiliser (15% N, 8% P, 12% K plus micronutrients). They were grown naturally at the Catholic University in Santiago (33° 27' S, 70° 38' W). Fertilisation was applied weekly via irrigation for four months, using 1 gr/plant/week of Ultrasol fast-acting fertiliser (25% N, 10% P, 10% K).

When the plants reached 50 cm high (December 15), night-time cold treatments were conducted during the dark hours (approximately 9 hours per the seasonal photoperiod). For this, the plants were transferred to cold chambers at 2°C every day at dusk, maintaining the same regimen of light hours as in natural conditions. The treatments were applied for 0, 15, 30 and 60 nights. The 60-night treatment was only conducted during the second season of evaluation in the floricane cultivars.

The following flowering parameters were evaluated weekly: number of days to the first visible flower bud, number of floral laterals, number of flowers per plant, and height and number of knots. During the second year of the trial, at the end of April before the winter recess, the differentiation state was also evaluated by meristematic analysis of the undeveloped buds. This analysis was performed under a magnifying glass at 63× (increase), using the methodology of Neri *et al*. (2012), which scores the meristem's morphological changes during floral initiation on a scale of 0 to 8.

2.2 Molecular analysis

Molecular analysis was performed during the second evaluation season. For the RNA extraction and cDNA synthesis, 3 samples were collected per treatment, using the first five buds from the apex downwards. Each sample consisted of approximately 40 mg of tissue from the 5 buds. The samples were collected at the inception of each treatment, on nights 0, 15, 30 and 60 at 11:00 AM, and the samples

were immediately frozen with liquid nitrogen and stored at -80°C. To study the genic expression during the cultivar's normal development and compare it among the treatments, samples from three plants kept outside the cold chambers were also obtained at each sampling.

The total RNA was extracted from each sample using the 3% cetyl trimethylammonium bromide (CTAB) protocol (Yu *et al*., 2012), quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and visualised by electrophoresis to ensure its integrity. The RNA was then treated with DNase (Promega) using 1.6 mg of RNA as a template to synthesise the cDNA using Moloney murine leukaemia virus reverse transcriptase (RT-MMLV; Promega) and Oligo primers (dT). The total RNA from each biological replicate was used as a template for two 25-µL reaction mixtures.

To obtain the TFL1 gene sequence from the raspberries based on orthologous genes from *Fragaria vesca* (JN172097.1, National Center for Biotechnology Information [NCBI]) and *Fragaria* x *ananassa* (AB822995.1, LC017718.1, NCBI), primers were designed with the sequences, 5'-ATGGCAAGAATGTCGGAA-3' and 5'-CTAGCGTCTTCTTGCTGC-3'. A band of approximately 500 bp was amplified by PCR from the cDNA of the 'Heritage' and 'Meeker' cultivars. This band was then purified using the Wizard®SV Gel and PCR Clean-Up System kit (Promega) to be cloned in bacteria with the Topo vector, pUC18. Three transformed colonies were randomly selected and, using a miniprep, the plasmid was extracted with the segment of interest, which was then sequenced.

The TFL1 sequence for *Rubus idaeus* (*Ri*TFL1) was recorded in NCBI with the code MF156853. This sequence was used to design primers for expression analysis (qRT-

PCR) using the Primer3 Program. Each primer's secondary structures and energy diagram were tested on the mFold webserver. Finally, BLAST search was performed in NCBI to ensure each primer's specificity with the sequences, 5'-ATCGGCATCCACAGGTTT-3' and 5'-CTCTCTGCGCATTGAAGT-3'.

For transcriptional analysis, 1 µL of cDNA was used in the SYBR Green RT-PCR, with 10 µL of Brilliant® II SYBR® Green QPCR Master Mix (Stratagene, Agilent Technologies) and 5 M of each primer in the Mx3000 thermal cycler (Stratagene). The thermal profile used for the amplification was 95°C for 10 min, 40 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and a melting curve from 55°C to 95°C with 0.5°C increases. Each reaction was performed with two technical replicates, and a water sample was included as the negative control.

The Ct value (threshold cycle value) was determined for each sample. Thus, Ct values were normalised using the Ct values of the raspberries' histone3 (H3) gene (AF304365.1). The primers used for this gene were 5'-GAACTGTTGCTCTCCGTG-3' and 5'-GAAACGCAGGTCAGTCTT-3', yielding a nonsignificant variation in the gene expression after the treatments were applied to the plants. The normalised Ct values were used to determine the times when the *Ri*TFL1 gene expression changed (Pfaffl, 2001).

2.3 Statistical analysis

The experimental design used was completely randomised with four replicates in the first year of evaluation and five replicates in the second year, with one plant as an experimental unit in both cases. For the molecular analysis, three replicates were used with one plant as an experimental unit. An analysis of variance was applied for each parameter evaluated per genotype. The means between the genotypes

according to treatment were compared using the least significant difference (LSD) test with a significance level of p=0.05. The programme Statistic-9 (Thomas and Maurice, 2009) was used for statistical analysis.

3. RESULTS

3.1 Flowering parameters

The evaluated parameters (Table 1) showed that the low nocturnal temperatures during the two seasons favourably affected the two primocane cultivars, ‘Autumn Bliss’ and ‘Heritage’. For ‘Autumn Bliss’, the numbers of floral laterals and total flowers were greater than those of the control at 15 and 30 cold nights. In addition, the number of days until the first floral bud appeared could be shortened for the first evaluation season.

In ‘Heritage’, the 30-night treatment increased the numbers of floral laterals and total flowers and decreased the number of days until the first floral bud appeared. However, this favourable response was not significant in the 15-night treatment, that is, in contrast to ‘Autumn Bliss’, ‘Heritage’ required more cold nights to improve and anticipate flowering.

For the floricane cultivars, ‘Meeker’ and ‘Tulameen’, only ‘Meeker’ responded to the 60-night treatment by flowering before the end of the season. For this treatment, ‘Meeker’ plants produced an average of 13 floral laterals and 63.5 total flowers per plant. The first visible floral bud was obtained 95 days after planting. Although flower buds could be observed at the end of the season, flowering occurred only in the upper part of the shoot, which is typically referred to as peak flowering (Figure 1).

