ROBERT YONEL MÁRQUEZ GUTIÉRREZ

PHENOTYPIC, BIOINFORMATICS AND MOLECULAR ANALYSES RELATED TO THE DEVELOPMENT AND REPRODUCTIVE TRANSITION OF *HUMULUS LUPULUS* L. (HOP) IN SUBTROPICAL REGIONS OF BRAZIL

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Dissertação apresentada à Universidade Federalde Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fisiologia Vegetal, para a obtenção do título de Mestre.

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Robert Yonel, Márquez Gutiérrez.
Phenotypic, bioinformatics and molecular analyses related to the development and reproductive transition of *Humulus lupulus* L. (Hop) in subtropical regions of Brazil / Robert Yonel Márquez Gutiérrez . - 2022.
1-88 p.

Orientador(a): Antonio Chalfun Junior.
Coorientador(a): Raphael Ricon de Oliveira.
Dissertação (mestrado acadêmico) - Universidade Federal de Lavras, 2022.
Bibliografia.

1. Development. 2. *Humulus lupulus*. 3. MADS-box genes. I. Chalfun Junior, Antonio. II. de Oliveira, Raphael Ricon. III. Título.

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ANALISES FENOTÍPICOS, BIOINFORMÁTICOS E MOLECULARES RELACIONADOS AO DESENVOLVIMENTO E À TRANSIÇÃO REPRODUTIVA DO LÚPULO (*HUMULUS LUPULUS* L.) EM REGIÕES SUBTROPICAIS DO BRASIL

Dissertação apresentada à Universidade Federalde Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fisiologia Vegetal, para a obtenção do título de Mestre.

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AGRADECIMENTOS

À Universidade Federal de Lavras, especialmente ao Programa de Pós-Graduação em Fisiologia Vegetal/Agronomia pela oportunidade.
A mi familia por apoyarme siempre en cada uno de mis sueños y por estar siempre a mi lado, inclusive en la distancia. Cada logro de mi vida es para ustedes.
Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela concessão da bolsa.
Ao meu orientador, o Prof Dr. Antonio Chalfun Júnior pela ajuda e confiança a mim depositada na execução deste trabalho.
Ao meu Co-orientador, Dr. Raphael Ricon de Oliveira, pela ajuda, amizade e ideias durante a execução deste trabalho.
Ao Thales Henrique Cherubino, pela ajuda e por todo o novo aprendizado.
À banca pelas sugestões neste trabalho.
Aos professores do Setor de Fisiologia Vegetal, pelos ensinamentos que auxiliaram no meu processo de formação.
A Yulimar, Santiago, Paolo e Alejandra, pela amizade e companheirismo como família.
Aos meus amigos, Mayra, Zulma, Hector, Andrea, Viviana, Karen, Janet por sempre fazer das sexta-feiras especial.
Aos colegas e amigos do laboratório de fisiologia molecular de plantas, Marlon, Andrea, Carlos, Dawyson, Lilian, Thales, Kauanne, Iasminy, Juliana, Gabriel, Gabriela, Manoel, pela ajuda nos momentos que eu precisei.
À Indira, Lizzete, Marlon, Andrea, Alexander e Daniela Perez, por sua amizade na fisiologia vegetal.

“Generating data is not the way forward, extensive interpretation with minimal testing is the key”

Sumanth Kumar Mutte

ABSTRACT

Plants have evolved to perceive endogenous and environmental signals to better adjust their phenological cycle, increasing the chance of reproductive success. These processes vary considerably between plants from different regions subject to variable climatic conditions. With the aim to establish the hop culture in subtropical regions of Brazil, it is indispensable to understand the plant development and plasticity in response to environmental changes and determine the factors that dictate the floral transition. To achieve this goal, first we performed a review (first chapter) and then two experimental works. In the first, we study the hop phenology subjected to Brazilian conditions and focusing on the floral transition. In detail, we analyzed the growth pattern and some environmental cues and related these data to the expression pattern of microRNAs 156/172 during the development. The phenotypic evaluations (number of nodes, heteroblasty, flowers) were followed by a decrease of miR156 expression levels and increase of miR172 levels. This suggested that the flowering in hop plants subjected to subtropical Brazilian conditions but irrigated is dependent on the age and can occur at any time of year. In the second work, since MADS-box is an important family of transcription factors involved in many aspects of plant development, we performed a genome-wide and transcriptional analysis. We found 65 MADS-box genes in the hop genome being them classified into 11 subfamilies within type II group (MIKCc) and in three subfamilies of type I. In the MIKCC group, we did not find members of the FLC and AGL17 subfamilies, involved in flowering repression and activation, respectively. FLC is related to vernalization thus, these results together with the fact that hop bloomed in our experiments suggest that cold periods are not necessary for hop flowering, whereas other floral activators than AGL17 are able to promote phase transition. The presence and expression profile of A-class, B-class, C/D-class, and E-class genes allowed us to propose how these influence the hop floral architecture. Moreover, it was identified a MADS-box gene expressed exclusively in the lupulin glands and involved in the secondary metabolism in the lupulin glands. Thus, our work contributes to understanding hop development and molecular aspects related to floral transition open perspectives to breeding programs and cultivation in different regions than temperate zones.

Key words: development, *Humulus lupulus*, MADS-box genes, phenology

RESUMO

As plantas evoluíram para perceber sinais endógenos e ambientais para ajustar seu ciclo fenológico, aumentando a chance de sucesso reprodutivo. Esses processos variam consideravelmente entre plantas de diferentes regiões sujeitas a condições climáticas variáveis. Com o objetivo de estabelecer a cultura do lúpulo nas regiões subtropicais do Brasil, é indispensável entender o desenvolvimento e a plasticidade das plantas em resposta às mudanças ambientais e determinar os fatores que ditam a transição floral. Para atingir este objetivo, primeiro realizamos uma revisão (primeiro capítulo) e depois dois trabalhos experimentais. Na primeira, estudamos a fenologia de três cultivares de lúpulo submetido às condições brasileiras e com foco na transição floral. Analisamos o padrão de crescimento e algumas pistas ambientais e relacionamos esses dados com o padrão de expressão dos microRNAs 156/172 durante o desenvolvimento. As avaliações fenotípicas (número de nós, heteroblastia, flores) foram acompanhadas da diminuição dos níveis de expressão de miR156 e aumento dos níveis de miR172. Isso sugere que a floração em plantas de lúpulo submetidas às condições subtropicais brasileiras, é dependente da idade e pode ocorrer em qualquer época do ano. No segundo trabalho, uma vez que MADS-box é uma importante família de fatores de transcrição envolvidos em muitos aspectos do desenvolvimento da planta, realizamos uma identificação geral dessa família no genoma do lúpulo. Encontramos 65 genes MADS-box, sendo eles classificados em 11 subfamílias dentro do grupo tipo II (MIKCc) e em três subfamílias do tipo I. No grupo MIKCC, não encontramos membros das subfamílias FLC e AGL17 envolvidos na repressão e ativação do florescimento, respectivamente. FLC está relacionada à vernalização, portanto, esses resultados juntamente com o fato de o lúpulo florescer em nossos experimentos sugerem que períodos frios não são necessários para a floração do lúpulo, enquanto outros ativadores florais além do AGL17 são capazes de promover a transição de fase. A presença e o perfil de expressão dos genes da classe A classe B, classe C/D e classe E nos permitiu propor como estes influenciam a arquitetura floral do lúpulo. Além disso, foi identificado um gene MADS-box expresso exclusivamente nas glândulas de lupulina, o qual poderia estar envolvido no metabolismo secundário nas glândulas de lupulina. Assim, nosso trabalho contribui para a compreensão do desenvolvimento do lúpulo e aspectos moleculares relacionados à transição floral abrem perspectivas para programas de melhoramento e cultivo em regiões diferentes das zonas temperadas.

Key words: desenvolvimento, fenologia, genes MADS-box, *Humulus lupulus*.

SUMÁRIO

CHAPTER I

General introduction

1. GENERAL INTRODUCTION

Hops (*Humulus lupulus* L.) is a herbaceous, dioecious and perennial liana belonging to the Cannabaceae family (Shephard et al., 2000). Its origin is China (Boutain, 2014), but currently its cultivation extends to different regions, mainly located at latitudes between 35 and 70 degrees in the northern hemisphere and between 35 and 43 degrees in the southern hemisphere. The main product of this plant is the female inflorescences (cones or strobila) that, inside, develop lupulin glands, which is rich in a resin with aromatic compounds and essential oils used in the manufacture of various products, including beer (Verzele and Keukeleire, 1991).

Hops have a longevity of approximately 14 years, being the rhizome perennial and the aerial part (represented by stem, leaves and flowers) annual (Fric et al., 1991). Ontogenesis is divided into seven phases, with morphological differences in underground structures and production. However, the ontogenic development is little known in regions with tropical and subtropical climates, and its life cycle may be altered due to environmental characteristics (Fric et al., 1991).

Hop phenology in temperate regions is well known (Roßbauer et al., 1995). Plants begin to grow after 6 months of inactivity (winter) at the time corresponding to early to mid-spring. Vegetative growth continues in late spring and throughout the summer. When the days begin to shorten in late summer and early autumn, vegetative growth ceases and the first reproductive structures begin to develop, which is followed by very rapid growth of the cones for at least a month. In early autumn, the plants translocate all nutrients to the roots and the aerial part senesce.

Regarding vegetative growth, Thomas and Schwabe, (1969) determined that hop plants have an optimal growth rate in photoperiods above 13 light hours. And when plants reach a minimum size (maturity to flower), which is seen as the number of nodes, they become competent to perceive the short-day signals and then begin the flowering development.

Hops are grown as an annual plant in countries in temperate regions, with the United States being the largest producer followed by Germany (Barth-Haas Group, 2019). It is curious that Brazil does not produce hops considering the country's long tradition in diverse agrarian cultures. Therefore, it is important to know the pattern of development of this culture in order to establish production in Brazil.

In this sense, it is important to understand the development of hop in function of internal factors, in order to have a broader vision of its development. Therefore, in the first article, it

was explored the development of three hop cultivars in Lavras-Brazil accompanied by the expression analysis of microRNAs (156 and 172) related to plant aging and reproductive transition (Teotia and Tang, 2015). In the second article, we identified through a wide-genome analysis the family of transcription factors named MADS-box and its expression profile in different tissues, The MADS-box genes are present in many organisms and important for plant development, specially floral transition and organogénesis, thus, this work is useful to better understand hop development and propose crop breeding strategies.

CHAPTER II:

Bibliographic review

1. BIBLIOGRAPHIC REVIEW

1.1. Taxonomy and phylogeny of *Humulus lupulus* L

H. lupulus is a species belonging to the Cannabaceae family, which also contains the Cannabis genus, widely known for grouping species of medicinal and psychoactive use (Verzele and Keukeleire, 1991). The genus *Humulus* has three species: *H. lupulus, H. japonicus and H. yannanensis*. The species *H. lupulus* contains three taxonomic varieties separated into two clades. The clade *H. lupulus* var. hops correspond to European wild hop and all cultivated varieties. The Asian-North American clade is divided by the clade var. cordifolius

corresponding to wild Japanese hops; and clades var. neomexicanus and var. pubescens correspond to North American wild hops. Haplotype studies corroborated the grouping of these taxonomic varieties (Murakami et al., 2006)

According to analyzes of the plastid genome, it is estimated that the divergence of the *Humulus* genus with the *Cannabis* genus occurred approximately 87.28 million years ago (mid-Cretaceous) in what is now China (Boutain, 2014). Whereas the divergence of the genus *Humulus* occurred 44.43 million years ago, giving rise to the ancestor of all taxonomic varieties of the clade var. *lupulus* and the ancestor of the species *H. yunnanensis* and *H. japonicus* (Boutain, 2014). The divergence of the clade var. hop with the other three taxonomic varieties occurred approximately 26.1 million years ago (Boutain, 2014).