To evaluate whether the buds under the last developed floral lateral differentiated to reproductive buds, a morphological meristematic analysis was conducted for each treatment, using a magnifying glass. Table 2 shows the number of reproductive buds, but without development at the end of season, as well as total reproductive buds, that is to say, the number of floral laterals plus the number of differentiated buds, per treatment per cultivar.

In ‘Autumn Bliss’, the treatments did not differ by the number of differentiated buds. When considering the differentiated buds and floral laterals, the 15- and 30-night treatments showed increased proportions of reproductive buds developed as floral laterals and undeveloped buds but with the meristem differentiating to a reproductive stem, where only the 30-night treatment showed a significant difference relative to the control (Table 1).

‘Heritage’ differed significantly in the number of differentiated buds, which was greater in the 15- and 30-night treatments compared with the control. However, the number of floral laterals only differed from the control for the 30-night treatment.

For the floricane cultivar, ‘Meeker’, all treatments presented differentiated buds, but only the 60-night treatment differed significantly from the other treatments. In addition, the 60-night-treated plants exhibited peak flowering; therefore, considering the buds that differentiated to reproductive and floral laterals, this difference was also significant. ‘Tulameen’ only presented differentiated buds in the 60-night treatment.

Figure 2 shows a schematic of the buds’ states throughout the entire season. In the remontant cultivars, the floral laterals developed, but the differentiated reproductive

buds did not develop, and a proportion of the buds remained vegetative upon entering recess.

The nonremontant clearly cultivars differed in that ‘Meeker’ was taller with differentiated buds and flowering occurred in the tip, while ‘Tulameen’ presented fewer differentiated buds for the 60-night cold treatment.

3.2 Vegetative growth

Growth in all cultivars featured a simple sigmoid curve. ‘Autumn Bliss’ and ‘Heritage’ reached nearly 2 and 2.5 m tall, respectively, during the first season, while during the second season, both cultivars reached approximately 1.60 m. In general, the plants subjected to cold treatments grew slightly less than did the control plants. Figure 3 features the primocane cultivar growth by treatment; a transversal line shows the moment at which the first floral bud appeared at the apex.

The floricane cultivar, ‘Meeker’, was the tallest, with individuals reaching more than 5 m in the first season, and growth arrest coming late in the season (Figure 4). The plants subjected to the 60-night cold treatment were shorter and flowered before entering recess. ‘Tulameen’ was shorter than ‘Meeker’, and the control plants were the shortest.

‘Autumn Bliss’ differed significantly from the control treatment for each cultivar’s final height in the first season (Table 3), where the plants submitted to the 15- and 30-night treatments were shorter, contrasting with the second season, where the control-treated plants were the shortest. The same trend occurred for the number of buds.

‘Heritage’ grew significantly less only for the 30-night treatment in the first season, while the second season showed no differences between treatments. The number of buds did not significantly differ between seasons (Table 3).

In ‘Meeker’, the cold clearly affected the bud height and numbers. For the first season, the 30-night-treated plants were significantly shorter than those of the other treatments. This effect was not seen in the number of buds (Table 3). In the second season, only the 60-night-treated plants were significantly shorter. The number of buds was significantly lower only in the 60-night treatment in the second season.

In ‘Tulameen’, the control treatment had the shortest plants, while the 30-night treatment presented the tallest plants; the 15- and 60-night treatments did not significantly differ, nor did the number of buds (Table 3).

3.3 Analysis of the TFL1 (flowering) repressor gene

Initially, the TFL1 gene sequence was identified for the raspberries, using primers based on the gene’s orthologous sequences in *Fragaria vesca* and *F. ananassa*. RNA samples from the ‘Heritage’ and ‘Meeker’ cultivars were used, being 100% homologous sequences in both cases. The obtained sequence was recorded under the code MF156853 in the NCBI database.

This sequence was compared with other TFL1 sequences in different species of the *Rosaceae* family, yielding the phylogenetic tree in Figure 5, which shows similarities mainly in the genus, *Fragaria*.

The mRNA sequence alignments, translated to proteins, were highly homologous (91%) between the sequences *Fragaria* x *Ananassa*, *Fragaria vesca* and *Prunus serotina*, relative to the sequence of *Rubus idaeus* (Figure 6).

Figure 7 shows the gene expression levels over time for the TFL1 gene sequence in *Rubus idaeus* for each cultivar and treatment. In 'Autumn Bliss', *Ri*TFL1 was repressed, and the cold-treated plants did not significantly differ compared with the controls. In 'Heritage', the gene was expressed in the control plants, while it was repressed in the treated plants; however, genic expression did not differ between the 15- and 30-night treatments.

In the floricane cultivars, 'Meeker' presented an initial *Ri*TFL1 expression that decreased after 60 days, with increased gene repression in the cold-treated plants. In 'Tulameen', the *Ri*TFL1 gene expression was only detected in the control treatment. In the 15-, 30-, and 60-night cold-treated plants, as well as in the 30- and 60-night control plants, the gene was repressed with no significant differences.

4. DISCUSSION

The results indicate that low nocturnal temperatures impacted the flowering and growth of the seasonal shoot. Low temperatures positively affected the red raspberries, mainly by shortening the vegetative period and accelerating floral induction and differentiation (López-Medina and Moore, 1999, Takeda 1993, Vasilakakis *et al*., 1980); however, this study obtained a positive flowering effect only under cold conditions at night, simulating a greater thermal day-night oscillation.

Within cultivars, the onset of flowering can vary significantly from year to year and from one location to another. In red raspberries, the cycles are controlled mainly by photoperiod and temperature (Carew *et al*., 2000). 'Heritage' loses its primocane-fruiting trait in temperate areas with low thermal oscillations (Gambardella *et al*., 2016). This is consistent with the results of the present study, since we found that

this cultivar responded better to cold treatments (Table 2). ‘Autumn Bliss’ behaves more uniformly in terms of flowering, and while cold treatments have enhanced its flowering, this effect is less marked than in ‘Heritage’.

Several authors have indicated that floricane cultivars require low temperatures for floral induction (Heide and Sønsteby, 2011, Sønsteby and Heide, 2008, Dale and Daubeny, 2008, Carew *et al*., 2000 Williams 1960), while primocane cultivars can flower at high temperatures (Sønsteby and Heide, 2010, Sønsteby and Heide, 2009, Carew *et al*., 2003). We found that while ‘Autumn Bliss’ and ‘Heritage’ are classified as primocane cultivars, their response to the cold differed since ‘Heritage’ adapted better to the cold treatments.

In the floricane cultivars, only ‘Meeker’ was enhanced by the cold, but it needed a longer exposure time at low temperatures to achieve significant results. With the 60-night cold treatment, it behaved like a primocane cultivar, which is similar to findings by Neri *et al*. (2012), in which the cultivar, 'Lagorai', behaved intermediately between the primocane cultivar, 'Erika', and the floricane cultivar, ‘Tulameen’. Meristematic evaluation of the undeveloped buds at the end of the season showed that in ‘Heritage’, ‘Meeker’ and ‘Tulameen’, the cold favoured bud differentiation (Table 2). Nocturnal cold in the middle of the growing season favours both primocane and floricane cultivars in different flowering aspects. Low temperatures enhance flowering in red raspberries, that is, they respond to vernalisation (Carew *et al*., 2000). This process may have quantitative cold requirements since the plants responded differently depending on the cold treatment applied to the cultivar. The different cold requirements for vernalisation may generate the two flowering traits known in raspberries.

Red raspberry flowering is followed by growth arrest. In primocane cultivars, this growth arrest occurs early in the season, while in floricane cultivars, growth cessation is due to shorter days and decreased temperatures (Heide and Sønsteby, 2011). In deciduous plants, nocturnal temperatures impact growth arrest relative to day temperatures, since low temperatures induce growth arrest under long-day conditions [35]. This dynamic could be related to the shorter height of the treated plants, since both primocane and floricane cultivars were shorter at the end of the evaluation period in the first season (Table 3). This effect was also observed by Carew *et al*. (2001) in the 'Autumn Bliss' cultivar, as these authors pointed out that low temperatures would act in two ways, affecting the floral induction time and the shoots' growth rate. However, this difference was less marked in the second season, likely due to the difference in temperature/length of time between the planting and growth arrest in the second season (Supplementary Table 2). In the second season, the control treatments in 'Autumn Bliss' and 'Tulameen' exhibited the shortest plants; therefore, this growth arrest occurred earlier than those of the other treatments; however, this was unrelated to flowering, and the 15- and 30-night cold treatments increased the number of floral laterals in 'Autumn Bliss' (Table 1), while in 'Tulameen', only the 60-night treatment had differentiated buds (Table 2) despite having a late growth arrest, reflected in its greater height.

In addition to the photoperiod and temperature effects on flowering (Carew *et al*., 2000), an important genetic factor would produce different responses to environmental factors. In the primocane cultivar, 'Autumn Treasure', high temperatures prior to flowering, delayed and suppressed this process; therefore, this cultivar would require lower temperatures for flowering. The authors related this

phenomenon to the gene introgression of the species, *Rubus arcticus*, into this cultivar's pedigree (Sønsteby and Heide, 2010). This is similar to that observed in 'Heritage' in climates with maritime influence and low thermal oscillation, where the lack of cold, rather than high temperatures, suppresses flowering. Temperature would have a dominant effect on photoperiod, and floral induction may occur within a certain temperature range (Kurokura *et al*., 2013).

When studying the influence of cold nights on *Ri*TFL1 expression levels, we observed that in all cultivars, the cold repressed *Ri*TFL1. However, in high primocane-fruiting cultivars such as 'Autumn Bliss', this gene is repressed regardless of cold treatment. In 'Heritage', a low primocane-fruiting cultivar, the gene was expressed in the control plants, thus repressing its expression via the cold treatment. *Ri*TFL repression may have favoured the early flowering in this trial, since the plants developed more reproductive buds before entering recess. This agrees with the results of (Koskela *et al*., 2017), who demonstrated that low temperatures are necessary to suppress TFL1 in *Fragaria vesca*, enabling the plants to induce flowering.

5. CONCLUSION

This study demonstrated that low night-time temperatures during the growth season favourably affected flowering, mainly in 'Heritage', a cultivar with low primocane fruiting. This cultivar's behaviour is explained when grown in temperate climates with low day-night temperature oscillation. Heritage requires low temperatures during its development period to repress the *Ri*TFL1 gene, which enables floral induction to develop, achieving flowering in seasonal shoots before the winter recess. Thus,

different genotypes' cold requirements for vernalisation could generate the different phenotypes related to flowering, where low temperatures would diminish *Ri*TFL1 expression in individuals with intermediate behaviour between primocane and floricane fruiting traits. Cultivars such as 'Autumn Bliss' show more uniform flowering without being mainly affected by the environment, which allows expanding the cultivation areas with the certainty that the shoots will flower. Establishing a genetic difference between same-species cultivars requires further studies to determine whether *Ri*TFL1 expression is directly related to seasonal shoot flowering. Verifying whether this gene determines the degree of primocane-fruiting could enable its use as a selection marker in segregating populations, where high primocane-fruiting individuals are sought.

Obtaining genotypes that can flower without requiring exposure to low temperatures to trigger floral induction is a primary selection characteristic, both to expand the cultivation areas and for off-season production.

ACKNOWLEDGEMENTS

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TABLES

Table 1. Effect of low temperatures at night on flowering for two primocane cultivars in two evaluation seasons.

Cultivar	Treatment	No. of floral lateral shoots		Total number of flowers		No. of days to the first visible floral bud	
		2015	2016	2015	2016	2015	2016
Autumn Bliss	0	12.5 b	13.2 b	85.3 b	95.2 b	50.3 a	40.4 a
	15	15.0 a	14.0 ab	125.0 a	118.0 ab	40.8 b	45.8 a
	30	15.5 a	15.6 a	125.3 a	142.0 a	46.8 ab	44.4 a
Heritage	0	12.0 b	13.2 b	61.0 a	90.4 a	85.8 a	67.0 a
	15	12.8 ab	13.2 b	63.8 a	89.4 a	86.0 a	64.6 a
	30	14.5 a	15.0 a	89.8 a	114.0 a	63.5 b	57.6 a

* All data correspond to averages of four replications for first season and five replications for second season, each represented by one plant.
'Different letters within the columns indicate significant differences ($p<0.05$) according to the LSD test.