1.2. Botanical aspects of hop

Hops is a dioecious plant that grows by winding itself clockwise on any available support with the help of hook hairs, located at angles in the stem (Neve, 1991). In this plant, four organs can be distinguished, root and rhizome (underground) and vegetative and reproductive organs (aerial) (Fric et al., 1991). Hops are diploid with chromosomal load 2n=2x=18 + XY (Shephard et al., 2000)**.**

The rhizome includes all the organs produced by stem modifications that are below the ground. The underground stem system is composed of the rhizome and the stolons (Neve, 1991). Underground stems can remain dormant for periods of approximately 4 years, maintaining the perennial life of the plants (Fric et al., 1991). Rhizomes undergo changes in

their structures in response to the environment and have much shorter and thinner internodes than aerial stems (Fric et al., 1991). The main function of the rhizomes is to maintain the meristematic tissues during the unfavorable periods of winter.

Aerial vegetative organs include the axillary buds, stem and leaves. The shoot apical meristem (SAM) arises from the rhizome and is surrounded by several bracts which turn green once they emerge from the soil (Fric et al., 1991). The leaf buds emerge from the axils of the bracts and are differentiated along with the other aerial tissues at the apex. Each leaf is accompanied by three dormant buds and any suppression of apical dominance activates the central bud (Fric et al., 1991).

The hop stalks include the main stem (bine) and the secondary branches. In spring, the main stem originates as the first aerial organ and, during the growing season (summer), it can reach 8 or 9 meters (Neve, 1991). The lower (older) internodes stop growing in length and begin to grow in thickness, followed by changes involved in aging (Neve, 1991).

The leaves appear in pairs at each node, are petiolate, have a slightly serrated heart-shaped margin and, when mature, are usually trilobed (Neve, 1991; Verzele and Keukeleire, 1991). The leaves are green or yellow-brown and are hairy on both sides, and can reach 20 cm in length (Verzele and Keukeleire, 1991). Young leaves have more bent leaf blades, but well-grown leaves are gently wavy. The venation of the leaves is palmate and the main veins project from the lower surface of the leaves (Neve, 1991). On the abaxial surface of the leaves, glands that contain resins and essential oils develop, these glands are called lenticular glands (Fric et al., 1991).

Male plants produce inflorescences that are heavily branched panicles with small flowers on short stalks. At the time of flowering, they reach 5-6 mm in diameter. Each flower has 5 sepals with adherent stamens and anthers that contain yellow pollen. The setals are equipped with lupulin glands, but their content is small compared to female inflorescences (Fric et al., 1991).

Female plants produce inflorescences that contain 20 to 60 flowers on an axis called a crank. Each crank contains two pairs of flowers that are protected by a bracteole. In turn, the flowers have a small green perianth, which is close to the ovary of an ovule (Fric et al., 1991). The ovaries contain two filamentous stigmas that are free, fixed and close to the micropyle, and on their surfaces there are long papillae that capture pollen from the air. The stigmas are whitish and when they atrophy, they quickly turn brown and lose the ability to adhere the pollen (Fric et al., 1991). With this loss, its function ends, as well as the flower's, and at this point the entire

inflorescence changes to the syncarpia state, in which the carpels are fused (Fric et al., 1991). The cones (strobiles) of female hop plants are, morphologically, an inflorescence in which they originate from a catkin. From a developmental point of view, the strobiles are modified and shortened branches, which consist of covering bracts and involucral bracts (Fric et al., 1991).

Humulus lupulus develops two types of glands, peltate and bulbous, which are distributed on the abaxial surface of leaves, bracts and bracteoles. Meanwhile, non-glandular trichomes are in the veins (Oliveira et al., 1988). Pelted trichomes consist of four basal cells, four stem cells, and a long, single-layered, multi-celled flat head. The secretion accumulates in the subcutaneous space, leading to a more or less spherical trichome (Oliveira et al., 1988). These trichomes can have dimensions of about 100 x 120 µm, when secretions accumulate, and 135 x 150 µm, after the secretion is released by artificial pressure. Bracteole trichomes can measure 170 x 205 µm with secretion (Oliveira et al., 1988).

1.3. Phenology

The beginning of an annual cycle can be seen as the end of the transport of nutrients from the aerial part of the plant to the roots. Furthermore, with the removal of the entire shoot by decapitation at the time of harvest, the migration of the material is abruptly interrupted, causing a new cycle, precisely after harvesting (Roßbauer et al., 1995). During the annual cycle, the shoot of a hop plant continuously differentiates until the shoot senesces at the end of the cycle. Accordingly, two main periods can be defined in temperate regions:

A) Dormancy period: lasts approximately 6 months, from the second half of October to the beginning of April. This period can be subdivided into four phases (Roßbauer et al., 1995). **Pre-dormancy**: starts from the drop of the shoot. At this point, all the rhizome buds, especially the most active ones on the new stem, stop growing and go into dormancy. The epidermis of the upper buds thickens to protect the vegetative apex during winter. At the same time, the surface of the roots is covered with a brown layer. **Dormancy**: During hibernation, rhizome buds cannot grow under any conditions. No change takes place in the underground parts. Specifically, there is very little metabolic activity. This period ends in December. **Post-dormancy**: This period is characterized by the acquisition of endogenous competence of the buds to start the growth, but they are limited by external conditions, usually low temperatures. Although growth cannot be visualized, the transformation of storage substances (eg, polysaccharides to monosaccharides)

takes place in the rhizome, which is mobilized from the tuberous roots to the rhizome. Gradually, the absorption of water and nutrients by the roots is restored.

Period of germination and underground growth: begins with the activation of rhizome buds and ends with the appearance of buds above ground. During this period, the growth of the underground part is provided by the reserve accumulated in previous years. The fastest growth corresponds to the buds that are on the new stem and stolon. These tissues are close to the surface of the soil and are therefore exposed to sudden changes in temperature and humidity. The growth of the underground part differs according to the temperature, properties and thickness of the soil layer above the rhizome. Low temperatures and a thick layer of soil on top of the rhizome often delay the period of underground growth and therefore can delay the entire winter standstill period of the hop plant.

B) Vegetative growth period: begins in spring, with the buds of the bines, and lasts until the fall of the entire shoot in autumn. The entire period can be subdivided into eight phases (Fric et al., 1991).

Linear growth period of bines: begins with the emergence of shoots from the ground and ends when the growth of three internodes above the ground is completed. At first, the bines grow strongly upwards. Bines are characterized by being weak and with high water content. Each internode measures approximately 20 cm in length, so during this period, the bines measure approximately 60 cm. The activity of the root system increases and the roots begin to receive nutrients. Likewise, a gradual increase in photosynthesis begins, however, the tubers' reserves still maintain the plant's growth.

Establishment period: This period begins when the three internodes are formed and ends when the secondary branches begin to form. After the formation of the three internodes, the bines grow so that the apex moves clockwise in space, circumscribing circles of 10 to 15 cm in diameter until it finds a support. A spiral turn is achieved in approximately 1 h. Once the bines find a support, the winding speed slows down and its diameter becomes the thickness of the support. Under favorable temperature conditions, bines can grow very fast and this can be difficult to manage in hop gardens. During this period, the root system grows intensely.

Branching period: begins when secondary branches appear and ends when inflorescences begin to become visible. There is an intense growth of bines and branches. Under favorable temperature conditions (24 °C), they can grow up to 36 cm per day. At the top of the bines, at a height of more than 3 m, the branches appear simultaneously with the leaves and

grow concomitantly with them. Leaf surface and photosynthetic activity increase rapidly. However, the reserves accumulated in subterranean organs during the previous year are involved, as well as in the shoots, in the initial stages of rapid growth of the bines.

The “butonization” period begins when the catkins on the inflorescences become visible. The catkin grows concomitantly with the branch that has it. The lower part of the bines produces a small number of fertile branches that do not branch much and produce a low number of individual catkins in the apical part. Fertile branches, with groups of catkins in the axils of their leaves, are produced on top of the bines at each node. Photosynthetic and root activity increases considerably during this period and underground reserves begin to decrease.

Inflorescence period: This period begins when the stigma clusters on the inflorescences become visible and ends when the stigmas wither and turn brown. Branches in the central part flower first, but flowering on these branches begins at different times. That is, from the axils of the second and third pair of leaves they bloom first, the others bloom later. The growth rate in length decreases considerably during this period, but the growth of branches is very intense, along with the growth of young leaves.

Cone formation period: begins with the wilting of the stigmas and ends with the maturity of the crop. In the beginning, the cones grow longitudinally, but in the end they begin to become more closed, colorful and heavy. Towards maturity, the water content of the cones decreases and this is important for the brewery because bitter substances and essential oils reach their optimal quality and quantity. These substances reach their ideal state at the time of technical maturity (23% dry matter) (Dodds, 2017). During this period, the growth of branches and bines ceases and the growth rate of younger leaves is very low. However, the cones continue to grow vigorously and, at the same time, the deposition of reserve substances in underground organs increases, reaching approximately half of the final amount deposited. In the root system, the weight of tuberous roots increases, but root activity and number of roots decrease.

Physiological maturity period: in this period the cones mature, forming fruits, if pollination occurs earlier. This period begins with the technical maturity of the cones and ends with the full maturity of the seeds. The cones turn brown, the bracts turn outward, detaching easily, and the quality and quantity of the main substances decrease. Aerial growth ceases completely, starting with leaves and branches.

Period of decomposition of bines: during this period, all the aerial parts perish. It begins with the physiological maturity of the cones and ends with the complete shedding of the bines.

The lower leaves of the bines interrupt their functions and finally fall off along with the dormant buds in the armpits. The transfer from the reserve to the underground part also ends.

1.4. Hop flowering

Flowering time in plants is controlled by complex pathways that interact with each other and that integrate environmental signals (light, temperature, photoperiod) with endogenous factors (hormones and age) to ensure that flowering occurs at the right time (Gazzarrini and Tsai, 2014)**.** The onset of flowering in hop is cultivar dependent. Consequently, flowering can be classified into three categories of cultivars: those that flower early, in a cycle of approximately 1.5 months from the beginning of vegetative growth; those that bloom in the middle of the cycle (approximately 2 months from the beginning of vegetative growth) and the late variables that flower at approximately 3 months after the beginning of vegetative growth (Thomas and Schwabe, 1969)**.**

In several reports, it has been established that hops, being a plant native to temperate regions, require vernalization to flower (Thomas and Schwabe, 1969). However, Bauerle, (2019) found that the production yields and the concentration of α and β acids did not vary in vernalized and non-vernalized plants. Interestingly, the same author found that plants rooted from non-vernalized cuttings had the same yields and production quality as plants with vernalized or non-vernalized rhizomes, all growing in a greenhouse. This indicates that the age of the rhizome does not play a critical role in production, as plants approximately 4 months old and propagated from cuttings had production yields comparable to those of older plants. In addition, Bauerle (2019) found that under controlled conditions of temperature and photoperiod (18 h of light during vegetative growth), plants of all varieties studied showed the same growth pattern (length and number of nodes) as plants cultivated in the field, in addition to having been possible to obtain four cycles of this culture per year. These findings would undoubtedly help to study the flowering of this plant under controlled conditions.