Table 2. Number of reproductive buds at the end of the season, for each cultivar per treatment. The first column shows the number of reproductive buds without development and the second column presents the total number of reproductive buds (floral laterals and reproductive buds without development)

Treatment	Autumn Bliss		Heritage		Meeker		Tulameen	
	Without development	Total	Without development	Total	Without development	Total	Without development	Total
0	8.7 a	22.7 b	1.0 b	14.3 b	20.7 b	20.7 b	0.0 b	.
15	9.0 a	23.0 ab	2.7 a	15.7 ab	18.3 b	18.3 b	0.0 b	.
30	10.0 a	26.7 a	3.0 a	18.3 a	23.0 b	23.0 b	0.0 b	.
60	.	.	.	.	30.0 a	38.7 a	4.3 a	.

* All data correspond to averages of three replications for second season, each represented by one plants.
'Different letters within the columns indicate significant differences ($p<0.05$) according to the LSD test.

Table 3. Effect of low night temperatures for the height, and nodes numbers, at the end of the season for each cultivar per treatment during the growth stage.

Cultivar	Treatment	Height (cm)		No. of nodes	
		2014-2015	2015-2016	2014-2015	2015-2016
Autumn Bliss	0	229.8 a	142.2 b	47.8 a	36.4 b
	15	197.0 b	164.8 a	42.0 b	39.0 ab
	30	194.3 b	165.2 a	42.8 b	39.8 a
Heritage	0	279.5 a	165.2 a	73.8 a	49.0 a
	15	251.8 a	160.8 a	67.0 a	49.2 a
	30	186.3 b	148. 0 a	53.0 b	44.0 a
Meeker	0	504.3 a	399.0 a	94.8 a	86.6 a
	15	413.5 ab	396.0 a	85.3 a	85.2 a
	30	370.3 b	375.4 a	80.8 a	81.6 a
	60	.	260.0 b	.	67.6 b
Tulameen	0	.	208.4 b	.	60.2 a
	15	.	240.0 ab	.	69.4 a
	30	.	260.6 a	.	72.4 a
	60	.	237.6 ab	.	67.0 a

* All data correspond to averages of four replications for first season and five replications for second season, each represented by one plant.
'Different letters within the columns indicate significant differences ($p<0.05$) according to the LSD test.

SUPPLEMENTARY TABLES

Supplementary table 1. Temperatures recorded during the trial evaluation period in the location of Santiago

Temperature	Nov		Dic		Jan		Feb		Mar		Apr	
	2014	2015	2014	2015	2015	2016	2015	2016	2015	2016	2015	2016
Max	23,6	23,1	24,7	25,7	26,4	26,3	26,7	24,8	24,9	23,2	17,3	17,5
Min	14,1	13,4	13,8	17,7	20,1	18,4	17,8	20,9	18,1	16,6	10,9	9,3
Mean	20,3	18,8	21,7	22,2	24,3	22,7	22,8	23,1	21,8	20,1	14,5	14,5

* Evaluation period: Nov. 2014 to Apr. 2015 first season and Nov. 2015 to Apr. 2016 second season

Supplementary table 2. Thermal sum from the establishment of the trial to the cessation of growth, for each cultivar in both seasons, according to the cold treatment.

Cultivar	Year	Treatment		
		0	15	30
Autumn Bliss	2015	1411.2 a	1305.5 a	1195.6 a
	2016	1338.6 a	1275.7 a	1241.4 a
Heritage	2015	1823.5 a	1677.3 a	1379.8 a
	2016	1512.0 b	1339.4 b	1260.1 b
Meeker	2015	1881.1 a	1826.5 a	1716.5 a
	2016	1802.4 b	1719.1 b	1619.4 b

* All data correspond to averages of four replications for first season and second season, each represented by one plant.
'Different letters within the columns indicate significant differences ($p<0.05$) according to the LSD test.

FIGURE CAPTIONS

Figure 1. ‘Meeker’ cultivar after the 60-night cold treatment featuring flowering and fruiting before winter recess.

Figure 2. Bud condition along the seasonal shoot, from the apex to the base, at the end of the season and beginning of winter recess according to each cultivar treatment. The meristematic analysis was carried out in three plants for each cultivar according to treatment

Figure 3. Height reached by both primocane cultivars according to treatment for the two evaluation seasons (A and C 2015, B and D 2016). The lines indicate the moment when the first visible floral bud appeared.

Figure 4. Height reached by the two floricane cultivars according to treatment for the two evaluation seasons for ‘Meeker’ (A 2015 and B 2016) and season 2016 for ‘Tulameen’ (C). The line indicates when the first visible floral bud appeared for the second evaluation season in ‘Meeker’.

Figure 5. The phylogenetiv analysis of TFL1 mRNA nucleotide sequence in Rosaceae species and Arabidopsis thaliana (KX139000.1). The abbreviations correspond to Rosa chinensis (JQ008813.1), Malus domestica (NM_001293865.1 and NM_002293958.1), Prunus serotina (KY435487.1), Rubus idaeus (MF156853), Fragaria x ananassa (KR139757.1 and LC017718.1) and Fragaria vesca

(NM_001280077.1). Phylogenetic relationship tree constructed using Geneious program v. 9.1.4, UPGMA tree.

Figure 6. The protein aligment of traduction of TFL1 mRNA nucleotide sequence in *Rosaceae* species. The abbreviations correspond to *Fragaria x ananassa* (AMR34693.1), *Fragaria vesca* (NP_001267006.1), *Malus domestica* (NP_001280794.1), *Pyrus pyrifolia* (BAJ33557.1), *Prunus serotina* (AQX77700.1), *Rosa chinensis* (JQ008813.1) and *Rubus idaeus* (MF156853). Protein aligment was constructed using *Geneious* program v. 9.1.4.

Figure 7**.** TFL1 gene expression over time in the 'Autumn Bliss', 'Heritage', 'Meeker' and 'Tulameen' cultivar apices. Gene expression was measured during the plants' growth period from the beginning of the cold treatments; thus, the gene expression was evaluated at 15, 30 and 60 days (the latter only in the floricane cultivars) with and without cold nights. Relative gene expression with the same letter indicates no significant difference, with $p \geq 0.05$.

Figure 1

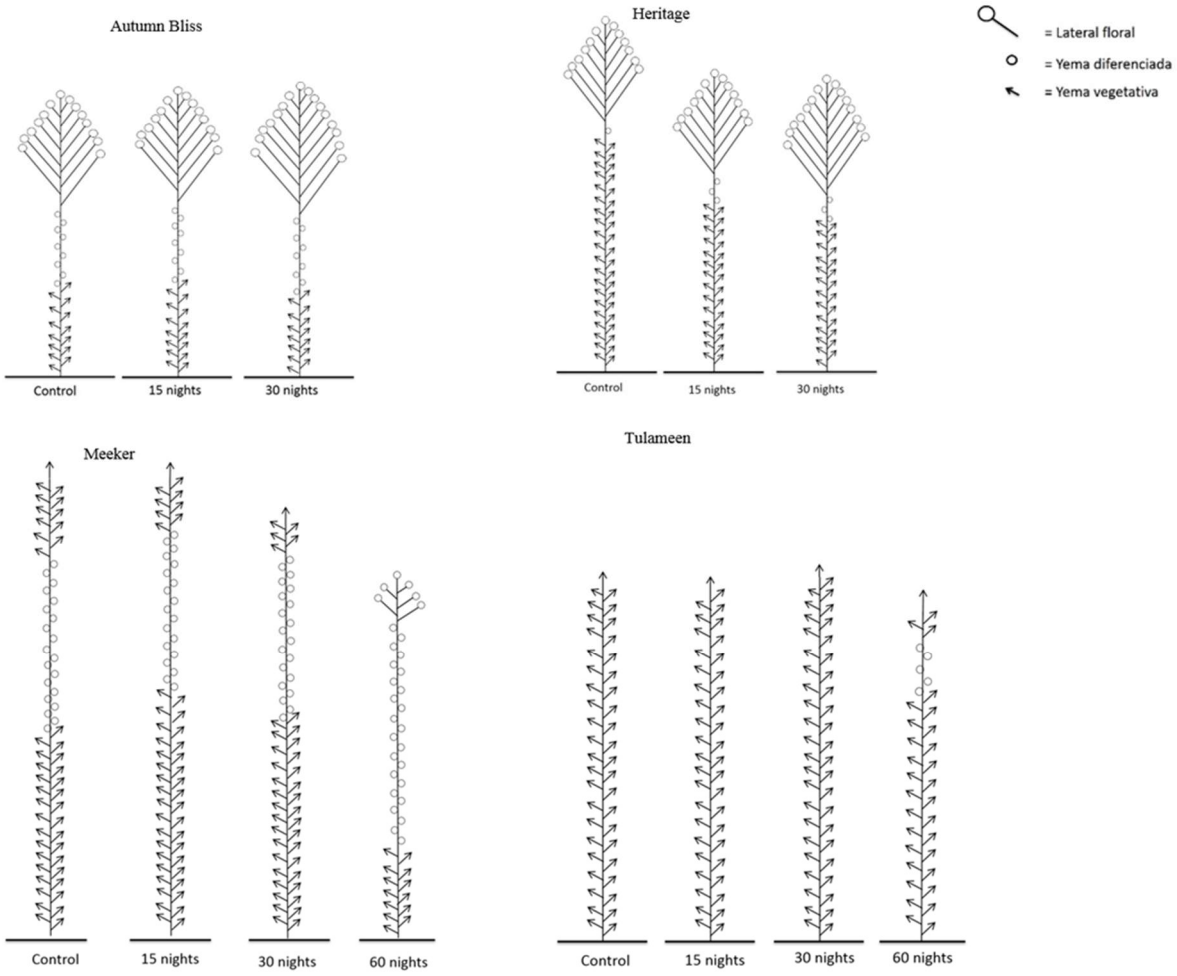

Autumn Bliss
Control
15 nights
30 nights
Heritage
Control
15 nights
30 nights
= Lateral floral
= Yema diferenciada
= Yema vegetativa
Meeker
Control
15 nights
30 nights
60 nights
Tulameen
Control
15 nights
30 nights
60 nights