Previously, Thomas and Schwabe (1969) found that hop plants are short-day plants, because they flower once the photoperiod begins to decrease from the critical light hours (16.5), and that vegetative growth ceases entirely if plants grow under very short photoperiod conditions (8 hours light). These researchers also found that interrupting darkness with flashes of light for half an hour on short days prevented flowering. In addition to these findings, they found that low temperatures and minimum hop plant size are key parameters for flowering.

Consequently, these researchers found that plants with fewer nodes than needed to flower (maturity to flower) did not flower under short-day conditions. Based on this, the plant's maturity for flowering, expressed in number of nodes, changes according to the variety, with early varieties requiring the fewest number of nodes to flower.

1.5. MADS-box family genes

The MADS-box family genes have been found in animals, plants and fungi and participate in several regulatory processes of development. These genes encode transcription factors that bind to the CArG domain of target genes (Ng and Yanofsky, 2001). In plants, MADS-box genes have many functions, such as determining floral organs, floral transition, determining flowering time, embryonic development and seed pigmentation. These genes are classified into two groups:

Ttype I and Type II. Type I are divided into Mα, Mβ and Mr, while type II are divided into $MIKC^C$ and MIKC* (Masiero et al., 2011). Those from the $MIKC^C$ are classified, in turn, into 12 subfamilies (Pařenicová et al., 2003). MADS-box genes have a conserved MADS(M) domain with sequences of about 60 amino acids in the N-terminal regions. In addition to this domain, types II contain the keratin-like domain (K), the intervention domain (I), and a C-terminal region. In turn, type I MADS-boxes have a relatively simple structure, usually containing 1 to 2 exons (Smaczniak et al., 2012).

1.6. Economic aspects of hop

From 2017 to 2018, the world demand for α-acid went from 11,399 t to 11,677 t and production remained below (Barth-Haas Group, 2019). For the year 2018, the main producer of hops worldwide was the United States, with a total of cultivated land of 22,272 ha. Germany ranked second in cultivated area, having 20,144 hectares. In this order, it is followed by the Czech Republic, with 5,020 ha, and China, with 2,608 ha. World production followed the same trend, with the United States producing 49,173.7 t and 5,229 t of α-acid in 2018; and Germany with 41,794.3 t and 3,828 t of α-acid. For South America, Argentina was the largest producer, cultivating 160 ha and producing 269.6 t and 25 t of α-acid, for the year 2018 (Barth-Haas Group, 2019). By 2015, there were 51,512 hectares cultivated with hops worldwide. This parameter increased by 9.0% in 2016, when it was reported that the total area cultivated with

hops was 56,141. In 2015 there was a decrease in hop production of 9.6%, representing a total yield of 87,415 t (tons). For the year 2016 there was a recovery in hop production, compared to 2015, of 28.0% (Barth-Haas Group, 2017). From 2012 to 2016, the world demand for α-acid increased linearly, going from 9,369 t to 10,993 t. While the production remained below the demand, showing an oscillating behavior (Barth-Haas Group, 2017) and necessity to improve production.

The world beer market is substantially dependent on hop production. The global beer market is worth around $500 billion annually, and almost 5% of this is due to the craft brewing sector. There is a global demand for new beers with novel flavors, and craft brewers are focused on identifying new hop varieties that have these desired flavors and aromatic characteristics. The most important countries in the production of beer are China, the United States, Brazil, Mexico, and Germany. These countries accumulate 52% of world beer production (Barth-Haas Group, 2019). Compared to the volume produced in 2015 (1,960,991,000 hl), world beer production in 2016 fell by 3.5 million hectoliters (Barth-Haas Group, 2017). European production remained stable between 2015-2016, with a slight increase in some countries, North America presented an increase in beer production of 7,900 hl, while South America, Brazil and Venezuela decreased by 5,200 and 10,200 hl, respectively (Barth-Haas Group, 2017). World beer production for the year 2017 was 1,942,402,000 hl and for the year 2018 it was 1,904,602,000 hl, representing a drop of 0.8% between these two years. This was mainly due to the drop in production in China, which was 59,000 hl. In 2018, it was reported that 85 countries, of the 171 beer-producing countries, increased their production and that only 34 reported a drop (Barth-Haas Group, 2019). Thus, all these numbers reflect the economic importance of the hop market that could be explored by other countries generating income and employment.

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CHAPTER II

Article 1: Less environment, more endogenous control: the flowering of *Humulus lupulus* L. (hop) in Brazilian subtropical regions

Less environment, more endogenous control: the flowering of *Humulus lupulus* L. (hop) in Brazilian subtropical regions

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ABSTRACT

Hop cultivation has been restricted to temperate regions and countries of the north hemisphere. This occurred because it was believed that the vernalization was necessary to hop floral development. However, recent works has demonstrated that this is not true since hop plants present a complete phenological cycle in tropical and subtropical regions, which opens perspective to cultivation once the developmental differences are comprehended and adjusted for production. Here, we explored the phenological and molecular aspects of the hop development at subtropical Brazilian conditions to understand hop plasticity and to enable its cultivation. In this work, we observed that the development of hop at Minas Gerais (Brazil) does not correspond with the reported for temperate regions since hop plants bloom at different times of the year and in different seasons. This could be due to photoperiod, since in these regions is always inductive, because the hop is a short-day plant with 16 h critical of light. Therefore, we hypothesized that the control of flowering in these regions could be due to endogenous factors as the age of plants, once they bloomed when a determined number of nodes was formed. Associated with morphological characteristics, we quantified the expression of microRNAs 156/172 which are related to aging and floral transition. Hop plants with different numbers of nodes showed that the level of miR156 decreased in older plants, whereas the level of two miR172 was greater in plants with 25 nodes. This suggests that microRNAs 156 and 172 participate in the phase transition during hop development, supporting that hop flowering is controlled by aging or number of nodes, according to observations in other plants.

Key words: flowering, *Humulus lupulus*, hop development, microRNAs

1. Introduction

Floral transition of the vegetative meristems is the starting point of plant reproductive development, an important stage in which plants have to cope with environmental changes to guarantee reproduction (Wang et al., 2020). Hops (*Humulus lupulus* L.) is a herbaceous, dioecious and perennial liana belonging to the Cannabaceae family (Shephard et al., 2000). The main product of this plant is the female inflorescences (cones or strobila) that develop inside the lupulin glands, a resin rich in aromatic compounds and essential oils used in the manufacture of various products, including beer (Verzele and Keukeleire, 1991). The α and β acids present in the resins provide bitterness to the beer.

In subtropical regions, the the blooming seems to depend more on plant height than other factors (Sun and Frelich, 2011), therefore, it is possible that in hop, this phenomenon occurs. However, despite its economic importance, it has been cultivated in temperate zones and few works have explored hop development in tropical and subtropical regions. Currently, hop cultivation extends mainly at latitudes between 35 and 70 degrees in the northern hemisphere and between 35 and 43 degrees in the southern hemisphere (Dodds, 2017; Fric et al., 1991). The rhizome is perennial, with a longevity of approximately 14 years, and the aerial part (represented by stem, leaves and flowers) is annual (Fric et al., 1991). Hop phenology in temperate regions is well known. Plants begin to grow after 6 months of inactivity (winter) at the time corresponding to early to mid-spring. Vegetative growth continues in late spring and throughout the summer. And when the days begin to shorten in late summer and early autumn, vegetative growth ceases and the first reproductive structures begin to develop, which is followed by very rapid growth of the cones for at least a month. In early autumn, the plants translocate all nutrients to the roots and the aerial part dies (Roßbauer et al., 1995). In relation to the development physiology, the hop is a plant that grows optimally in photoperiod above 13 h light, it is a short-day plant (16.5 h critical), and the flowering is achieved once the plants reach to a minimal size (Thomas and Schwabe, 1969). Moreover, recently it was demonstrated tha hop plant does not require vernalization to floral transition and rhizome age does not influence hop yields (Bauerle, 2019).

In agreement, the phenology of four hop cultivars was described in Santa Catarina/Brazil (Mendes, 2020). This work shows that vegetative growth occurred between the months of September and December, the reproductive phase began to the end of December, and the cones development occurred between January and February. Interestingly, some of the phenology stages proposed for hop are not found in subtropical regions, for example, plants are

able to bloom before the established time with a variable vegetative growth, which causes unevenness in the cones and therefore impairs the harvesting (Acosta et al., 2021).

During plant development the dynamic of expression of microRNAs is related to floral transition (Schwab, 2012). The microRNAs are a type of small non-coding RNA (20-22 nt) that regulates the expression at post-transcriptional levels through RNA–RNA interactions with the mRNAs of their target genes (Teotia and Tang, 2015). During the juvenile phase the high level of miR156 in leaves of *Arabidopsis* regulates the expression of *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* (*SPL*), a family of transcription factors involved in the transition from vegetative to reproductive meristem (Wang, 2014; Wu et al., 2009). The phase transition is visible in leaves since its morphology changes concomitant with a diminution of miR156 level (Silva et al., 2019; Wu et al., 2009). During early development of plants, the identity of leaves is controlled by the shoot apical meristem (SAM), through levels of SPL transcription factors (Fouracre and Poethig, 2019). Recently it has been demonstrated that diminution of expression of miR156 is due to cellular division in the SAM, through epigenetic repression in the miR156 locus (Cheng et al., 2021). Therefore, the expression of miR156 in leaves reflects the expression of miR156 in the SAM, since all aerial organs originate in the SAM. Another mirRNA involved in the phase transition is miR172, which is expressed in the late developing stages and related to floral transition (Schwab, 2012; Teotia and Tang, 2015). This microRNA regulates the expression of *APETALA2* transcription factors, a family of transcription factors involved in flowering represion (Aukerman and Sakai, 2003). Although the interplay between microRNAs expression and phase transition were described in model plants, it is poorly explored in crop plants.

In this work, we demonstrated that hop phenology does not depend on seasons as described in temperate regions (Fric et al., 1991), but instead it seems that it depends on the plant aging and developmental stage (number of nodes formed). Also, we observed that the sprouting phase occurs at any time of year, and the new bines development depends on the presence of preexisting bines. We demonstrate that miR156 levels are greater in juvenil plants and lower in adult plants. Also, the levels of two miR172 are greater in plants with 25 nodes, of which were observed flowering.

2. Materials and methods

2.1. Plant material and field conditions

To evaluate the phenological phases and the expression of miR156 and miR172, female plants of three hop cultivars were used: Chinook, Millenium, and Northern Brewer, classified as medium-late, late and early flowering, respectively. The experiment was conducted in one years-old plants (during the years 2019-2021) in a locality of Lavras/MG-Brazil (-21.246732, -45.002251; 918 m.s.n.m). Lavras is a region considered to have a subtropical climate marked by a rainy temperate (mesothermal) with dry winters and rainy summers, and temperature of the hottest month above 22 °C in February (Dantas et al., 2007). The soil was classified as clayey (36 % of sand, 18 % of silt and 46 %of clay), with a content of organic matter of 1.23 %, a cation exchange capacity of 7.20 cmolc/dm^3, and pH of 6.4, according to the soil test performed by the soil laboratory, soil department, Federal University of Lavras. Each cultivar was planted in rows separated by 1.5 m and each plant distant 1 m apart. Three stems per plant grew over natural fiber strings of 0.5 cm of diameter. The crop management was done according to Dodds, (2017). To analyze the expression of mir156 and mir172, pairs of leaves of the 5th, 10th, 15th, and 20th nodes were collected from plants with size of 10, 15, 20 and 25 nodes, respectively. The leaves were immersed immediately in liquid nitrogen and stored to -80°C.

2.2. Phenological parameters

The growth cycle of plants started in November and finished in March. In this period, phenotypic analysis was carried out by observing the plant size (number nodes) and appearance of inflorescences. The nodes were quantified from the base of the plant to the last visible node prior to the apical meristem. The phenological stage of plants was classified according to the BBCH scale for hop (Roßbauer et al., 1995). The phenological phases were accompanied with climatic data from INAMET (http://www.inamet.gov.ao/ao/).

2.3. Identification of microRNA in the hop genome

The *Humulus lupulus* assembly genome and genome annotation files were obtained from hopbase (http://hopbase.cgrb.oregonstate.edu/; Hill et al., 2017). The identification of microRNAs was done according to de Souza Gomes et al., (2011). Using the NCBI BLASTn and Einverted EMBOSS tools (Altschul et al., 1997; Li et al., 2015) it was found the potential hairpin sequences (similar to miRNA precursor) with the following parameters: 336-nucleotides maximum repeat and threshold value of 25. The filters as GC (guanine and cytosine)

content between 20 and 60 %, minimum free energy (MFE), homology with conserved mature miRNAs, homology with repetitive regions and non-coding RNAs (except miRNAs), were applied.

The miRBase database (version 22.1 https://www.mirbase.org/; Kozomara et al., 2019) was used for comparisons through an alignment with the putative sequences, accepting no more than 3 mismatches in whole mature miRNAs, 0 mismatch in seed region (2-8 nt) and 1 nucleotide overlapping in the hairpin loop. Subsequently, it was removed the sequences similar to the known non-coding RNA sequences, such as rRNAs, snRNAs, slRNAs, SRP, tRNA, and RNAaseP, using RFAM database version12.0 (Gardner et al., 2009). Finally and using repeatmasker 2.0.2 (http://www.repeatmasker.org/) the repetitive sequences were removed.

2.4. *In silico* analysis of targets of mir156 and mir172

In order to know which members of microRNAs 156 and 172 found in the hop genome are targeted to *SQUAMOSA PROMOTER BINDING LIKE PROTEIN* and *APETALA2* genes, respectively, we used the psRNATarget tool (https://www.zhaolab.org/psRNATarget/ Dai et al., 2018; Dai and Zhao, 2011). The mature sequences were used as queries against candidate targets which were retrieved from Hopbase (http://hopbase.cgrb.oregonstate.edu/; Hill et al., 2017). It was considered those microRNA that presented a "Maximum expectation (Exp)" (number of mismatches) between 0 and 0.5 to target genes previously mentioned (verifying the hits in the hopbase). While the others parameters were standard. Candidate targets were retrieved from Hopbase (http://hopbase.cgrb.oregonstate.edu/; Hill et al., 2017).

2.5. RNA extraction, cDNA syntheses and quantitative PCR (qPCR)

Total RNA from leaves were extracted according to de Oliveira et al., (2015). To eliminate DNA contamination, RNA samples (5 µg) were treated with DNase I using the Turbo DNA-free Kit (Ambion). RNA integrity was analyzed in 1 % agarose gel, and RNA content, as well as purity, were accessed by spectroscopy ($2.2 < OD260/280$ and $OD260/230 > 1.8$) (NanoVue GE Healthcare, Munich, Germany).

The cDNA synthesis followed the protocol proposed by Varkonyi-Gasic et al., (2007), using the *ImProm-II*^{TM} *Reverse Transcriptase* (Promega), in which 1000 ng of treated RNA was adjusted in a volume of 8.5 µl with RNAse-free water. To the treated RNA was added

1.25 µl of oligo-dT primer, 2.5 µl of each specific stem-loop RT primer (10 µM) (Table S1) and 1.25 µl of the dNTP (10 µM). The samples were incubated at 70 °C for 10 min for denaturation of the secondary structures and later incubated at 4 °C for 10 min. Then, it was added 5 µl of Improm-II 5 × reaction buffer, 2.4 µl of $MgCl_2$ 25 mM, 0.6 µl of RNaseOut (Invitrogen), and 1 µl of the Improm-II Reverse. The reactions were incubated in a thermocycler at 16 °C for 30 min, followed by reverse transcription of 60 cycles at 30 °C for 30 s, 42 °C for 30 s, and 50 °C for 1 s. For inactivation of the Improm-II Reverse Transcriptase, the reaction was incubated at 70 °C for 15 min. Subsequently, the reactions were stored at −20 °C.

Real-time quantitative PCR was performed with the Rotor-Gene SYBR® Green PCR Kit (Qiagen), on a Rotor Gene-Q(R) thermocycler (Venlo, Netherlands) using three biological repetitions runned in technical triplicates. Reactions were carried out in 15 µL reaction volume: 7.5 µL of SYBR-green (QuantiFast SYBR Green PCR Kit - Qiagen), 1.5 µL of each forward and reverse gene-specific primers (10 µM; primers available in Table S1), 1.5 µL of cDNA, and 3 µL of RNase-DNase-free water. The amplification was performed with the following conditions: enzyme activation with 5 min at 95 ºC, then 40 cycles of 95 ºC for 5 s, followed by 10 s at 60 ºC, and completed by a melting curve analysis to assess the specificity of the reaction by raising the temperature from 60 to 95 ºC, with 1 ºC increase in temperature every 5 s. Relative gene expression was calculated based on the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001) and normalized against *S-ADENOSYLMETHIONINE SYNTHETASE* (*HlSAMS*; g17085) and *TRANSLATION ELONGATION FACTOR ALPHA* (*HlTEFA*; g7597). These reference genes were selected based on their expression stabilities across public RNAseq libraries.

2.6. Statistical analysis

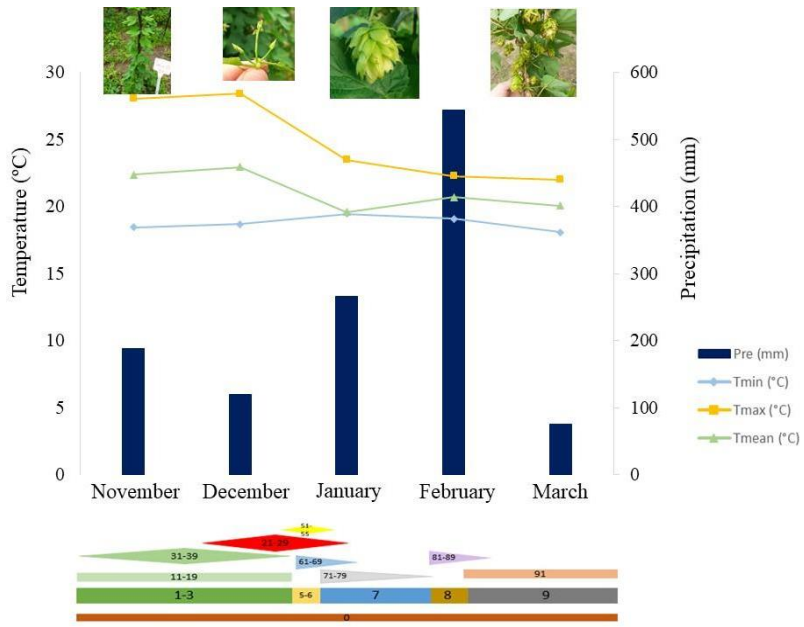
For the gene expression, statistical analyses were performed with the R software. The expression rate and the confidence intervals were calculated according to the method proposed by Steibel et al., (2009), which considers the linear mixed model given by the following equation: yijklm = µ + TGijk + Il + eijklm where yijklm is the Cq (Quantification cycle) obtained from the thermocycler software for the kth gene (reference or target) from the mth well, corresponding to the lth plant subject to the *i*th treatment (nodes number) at the *jth* tissues (Leaf); TGijk is the effect of the combination of the *ith* treatment (n10, n15, n20, and n25) at the *jth* tissues (Leaf). Graphics were performed with R.

3. Results

3.1. Phenological and climatological analysis during the hop growth

The climatological data and main phenological stages of three cultivars of hop were evaluated between November and March from 2019 to 2020 (Figure 1). The mean temperature varied between 20 and 22 ºC and the rainiest month was February according to Dantas et al. (2007). In Brazilian subtropical conditions (Lavras/MG), the vegetative development occurred between November 2019 and December 2020, occurring at the end of December the inflorescence emergence. This behavior pattern was similar between cultivars with few differences, for example, cv. floral development started for Chinook and Millenium cultivars within 10 days late. Also, the sprouting stage was observed during all year, and some stages overlap with principal stages (Table S2) suggesting that at this region the phenological scale must be modified.

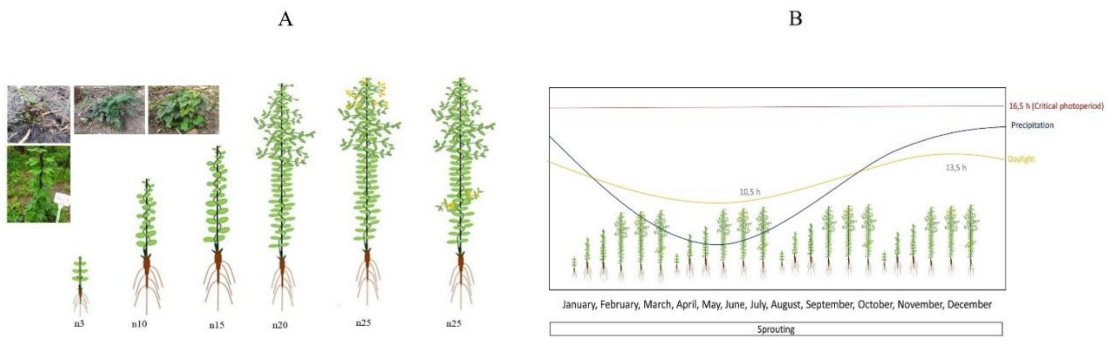
Figure 1 - Phenological and climatological analysis of hop growth during November and March (2019-2020). The phenological stages are presented in colored figures at the bottom of the figure. Brown: sprouting (0); dark green: leaf development, formation of side shoots and elongation of bines (1-3); yellow: Inflorescence emergence and flowering (5-6); blue: developments of cones; golden yellow: maturity of cones (8); gray: bines maturity (9). Inside: photos of the principal characteristics that plants presented during the development.



Source: From the author (2022)

Since the time for each stage of hop growth and development is different for the considered Brazilian condition (Figure 1) in relation to temperate zones (Fric et al., 1991), we evaluate in detail the nodes formation until the reproductive development (Figure 2A). The aerial part started to grow from buds which lay in the rhizome (beginning of shoot-growth). From this stage to the 3 nodes stage, the growth of plants is linear as a seedling, until they find a physical support to develop as lianas. From there, the plants grew rapidly (n3-n25) in 4 weeks on the support in a clockwise direction. In the 15-20 nodes stages some plants started to develop secondary branches, which was from the mid to the tip. The Northern Brewer cultivar began to flower when they reached 25 nodes, while the Chinook and Millenium cultivars started to flower once it reached 28 and 30 nodes, respectively. This beginning of flowering was observed independent of chronologic time, but dependent on size of plants with a minimum number of nodes. The first inflorescences emerged at the tip, either in principal or secondary branch, and continued emerging to the mid of the bines. The inflorescence stage lasted 1-2 week, which finished when the stigmas began to degrade. From there, the cone stage started lasting at least a month (Figure 1). Notwithstanding, the emergence of new inflorescences was concomitantly with this phase, therefore, there was no a separate stage of inflorescence and cones development. After one month, the cones began to degrade and fall, while the plants remained. The aerial parts died when they were removed manually, this allowed news buds to develop new aerial parts and start the cycle again at any time of year, and therfore to flower independet of time of year (Figure 2B). The formation of new bines, from previously formed buds (Figure 2), lasted approximately one week.