Figure 2

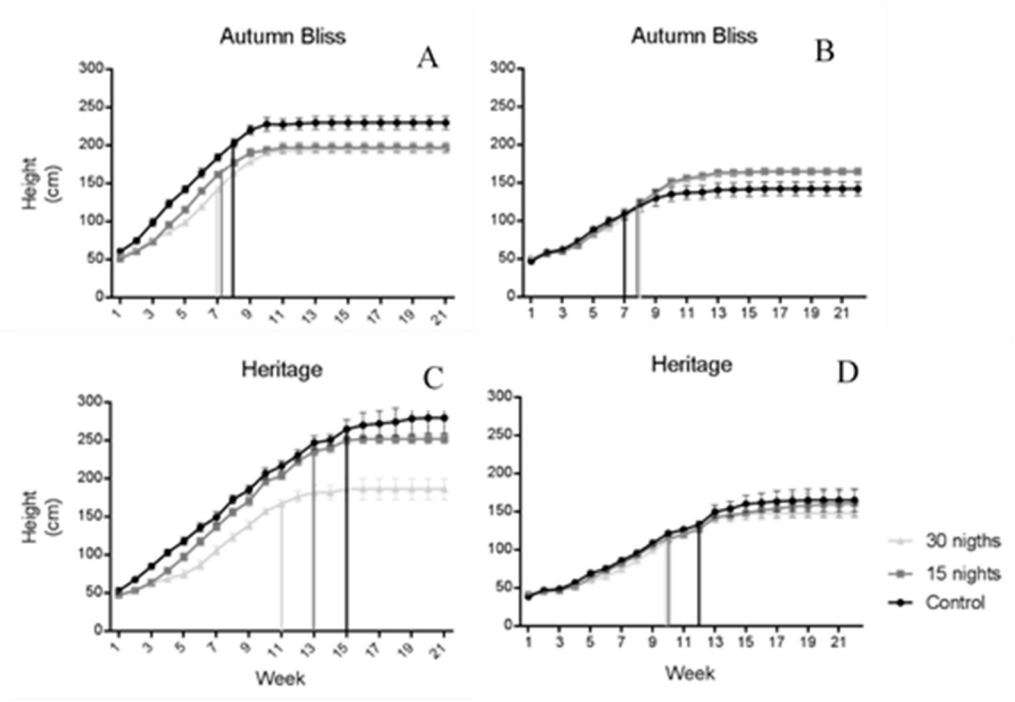


Figure 3

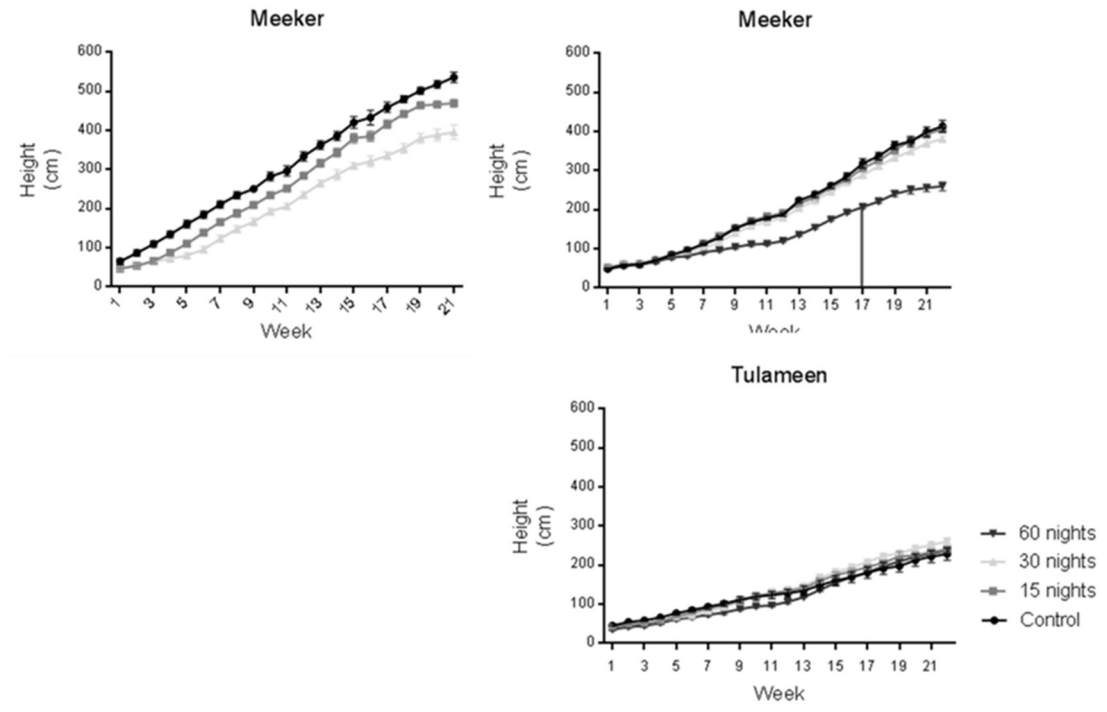
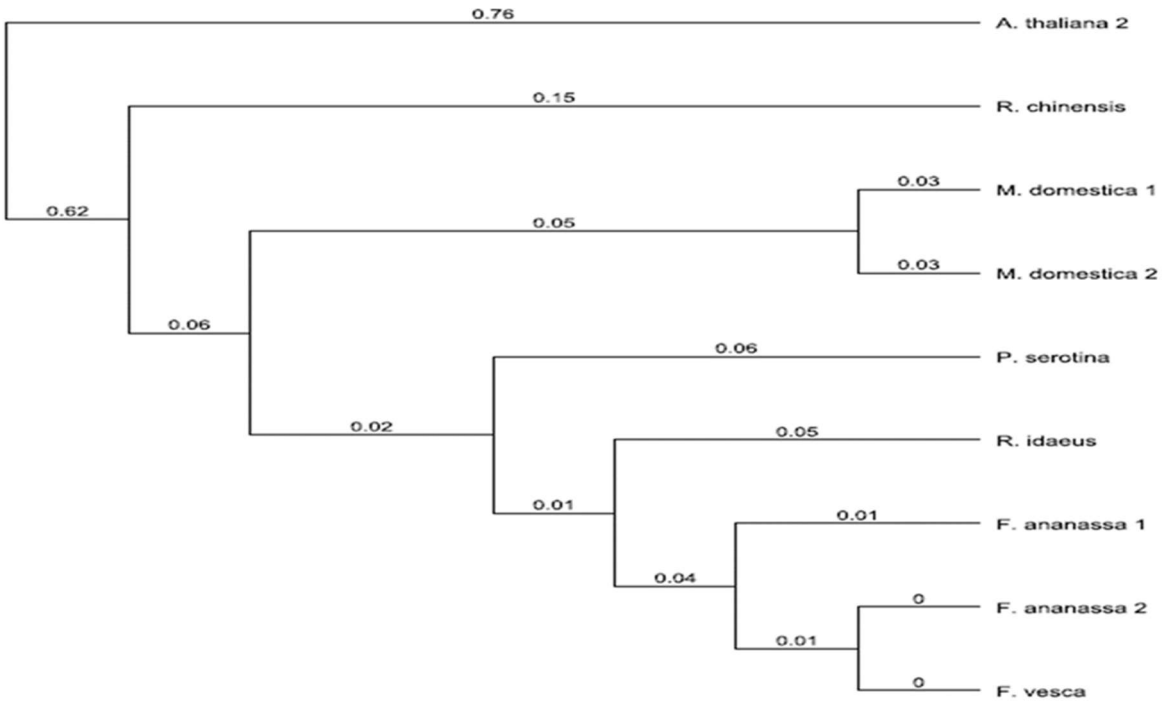


Figure 4


0.76
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0.15
R. chinensis
0.03
M. domestica 1
0.62
0.05
0.03
M. domestica 2
0.06
0.06
P. serotina
0.02
0.05
R. idaeus
0.01
0.01
F. ananassa 1
0.04
0
F. ananassa 2
0.01
0
F. vesca