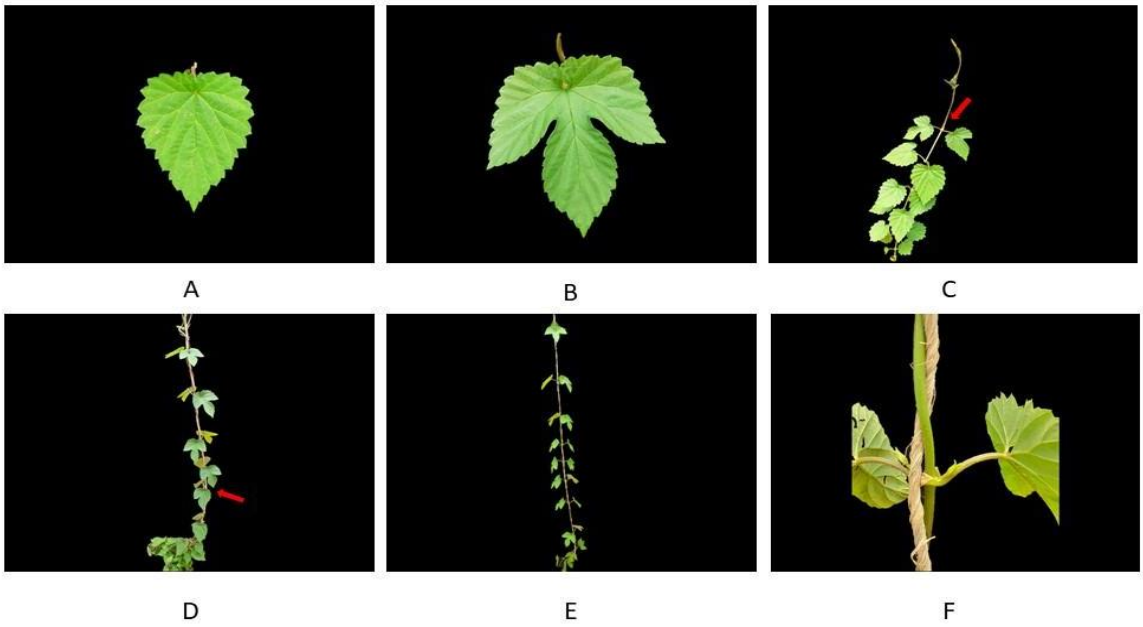
Figure 2- Forms that hop plants adopted during vegetative and reproductive development. Inside: photos of buds in the rhizome, and the first stages of vegetative development (A). Vegetative and reproductive development of hop plants in Brazilian subtropical regions (B).



Source: From the author (2022)

The hop is a plant that presents heteroblasty (Fric et al., 1991), which could be observed in the leaves of the cv. Northern Brewer during vegetative development (Figure 3). It is possible to see an entire leaf (Figure 3A) becomes trilobed (Figure 3B), which occurs usually, from the 7th or 8th nodes (Figure 3C and D) and marks the transition to the mature stage. When plants reached 25 nodes, they began to develop the inflorescences in the axillary in the principal branch or in secondary branches (Figure 3E).

Figure 3 - Heteroblasty in hop leaves observed for the cv. Northern Brewer during the vegetative and reproductive development. Entire juvenile leaf (A). Trilobed leaf (B). Change from entire to trilobed leaves in 7th node (red arrow) (C). Change of entire to trilobed leaves from the 7th node to the apical meristem (red arrow) (D). Trilobed leaves from the 7th node (E). Inflorescences in axillary in 20th node (F).



Source: From the author (2022)

3.2. Precursors and mature miRNAs 156 and 172 in *Humulus lupulus* L.

With the aim to know the involvement of microRNAs 156 and 172 in reproductive transition, it was identified members of these families in the *H. lupulus* genome. Regarding the MIR156 family, there are eight members that codified precursors with sizes between 96 and 263 nt, having three copies of hlu-miR156d in the hop genome (Table S3). From these, fifteen mature sequences are originated, with sizes that vary from 18 to 22 nt (Table 1). Being that the

precursor hlu-miR156a only gives rise to one mature sequence in its 3' arm. Respecting the MIR172 family, four members were found in the hop genome, which codify precursors with sizes between 177 and 253 nt, from those, the member hlu-miR172b is duplicated in the genome (Table 1, Table S3). These members of the MIR172 family give place to eight mature miRNAs (3p and 5p) that vary in size between 19 and 23 nt (Table 1).

Table 1- Precursors and mature sequences of eight miRNAs 172 and fifteen miRNAs 156 identified in the hop genome.

Precursor_name	Mature_Name	Mature_Sequence	Start_position	End_position	Orthology_mature_name	Mismatches	Gaps	Size_miRNA	Orthology_miRNA
hlu-miR172	hlu-miR172-3p	UGAGAAUCUUGAUGAUGCUGCAU	131	153	aau-miR172	0	0	23	23
	hlu-miR172-5p	GCAUCAUCAUCAAGAUUCA	26	44	bra-miR172c-5p	0	0	19	21
hlu-miR172b-2	hlu-miR172b-2-5p	GCAGCAUCAUCAAGAUUCUCA	12	32	csi-miR172b-5p	1	0	21	21
	hlu-miR172b-2-3p	UGAAUCUUGAUGAUGCUCCA	131	150	vvi-miR172b	1	0	20	21
hlu-miR172d	hlu-miR172d-5p	GCGGCAUCAUCAAGAUUCACA	16	36	csi-miR172d-5p	0	0	21	21
	hlu-miR172d-3p	GGAAUCUUGAUGAUGCUGCAU	220	240	bra-miR172d-3p	0	0	21	21
hlu-miR172b-1	hlu-miR172b-1-5p	GCAGCAUCAUCAAGAUUCUCA	12	32	csi-miR172b-5p	1	0	21	21
	hlu-miR172b-1-3p	UGAAUCUUGAUGAUGCUCCA	130	149	vvi-miR172b	1	0	20	21
hlu-miR156b-2	hlu-miR156b-2-5p	UUGACAGAAGAUAGAGAGCAC	11	31	aof-miR156b	0	0	21	21
	hlu-miR156b-2-3p	GCUCUCUAUUCUUCUGUCAUC	69	89	vca-miR156b-3p	1	0	21	21
hlu-miR156a	hlu-miR156a-3p	UGACAGAAGAGAGAGAGCACA	183	203	tcc-miR156a	0	0	21	21
hlu-miR156b-1	hlu-miR156b-1-5p	UUGACAGAAGAUAGAGAGCAC	9	29	aof-miR156b	0	0	21	21
	hlu-miR156b-1-3p	GCUCUCUGUUCUUCUGUC	78	95	vca-miR156b-3p	2	0	18	21
hlu-miR156g	hlu-miR156g-5p	CAGAAGAUAGAGAGCACAG	10	28	gma-miR156g	0	0	19	20
	hlu-miR156g-3p	GCUCUCUGUUCUUCUGUC	75	92	csi-miR156g-3p	1	0	18	21
hlu-miR156d-2	hlu-miR156d-2-3p	GCUCUCUAUGCUUCUGUCAUCA	92	113	stu-miR156d-3p	0	0	22	22
	hlu-miR156d-2-5p	UUGACAGAAGAUAGAGAGCAC	12	32	stu-miR156d-5p	0	0	21	21
hlu-miR156d-1	hlu-miR156d-1-3p	GCUCUCUAUGCUUCUGUCAUCA	93	114	stu-miR156d-3p	0	0	22	22
	hlu-miR156d-1-5p	UUGACAGAAGAUAGAGAGCAC	12	32	stu-miR156d-5p	0	0	21	21
hlu-miR156d-3	hlu-miR156d-3-3p	GCUCACUCUCUUUCUGUCA	88	106	cas-miR156d-3p	0	0	19	21
	hlu-miR156d-3-5p	UGACAGAAGAGAGUGGAG	25	42	pab-miR156d	2	0	18	21
hlu-miR156c	hlu-miR156c-3p	GCUCACUGCUCUAUCUGUCAG	69	89	cas-miR156c-3p	0	0	21	22
	hlu-miR156c-5p	UGACAGAAGAGAGUGAGCACA	8	28	rco-miR156c	0	0	21	21

Source: From the author (2022)

3.3. Prediction of the miRNAs 156 and miRNAs 172 targets

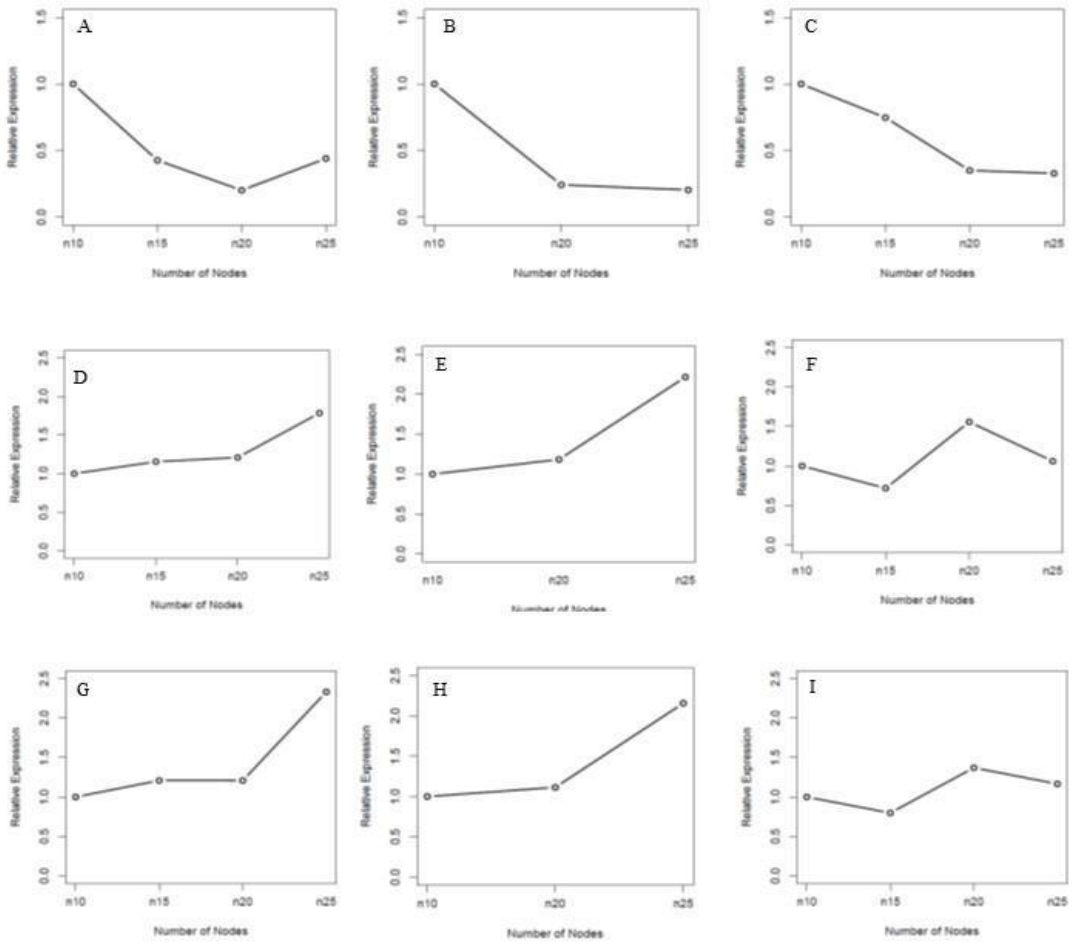
The prediction of mature miRNAs 156 and 172 targets in the hop genome revealed that fifteen mature miR156 are targeted to 2835 genes, with an expectation that ranged between 0-5 (Table S4). However, only hlu-miR156a-3p presented an expectation of 0 with five *SPL* of hop (according hopbase), which means that this microRNA has a match of 100 % with these genes, as expected since it was described as targets in other plants (Wu et al., 2009). Regarding the eight miRNAs 172, two out of these (hlu-miR172-3p and hlu-miR172d-3p) presented an expectation of 0.5 with six *AP2* of hop (Table S5), this means that only one nucleotide does not match with these genes. According to these results, these microRNAs were selected to analyze their expression during hop development.

3.4. Real time PCR of miRNAs in leaves of plants with different ages

The expression of three miRNAs (hlu-miR156a-3p, hlu-miR172d-3p and hlu-miR172-3p) was accessed by RT-qPCR in leaves with differents ages of the three hop cultivars (Figure 4). The expression level of hlu-miR156a-3p decreased significantly (Figs. S1 A,B), less than half, with respect to 10 nodes plants, in leaves from plants of 15, 20 and 25 (for the Chinook cultivar) and in plants of 20 and 25 nodes (for the Millenium cultivar) (Figs. 4A,B). In the comparison between 15, 20 and 25 nodes plants there were no significant differences. Respecto to Northern Brewer cultivar, the expression of hlu-miR156a-3p does not present significant differences between 10 and 15 nodes plants, although the expression decreased in plants with 15 nodes. However, the expression of hlu-miR156a-3p in plants with 10 and 15 nodes was statistically greater than in plants of 20 and 25 nodes (Figure 4C).

In the figure 4 D, E e F is possible to appreciate the relative expression respect to plants with 10 nodes, of hlu-miR172d-3p. Respect to the Chinook cultivar, only there was a statistically increase in plants with 25 nodes compared with plants of 10 nodes (Figure S2). While Millenium cultivar, the expression of hlu-miR172d-3p was significantly greater in plants with 25 nodes than in plants with 10 and 20 nodes. Finally, the expression of hlu-miR172d-3p did not present statistically differences in the Northern Brewer cultivar. The expression of hlu-miR172-3p was greater in plants with 25 nodes than in plants with 10 and 15 nodes for cultivar Chinook (Figure 4G, Figure S3). For other two cultivars, the expression of hlu-miR172-3p presented the same behavior as hlu-miR172d-3p.

Figure 4 - Relative expression of miRNAS in the hop cultivars Chinook, Millenium and Northern Brewer. Expression hlu-miR156a-3p (A-C), hlu-miR172d-3p (D to F) and hlu-miR172d-3p (G to H) in leaves from plants with 10, 15, 20 and 25 nodes of the cultivars Chinook (A, D and G), Millenium (B, E and H), and Northern Brewer (C, F and I).



Source: From the author (2022)

4. Discussion

In this work was evaluated the phenological stages and the expression pattern of microRNAs 156/172 of three hop cultivars during the development in this region. According to results, the start of growth began with the shoot development from buds in the rhizome. Unlike what has been reported for hop in temperate regions, in this region the buds do not enter in a stage winter arrest (endormancy). This period in temperate regions lasts approximately 6 months and finishes in the spring (Fric et al., 1991). Instead of that, in this region the buds are developing all year, and when they reach a maximum size, they enter in a stage of inactivation,

which is ended once the aerial parts are removed, allowing the development of new aerial parts. This phenomenon corresponds, instead, to a state of paradormancy, or dormancy by correlation in which the buds are inhibited by the presence of other organs (Falavigna et al., 2019). After buds activation, the formation of the new bine lasted one week, from there, the growth was linear to the 3 node stage; and when the plants found the support, they started to grow in a clockwise direction.

The vegetative growth occurred between the months of November and December, similar to other works in Brazil (Mendes, 2020), South Africa (De Lange et al., 2015) and Australia (Dodds, 2017). However, in this region the start of flowering seems to depend on the size of plants in terms of number of nodes. Since the flowering, once some plants achieved 25 nodes, occurred as early as 30 or 60 days after the activation of rhizome buds. Even some plants flowered in July or October (data not shown). This does not agree with the established for hop in temperate regions of the North hemisphere, where the flowering starts 90 days after the sprouting (Fric et al., 1991; Roßbauer et al., 1995). This indicates that, first: the flowering of hop in subtropical regions does not depend on season, but on the state of development of plants (number of nodes); second: the photoperiodic conditions of these regions always will be inductive to promote the flowering in hop plants. Because of this, our data suggest that hop is a short-day plant, instead of a shortening day plant, since from July to December, in this region the photoperiod prolonged (Figure S4). In accordance, it has been previously demonstrated that the hop is a short day plant, that flowers only if the photoperiod is less than 16.5 h, and that the plants are competent to perceive that stimulus once they reach a minimum size (number of nodes) (Thomas and Schwabe, 1969). These investigators also reported that flowering time is variety dependent, being that early flowering plants require less number of nodes to perceive the short day stimulus. In addition, Bauerle, (2019) reported that all cultivars in his study are ‘ripe to flower’ when ≥25 nodes are visible to the eye.

Similar results to ours were obtained by Acosta et al., (2021), where it was evaluated the phenology of hop in a subtropical region in Florida, United States. These investigators observed that in that region, the Cascade cultivar bloomed as early as 26 days after sprouting when the plants reached 15 nodes. Furthermore, the flowering was more prolonged, the senescence of aerial parts overlaps with new flowering, unevenness in the cones development and absence of a distinct lateral shoot formation stage were observed (Acosta et al., 2021). Moreover, they attributed those differences in phenology, with other works, to photoperiod, since the works showing the phenology of hop has been developed in regions with high latitude

(45 -50 ºN), where the daylight can achieve 17 h. In this locality the longest day has 13 h light (Figure 2B). These results show a challenge to establish the crop in this region, since this leads to a lot of unevenness in the maturity of the cones and therefore it is a challenge for the harvest of the cones. Therefore, it is important to know the endogenous factors that trigger flowering in hop.

The dynamics of expression of microRNAs 156/172 during plant development defines the age pathway (Wang, 2014; Wu et al., 2009). In this work, it has been observed that the expression of miR156 decreased in plants with 15, 20 and 25 nodes, with respect to plants with 10 nodes (Figure 4). This diminution corresponds with an intermediary stage between transition from juvenile to adult phase. In this stage, it was observed leaf morphology changes from entire to trilobed margins in the 7 th or 8 th nodes (Figure 3). In the same way, Silva et al., (2019) observed diminution of expression of miR156 in plants of *Passiflora edulis* when plants incremented the nodes number, they also observed that the phase transition was visible in the 8th or 9th nodes. Previously it has been reported that the determination of leaf identity depends on balance between SPL/miR156, which is determined in the shoot apical meristem (Fouracre and Poethig, 2019). These investigators also determined that the diminution of SAM size is due to an increase of SPL transcription factors, therefore, to a high level of miR156, the SAM is greater. In hop, Shephard et al., (2000) found diminution of SAM size during development of female and male plants.

In agreement, in our work it was observed the diminution of expression of miR156 also depends on the nodes, since the expression of the microRNAs was evaluated in leaves from 5th, 10th, 15th, and 20th nodes, in plants with 10, 15, 20 and 25 nodes, respectively. This agrees with a recent study where it was demonstrated that the diminution of expression of miR156 is due to cell division in the shoot apical meristem in *Arabidopsis thaliana*, explaining why the apical parts of a plant is older than the basal parts (Cheng et al., 2021). This phenomenon could explain the flowering near to the apical in hop plants and juvenile characteristics in first leaves.

The miR172 is another component involved in phase transition and it has been reported that the diminution of miR156 expression is concomitant with an increase of expression of miR172 (Teotia and Tang, 2015; Wang, 2014; Wu et al., 2009). In this work the increase of miR172 expression was observed only when plants have achieved 20 or 25 nodes (Figure 4D, E, I, H). This could explain the difference that exists in size between plants “ripe to flower” and flowering plants observed for hop previously (Bauerle, 2019; Thomas and Schwabe, 1969). This indicates that the expression of miR172 in plants with 25 nodes is needed, maybe to release

the expression of *FLOWERING LOCUS T (FT)* in leaves in short day conditions, since their targets in *A. thaliana*, AP2 or AP2-like transcription factors, inhibit the *FT* expression (Adrian et al., 2010; Hu et al., 2021; Teotia and Tang, 2015; Yant et al., 2010). Therefore, maybe in the hop the expression of miR172 and the perception of short days converged evolutionarily to ensure the flowering in plants with great size. This hypothesis must be demonstrated with more data, to understand better the development of the hop in subtropical regions like Brazil. From a practical point of view, the mechanism useful to delay flowering and control reproductive development of hop in Brazil, could be to silence the miR172 expression through antagonistic miRNA applications or gene editing tools.

5. Conclusion

The hop phenology in subtropical regions must be shown in terms of the developmental stages and not in terms of chronological dates. The formation of buds in the rhizome occurs throughout the year, and the activation of these buds depend on the absence of preexisting shoots. It was not observed a separate phase of formation of lateral branches, instead, it occurred while the shoot grew, in the stage of 15-20 nodes. The inflorescences started to appear, once plants achieved 25-30 nodes depending on hop cultivars. This result was correlated with a progressive decrease of miR156 expression in leaves from plants with different ages. In contrast, The expression of miR172 only was greater in plants with 25 nodes, which could explain the difference in size between plants with potential to bloom and bloomed plants. In agreement, the transition from juvenile to adult phase is visible through leaf morphology changes from entire to trilobed margins in the 7 th or 8 th nodes, which it was previously associated with miRNA 156 decrease and miR172 increase, together sugars contents, in other cultures (Schoor et al., 2021; Silva et al, 2019). Thus, our work contributes to comprehension of hop development and reproductive transition, opening perspectives to its cultivation in tropical and subtropical regions different from the traditional temperate regions and countries of the northern hemisphere.

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7. Supplementary Material

Table S1- Sequences of primers used for RT-qPCR analyses.

Primers	Sequences(5'-3')
hlu-miR156a-3P RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGTGCT
hlu-miR156a-3P Fw	GGCGTTGACAGAAGAGAGAG
hlu-miR172d-3p RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG
hlu-miR172d-3p Fw	ATGGGGGAATCTTGATGATG
hlu-miR172-3p RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG
hlu-miR172-3p Fw	GGGGTGAGAATCTTGATGATG
HlSAMS Fw	CGGTGAAGAATAGAGGTCTGG
HlSAMS Rv	CTCGGAGGTGAATAGGAAGG
HlEFALP Fw	ACCCCCCAAGTATTCTAAGG
HlEFALP Rv	CCTCTCAATCATGTTGTCACC

Source: From the author (2022)

Table S2- BBCH scale proposed for hop.