Figure 5

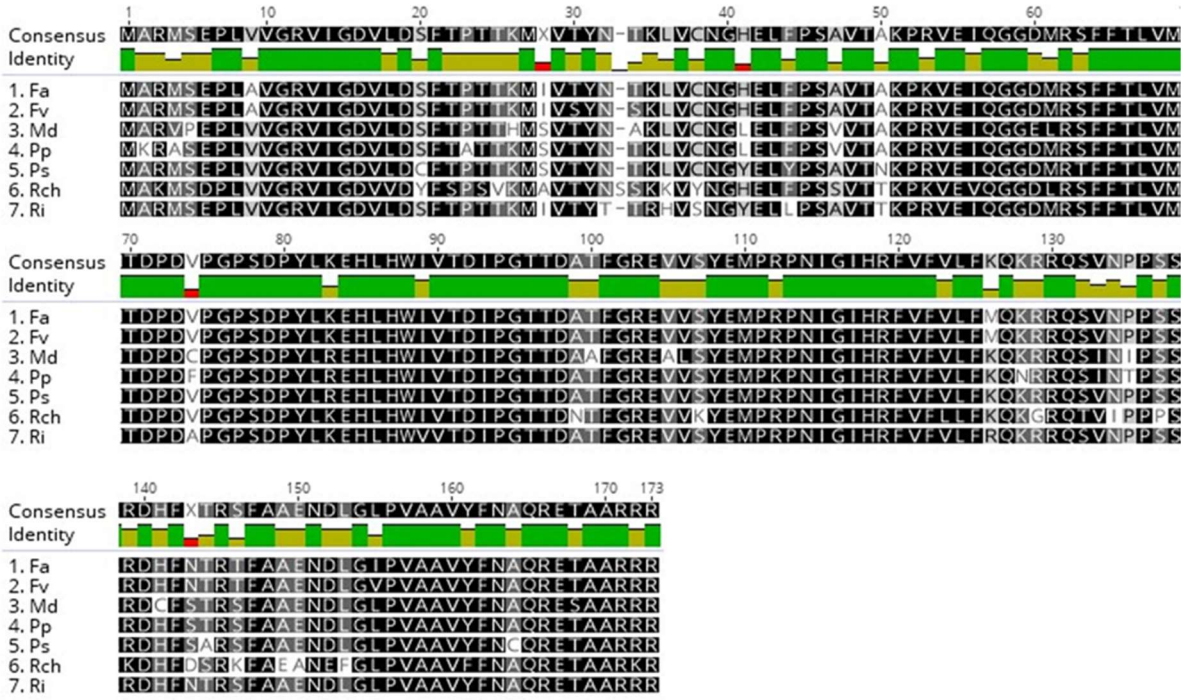

Figure 6

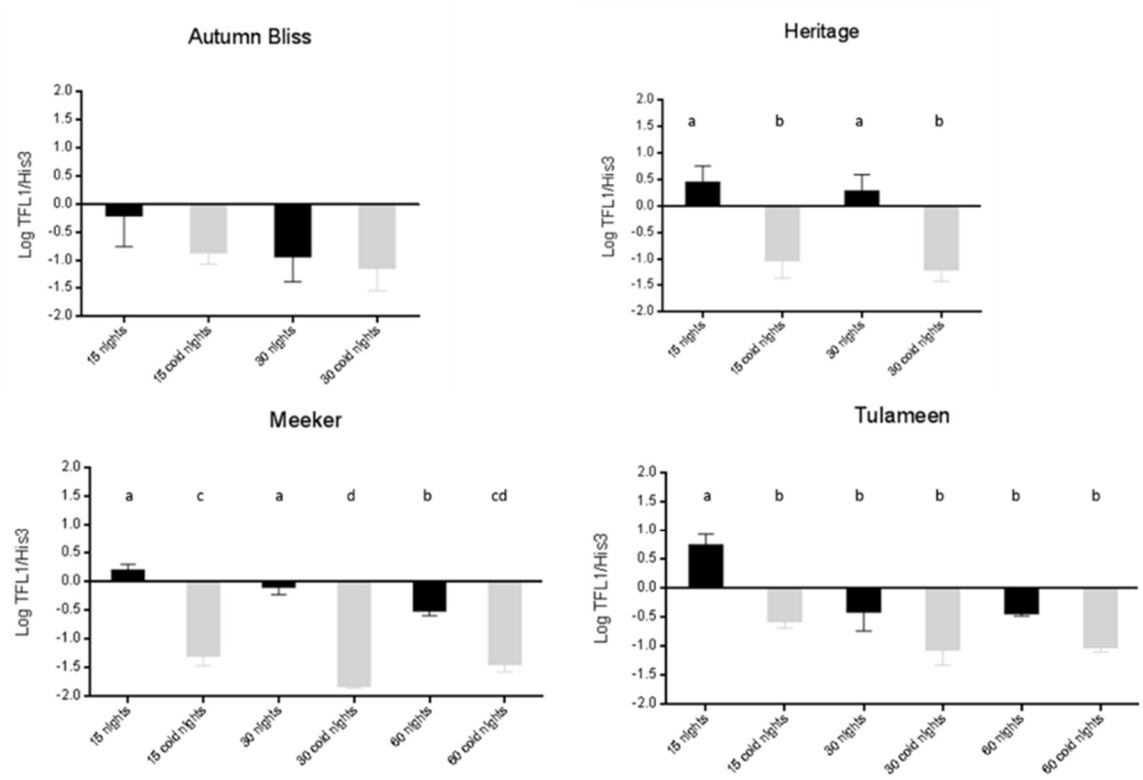
Autumn Bliss
Heritage
Meeker
Tulameen
Log TFL1/His3
15 nights
15 cold nights
30 nights
30 cold nights
60 nights
60 cold nights
a
b
c
d
cd

Figure 7

Chapter 5

General conclusions

The results in this study showed that there is a gradual expression of the character, in relation to percentage of flowering in the shoot of the season. A scale of remontancy was defined which can be used for the classification of individuals from segregating populations or new cultivar.

For this study was concluded that ‘Heritage’ is a poorly primocane cultivar and is responsive to cold treatments applied in the initial stages of plant development and during the night in the growing season.

Exist an effect of low temperature, the cold applied in early stages of plant development and the low night-time temperatures during the growth season, favourably affected flowering, mainly in ‘Heritage’. This cultivar requires low temperatures during its development period to repress the RiTFL1 gene, which enables floral induction to develop, achieving flowering in seasonal shoots before the winter recess. Thus, different genotypes’ cold requirements for vernalisation could generate the different phenotypes related to flowering, where low temperatures would diminish RiTFL1 expression in individuals with intermediate behaviour between primocane and floricane fruiting traits.