Growth stage	Code	Description
0: Sprouting	0	Dormancy: rootstock without shoots (uncut)

	1	Dormancy: rootstock without shoots (cut)
	7	Rootstock with shoots (uncut)
	8	Beginning of shoot-growth (rootstock cut)
	9	Emergence: first shoots emerge at the soil Surface
1: Leaf development	11	First pair of leaves unfolded
	12	2nd pair of leaves unfolded (beginning of twining)
	13	3rd pair of leaves unfolded
	1 .	Stages continuous till . . .
	19	9 and more pairs of leaves unfolded
2: Formation of side shoots	21	First pair of side shoots visible
	22	2nd pair of side shoots visible
	23	3rd pair of side shoots visible
	2 .	Stages continuous till . . .
	29	Nine and more pairs of side shoots visible (secondary side shoots occur)
3: Elongation of bines	31	Bines have reached 10% of top wire height
	32	Bines have reached 20% of top wire height
	33	Bines have reached 30% of top wire height
	3 .	Stages continuous till . . .
	38	Plants have reached the top wire
	39	End of bine growth
5: Inflorescence emergence	51	Inflorescence buds visible
	55	Inflorescence buds enlarged
6: Flowering	61	Beginning of flowering: about 10% of flowers open
	62	About 20% of flowers open
	63	About 30% of flowers open
	64	About 40% of flowers open
	65	Full flowering: about 50% of flowers open
	66	About 60% of flowers open
	67	About 70% of flowers open
	68	About 80% of flowers open
	69	End of flowering
7: Development of cones	71	Beginning of cone development: 10% of inflorescences are cones
	75	Cone development half way: all cones visible, cones soft, stigmas still presente
	79	Cone development complete: nearly all cones have reached full size
8: Maturity of cones	81	Beginning of maturity: 10% of cones are compact
	82	20% of cones are compact
	83	30% of cones are compact
	84	40% of cones are compact
	85	Advanced maturity: 50% of cones are compact
	86	60% of cones are compact
	87	70% of cones are compact
	88	80% of cones are compact
	89	Cones ripe for picking: cones closed; lupulin golden; aroma potential fully developed
9: Maturity of bines	92	Overripeness: cones yellow-brown discoloured, aroma deterioration
	97	Dormancy: leaves and stems dead

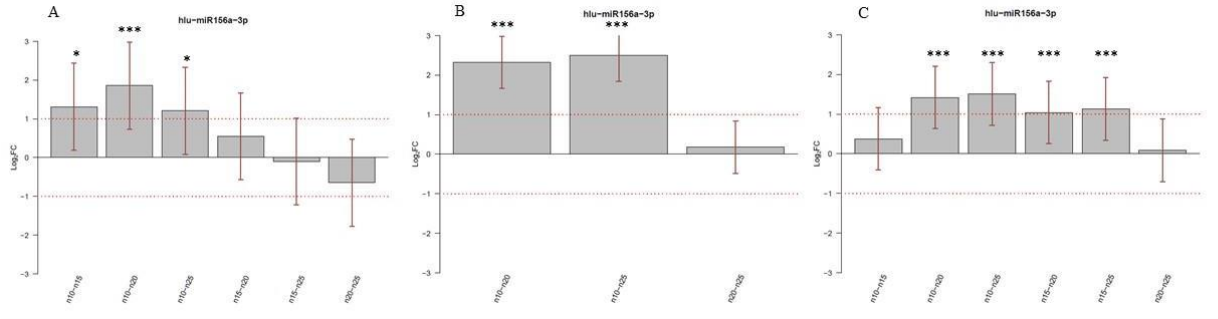
Source: Roßbauer et al., (1995)

Table S3. Localization of eight miR156 and four miR172 in the hop genome.

miRNA	Length	Scaffold	Inicial position	Final position	Strand
hlu-miR156a	263	000695F	873585	873847	plus
hlu-miR156b-1	105	000727F	858746	858850	Plus
hlu-miR156b-2	97	000052F	866379	866475	Plus
hlu-miR156c	95	001809F	636232	636326	Plus
hlu-miR156d-1	123	010706F	12838	12960	Plus
hlu-miR156d-2	122	003103F	298126	298247	Plus
hlu-miR156d-3	133	000244F	943613	943745	Plus
hlu-miR156g	99	000727F	859806	859904	Plus
hlu-miR172	177	007325F	61759	61935	Plus
hlu-miR172b-1	159	000851F	691588	691746	Plus
hlu-miR172b-2	160	003014F	355291	355450	Plus
hlu-miR172d	253	002838F	473697	473949	Plus

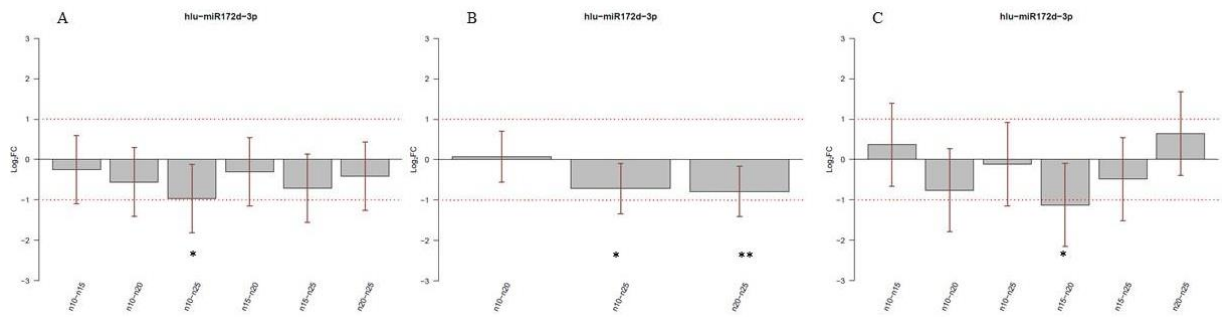
Source: From the author (2022)

Figure S1- Simultaneous tests for general linear hypotheses for expression of mir156a in the cultivar Chinook (A), Millenium (B), and Northern Brewer (C). Estimate Std. Error z value Pr(>|z|). Significant codes: 0.001 '***'; 0.01 '**'; 0.05 '*'; 0.1 '.'; ' ' 1.



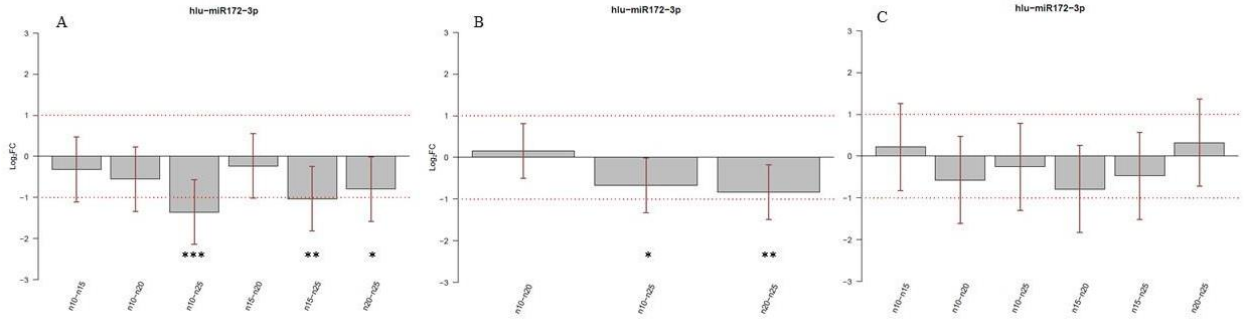
Source: From the author (2022)

Figure S2- Simultaneous tests for general linear hypotheses for expression of miR172d-3p in the cultivar Chinook (A), Millenium (B), and Northern Brewer (C). Estimate Std. Error z value Pr(>|z|). Significant codes: 0.001 '***'; 0.01 '**'; 0.05 '*'; 0.1 '.'; ' ' 1.



Source: From the author (2022)

Figure S3- Simultaneous tests for general linear hypotheses for expression of miR172d-3p in the cultivar Chinook (A), Millenium (B), and Northern Brewer (C). Estimate Std. Error z value Pr(>|z|). Significant codes: 0.001 ‘***’; 0.01 ‘**’; 0.05 ‘*’; 0.1 ‘.’; ‘’ 1.



Source: From the author (2022)

CHAPTER III

Article 2: Genome-wide analyses of MADS-box genes in *Humulus lupulus* reveal potential participation in plant development, floral architecture, and lupulin gland metabolism

Genome-wide analyses of MADS-box genes in *Humulus lupulus* reveal potential participation in plant development, floral architecture, and lupulin gland metabolism

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ABSTRACT

MADS-box transcription factors (TFs) are involved in multiple plant development processes and are most known during the reproductive transition and floral organ development. Very few genes have been characterized in the genome of *Humulus lupulus* L. (Cannabaceae), an important crop for the pharmaceutical and beverage industries. The MADS-box family has not been studied in this species yet. We identified 65 MADS-box genes in the hop genome, of which 29 encode type-II TFs (27 of subgroup MIKCC and 2 MIKC*) and 36 type-I proteins (26 α, 9 β, and 1 γ). Type-II MADS-box genes evolved more complex architectures than type-I genes. Interestingly, we did not find FLOWERING LOCUS C (FLC) homologs, a transcription factor that acts as a floral repressor and is negatively regulated by cold. This fact provides the molecular mechanism explaining a previous work showing that vernalization is not a requirement for hop flowering, which has implications for its cultivation in the tropics. Analysis of gene ontology and expression profiling revealed genes potentially involved in the development of male and female floral structures based on the differential expression of ABC homeotic genes in each whorl of the flower. We identified a gene exclusively expressed in lupulin glands, suggesting a role in specialized metabolism in these structures. *In toto*, this work contributes to understanding the evo-lutionary history of MADS-box genes in hop, and provides perspectives on functional genetic studies, biotechnology, and crop breeding.

Keywords: ABC model; hop; transcription factors; type-II MADS box; type-I MADS-box

1. Introduction

MADS-box proteins are transcription factors (TFs) that interact with the promoters of their target genes through the binding to CArG-box *cis*-elements (Riechmann et al., 1996). Phylogenetic data have classified MADS-box proteins into two groups: types I (e.g., SRF from human) and II (e.g., Mcm1 from yeast) (Becker and Theißen, 2003). A highly conserved sequence of about 60 amino acids called the MADS domain characterizes this family of TFs. In plants, MADS-box TFs have largely diversified and can be sub-classified into several clades. Type-I proteins are split into three groups: Mα, Mβ, and Mγ, whereas type-II proteins are classified into two groups: MIKC* and MIKC^C (Smaczniak et al., 2012). MIKC represents the protein structure of type-II MADS-box TFs, which has the conserved MADS-box for DNA-binding closed to the N-terminus followed by an intervening domain (I), a keratin-like domain (K) for protein-protein interaction, and the variable C-terminal domain. MIKC^C MADS-box proteins are sub-classified into 13 subfamilies, including the TM8 subfamily that is absent in *Arabidopsis* (Daminato et al., 2014).