Cultivars such as ‘Autumn Bliss’ show more uniform flowering without being mainly affected by the environment, which allows expanding the cultivation areas with the certainty that the shoots will flower. Establish a genetic difference between same-species cultivars, requires further studies to determine whether RiTFL1 expression is directly related to seasonal shoot flowering. Verifying whether this gene

determines the degree of primocane-fruiting could enable its use as a selection marker in segregating populations, where high primocane-fruiting individuals are sought.

Obtaining genotypes that can flower without requiring exposure to low temperatures to trigger floral induction is a primary selection characteristic, both to expand the cultivation areas and for off-season production.

Annex

Thermal regime for Santo Domingo (33°38′ S, 71°39’ W) and Santiago (33° 27' S, 70° 38' W) during the evaluation period.

Table 1. Temperatures recorded during the red raspberry trial evaluation period in Santo Domingo, Chile.

Temperature	Nov	Dec	Jan	Feb	Mar	Apr
Min.	3.9	4.0	6.0	4.0	4.1	3.8
Max.	26.9	27.2	27.1	29.4	30.4	30.9
Mean	14.2	15.9	17.8	16.0	16.8	14.6

* Evaluation period: Nov. 2014 to Apr. 2015.

Table 2. Temperatures recorded during the red raspberry trial evaluation period in Santiago, Chile.

Temperature	Nov	Dec		Jan		Feb	Mar	Apr
		Control	Treatment	Control	Treatment			
Min.	14.1	13.8	8.2	20.1	16.1	17.8	18.1	10.9
Max.	23.6	24.7	24.7	26.2	25.5	26.4	24.9	17.3
Mean	20.3	21.7	17.9	24.3	21.2	22.8	21.8	14.5

* Evaluation period: Nov. 2014 to Apr. 2015.

Table 3. Temperatures recorded during the red raspberry trial evaluation period in Santiago, Chile.

Temperature	Nov	Dec		Jan		Feb		Mar	Apr
		Control	Treatment	Control	Treatment	Control	Treatment		
Min.	16.4	17.7	15.6	18.4	12.8	20.9	14.9	16.6	9.3
Max.	23.1	25.7	23.2	26.3	17.5	24.8	24.8	23.2	17.5
Mean	19.1	22.2	19.0	22.7	15.6	23.1	20.4	20.1	14.5

* Evaluation period: Nov. 2015 to Apr. 2016

GenBank ▾ Send to: ▾

Rubus idaeus cultivar Heritage terminal flower 1 (TFL1) mRNA, complete cds

GenBank: MF156853.1

FASTA Graphics

Go to:

```
LOCUS       MF156853                519 bp    mRNA    linear   PLN 13-NOV-2018
DEFINITION  Rubus idaeus cultivar Heritage terminal flower 1 (TFL1) mRNA,
            complete cds.
ACCESSION   MF156853
VERSION     MF156853.1
KEYWORDS    .
SOURCE      Rubus idaeus
  ORGANISM  Rubus idaeus
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae;
            Pentapetalae; rosids; fabids; Rosales; Rosaceae; Rosoideae;
            Rosoideae unplaced; Rubus.
REFERENCE   1  (bases 1 to 519)
  AUTHORS   Contreras,E., Gambardella,M. and Pino,M.T.
  TITLE     Sequence and expression of RiTFL1 in raspberry homolog of TFL1
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 519)
  AUTHORS   Contreras,E., Gambardella,M. and Pino,M.T.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-MAY-2017) Fruticultura, Pontificia Universidad
            Catolica de Chile, Av. Vicuna Mackenna 4860, Santiago, Region
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                     EVVSYEMPRPNIGIHRFVFVLFRQKRRQSVNPPSSRDHFNTRSFAAENDLGLPVAAVY
                     FNAQRETAARRR"
ORIGIN
        1 atggcaagaa tgtcggaacc cttggttgtt ggaagggtaa ttggagatgt tcttgattcc
       61 ttcaccccaa ctaccaaaat gattgtcact tacactacga ggcacgtctc taatggatat
      121 gagctcttac cttctgcagt caccaccaaa cctagagttg agattcaagg cggtgacatg
      181 agatcattct tcactctggt gatgacagac ccagatgctc ctggccctag tgatccatat
      241 ttgaaggagc acctgcactg ggttgtgaca gacattccag gcaccacaga tgctacattt
      301 ggaagagagg tggtgagcta tgagatgcca aggcccaaca tcggcatcca caggtttgtg
      361 tttgttctct tcaggcagaa acgaaggcag tcagtgaacc ctccttcctc aagggaccac
      421 ttcaacaccc gaagcttcgc agcagaaaat gacctcggtc ttcctgtcgc tgccgtttac
      481 ttcaatgcgc agagagaaac agcagcaaga agacgctag
//
```

Figure 1. Sequence of TFL1 gene for red raspberry, Heritage cultivar

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Rubus idaeus cultivar Meeker terminal flower 1 (TFL1) mRNA, complete cds

GenBank: MF156854.1

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LOCUS       MF156854                519 bp    mRNA    linear   PLN 13-NOV-2018
DEFINITION  Rubus idaeus cultivar Meeker terminal flower 1 (TFL1) mRNA,
            complete cds.
ACCESSION   MF156854
VERSION     MF156854.1
KEYWORDS    .
SOURCE      Rubus idaeus
  ORGANISM  Rubus idaeus
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae;
            Pentapetalae; rosids; fabids; Rosales; Rosaceae; Rosoideae;
            Rosoideae unplaced; Rubus.
REFERENCE   1  (bases 1 to 519)
  AUTHORS   Contreras,E., Gambardella,M. and Pino,M.T.
  TITLE     Sequence and expression of RiTFL1 in raspberry homolog of TFL1
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 519)
  AUTHORS   Contreras,E., Gambardella,M. and Pino,M.T.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-MAY-2017) Fruticultura, Pontificia Universidad
            Catolica de Chile, Av. Vicuna Mackenna 4860, Santiago, Region
            Metropolitana 7820436, Chile
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
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//
```

Figure 2. Sequence of TFL1 gene for red raspberry, Meeker cultivar