MADS-box TFs orchestrate multiple developmental programs in plants, most notably vegetative and reproductive development programs. More recently, a novel MADS-box TF in apple was implicated in regulating dormancy cycles in response to environmental cues (da Silveira Falavigna et al., 2021). MADS-box TFs are also involved in maintaining the spike morphology of barley under high-temperature stress (Li et al., 2021), promoting bud break in ecodormant poplar (Gómez-Soto et al., 2021), and controlling nitrogen fixation symbiosis in common beans (Ayra et al., 2021). Flowering transition is another process governed by MADS-box genes. In *Arabidopsis*, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) integrates multiple flowering signals derived from photoperiod, temperature, hormone, and age-related signals (Hyun et al., 2016; Immink et al., 2012; Lee and Lee, 2010). SOC1 interacts with AGAMOUS-like 24 (AGL24) and FRUITFULL (FUL) to promote flowering (Liu et al., 2008; Torti and Fornara, 2012). In addition, the transition from the vegetative to the reproductive phase in *Arabidopsis* is controlled by the MADS-box protein, SHORT VEGETATIVE PHASE (SVP), which is a repressor of flowering under short days (Hartmann et al., 2000) alike FLOWERING LOCUS C (FLC) does prior to vernalization (Mateos et al., 2015; Michaels and Amasino, 1999). FLC is a TF that acts as a floral repressor and is negatively regulated by cold periods or vernalization, being essential to synchronize flowering and winter (Kim et al., 2009; Madrid et al., 2021). *FLC* homologs were described in the three major eudicot

lineages (Reeves et al., 2007; Ruelens et al., 2013), including sugar-beet, apple, and coffee (de Oliveira et al., 2014; Kagaya et al., 2020; Reeves et al., 2007). However, despite the role of FLC described in the Brassicaceae (Calderwood et al., 2021) and more recently cereal crops and grasses (Kennedy and Geuten, 2020; Sharma et al., 2017), the extent to which the molecular mechanisms underlying vernalization have been conserved during the diversification of the angiosperms remains elusive.

Some of the most studied MADS-box transcription factors are involved in the development of floral organs in angiosperms (Ma and dePamphilis, 2000). Such genes are called homeotic, since their misexpression in a given whorl leads to the formation of a different floral organ (Benedito et al., 2004; Bowman et al., 1989; Thomson and Wellmer, 2019). This led to the formulation of the ABC model (Coen and Meyerowitz, 1991), which encompasses the combinatorial transcription of MADS-box TFs that elicits the developmental program of specific organs in each whorl of the flower. In this model, a class-A gene expressed alone in the first whorl leads to the formation of sepals, the co-expression of class A and B genes in the second whorl leads to the development of petals, the co-expression of class B and C genes in the third whorl elicits the formation of anthers, and finally, the expression of a C-class gene alone in the fourth whorl leads to the formation of carpels.

Further research extended the ABC model to an ABCDE model, where D-class genes expressed in the carpel lead to the formation of ovules, and E-class genes expressed in all whorls form tetramers with ABC TFs that coordinate the development of each whorl (Riechmann et al., 1996; Theißen and Saedler, 2001). In *Arabidopsis*, the ABCDE model encompasses the A-class APETALA1 (AP1) (Irish and Sussex, 1990); the B-class APETALA3 (AP3) and PISTILLATA (PI) (Goto and Meyerowitz, 1994; Jack et al., 1992); the C-class AGAMOUS (AG) (Yanofsky et al., 1990); the D-class AGAMOUS-like 1, 5, and 11 (AGL1, AGL5, and AGL11) (Savidge et al., 1995); and the E-class SEPALLATA 1,2,3,4 (SEP1, SEP2, SEP3, and SEP4) (Pelaz et al., 2000). The ABCDE model has been conserved throughout angiosperm evolution. The genes encompassing each homeotic class have been determined in different species, from monocots, such as rice (Kyozuka et al., 2000; Wu et al., 2017), wheat (Kuijer et al., 2021), and Easter lily (Benedito et al., 2004); to dicots, such as soybean (Chi et al., 2011; Huang et al., 2014), coffee (de Oliveira et al., 2014), and the New Zealand endemic species, *Clianthus maximus* (Song et al., 2011), among many others. Moreover, studies have also revealed the significance of type-I MADS-box transcription factors in plant reproduction (Masiero et al., 2011).

Hop (*Humulus lupulus* L.) is a perennial crop that belongs to the Cannabaceae family. It blooms in short days once it develops a particular number of nodes. It has been phenotypically demonstrated that vernalization and dormancy do not influence flower yield and quality (Bauerle, 2019; Thomas and Schwabe, 1969). Moreover, hop is an economically important species because its cones (female inflorescences) are widely utilized in the pharmaceutical and beer industries (Benkherouf et al., 2020; Jiang et al., 2018; Yamashita et al., 2020). However, the mechanisms involved in the reproductive phase transition and flower development remain poorly explored at the molecular genetics level. We carried out a genome-wide approach, identified 65 MADS-box genes in the hop genome, and further studied their phylogenetic relationships, genetic structure, and gene expression profiles using publicly available RNA-Seq data. This study revealed TFs that potentially coordinate critical aspects of plant development, phase transition, and glandular metabolism. Therefore, this work advances our understanding of the evolutionary history of the MADS-box TFs in hop and opens new avenues for functional genetic research and crop breeding toward expanding its production zones in the world, especially in the tropics.

2. Materials and Methods

2.1. Gene prediction

H. lupulus L. gene prediction was performed using AUGUSTUS version 3.3.3 (Stanke et al., 2008). RNA-seq libraries retrieved from the NCBI's SRA database guided the proper identification of exon-intron gene boundaries (Table S2; accessed 07 Jan 2020). After quality evaluation with FastQC, the libraries were processed with Trimmomatic v.0.39 (Bolger et al., 2014) to remove adapter sequences and fragments with poor overall Phred quality. High-quality libraries were then aligned to the *H. lupulus* L., masked genome sequence from the HopBase platform (Hill et al., 2017) (accessed 07 Jan 2020) using HISAT2 v.2.1 (Pertea et al., 2016). During the training phase to establish AUGUSTUS metaparameters for the species, RNA-seq libraries from glands, leaf, cones without glands, and meristem (SRR575195, SRR10589377, SRR575201, SRR10320794, respectively) were assembled using Trinity v.2.11.0 (Haas et al., 2013). Candidate coding regions were identified with TransDecoder v.5.5.0 [47]. Subsequently, protein sequences were generated and utilized to train AUGUSTUS according to Alternate protocol 1 (Hoff and Stanke, 2019). The training was also enriched with EST and UTR coordinates utilizing coding sequences from Trandecoder and the PASA pipeline (Haas et al.,

2003). Sorted BAM files were used to generate exon (with Bam2wig) and intron hints. Finally, the trained metaparameters were fed into AUGUSTUS for gene prediction.

2.2. Identification of MADS-box genes in hop

We used the Basic Local Alignment Search Tool BLAST v.2.11.0 (Altschul et al., 1997) to scan the hop proteome searching for MADS-box proteins. A conserved domain sequence from Serum Response Factor (SRF) retrieved from the Pfam database (http://pfam.xfam.org/) was used as a query in BlastP against proteins obtained previously in the gene prediction step. In parallel, BlastP was carried out against the hop proteome retrieved from the HopBase platform. We only considered MADS-box proteins with sequences presenting a conserved domain with all three Pfam, SMART, and NCBI-BlastP analyses. Redundant proteins reported on the same locus were combined after manually curating genomic loci with IGV. Putative MADS-box protein sequences with less than 100 amino acid residues were re-submitted to AUGUSTUS, with UTR parameters turned off, until new sequences were no longer retrieved. Each non-redundant, putative MADS-box protein sequence identified in these analyses was named HlMADS01 to 65. Their physicochemical properties (length of amino acid sequence, molecular weight, and isoelectric point) were determined with the ExPASy Proteomics tool (https://web.expasy.org/protparam/).

2.3. Phylogenetic analysis

MADS-box protein sequences retrieved from species spanning the plant kingdom (*Chlamydomonas reinhardtii*, *Physcomitrella patens*, *Selaginella moellendorffii*, *Picea abies*, *Sorghum bicolor*, *Oryza sativa*, *Cucumis sativus*, *Malus domestica*, *Medicago truncatula*, and *Vitis vinifera*) were retrieved from the PlantTFDB database v.5.0 (Jin et al., 2014) along with those from *Arabidopsis thaliana* and *Solanum lycopersicum* retrieved from the NCBI database, along with those from *H. lupulus* identified above, were used in our phylogenetic analysis. For sequence types I, MIKC^{c}, and MIKC*, multiple sequence alignment jobs were performed separately with MAFFT v.7.475 (Katoh et al., 2002). The alignment quality was evaluated with GUIDANCE 2 v.2.02 (Sela et al., 2015). Both steps used default parameters. Phylogenetic trees were inferred with PHYLIP v.3.696 (Felsenstein, 1989) with 1,000 bootstrap replicates, using

the Jones-Taylor-Thornton matrix and neighbor-joining method (Saitou and Nei, 1987). Finally, the tree was visualized with FigTree, and the hop MADS-box proteins were classified into subgroups according to the *Arabidopsis* MADS-box subfamilies (Pařenicová et al., 2003) plus the subfamily TM8 first reported in tomato (Daminato et al., 2014, p. 8). When a subfamily was absent in a first search, tBLASTn was performed using the protein sequence from *Arabidopsis* of that subfamily as the query.

2.4. Gene structure and conserved motif of MADS-box analyses

The exon-intron structures of MADS-box genes were identified with the Gene Structure Display Server GSDS2.0 (Hu et al., 2015) using the GFF files generated from our gene prediction as well as the annotation available at the HopBase. The MEME suite online analysis tool (Bailey et al., 2015) was used to identify putative motifs of hop MADS-box proteins with the following parameters: maximum of 20 motifs to be identified and motif width between 6 and 60. In this case, we used sequences from *Chlamydomonas reinhardtii, Physcomitrella patens, Selaginella moellendorffii, Picea abies, Sorghum bicolor, Oryza sativa, Cucumis sativus, Malus domestica, Medicago truncatula,* and *Vitis vinifera*, as background normalization. Finally, the conserved motifs obtained were verified with PFAM, SMART, and NCBI conserved domain inference tools.

2.5. Gene ontology (GO) annotation of hop MADS-box genes

The MADS-box genes of hop were annotated into each of the three categories of Gene Ontology (GO: biological process, molecular function, and cellular component) using the Blast2GO software (Conesa et al., 2005), and the results were visualized with WEGO (Ye et al., 2018).

2.6. Expression analysis of MADS-box genes in hop tissues

Gene transcription profiling of hop MADS-box genes was generated with NCBI-SRA RNA-seq libraries of meristems (SRR10320793), stems (SRR10320795), leaves (SRR575205), young leaves (ERR2040411), cones without glands (SRR575201), bracts (SRR10541757), glands (SRR575193), and a sample of the whole plant during the growing season (SRR4242068). The data were aligned to the hop masked genome sequence with STAR v.2.7.7

(Dobin et al., 2013) using default parameters. The number of aligned reads was quantified with the htseq-count function in HTseq v.0.11.5 (Anders et al., 2014) assuming no strand specificity. The quantified reads were normalized as FPKM (Fragments Per Kilobase of transcript per Million mapped reads) with the edgeR package. Finally, a heatmap of MADS-box gene expression was generated in R v.3.6.3 using the gplots package. To corroborate these results, we used Samtools to filter all reads uniquely mapped to the genome. Subsequently, each locus was visualized with IGV, and the alignments for selected genes are reported in Figure S7.

3. Results

3.1. MADS-box genes encoded in the hop genome and Gene Ontology annotation

Using AUGUSTUS on RNA-Seq libraries, our bioinformatics pipeline identified 47 genes coding for proteins with canonical MADS-box domains. Moreover, the hop genome sequence has 69 genes annotated as coding for MADS-box proteins. The overlap of our results with the official hop genome annotation shows a set of 65 non-redundant genes (unigenes: HlMADS01 to HlMADS65), 7 of which exclusively from our prediction, 23 exclusively in the hop genome annotation, and 35 represented in both sets. All novel 7 genes identified in our *de novo* prediction pipeline were MIKC^c-type proteins (HlMADS28-33 and HlMADS65). The encoded protein length ranged from 135 to 547 amino acid residues, with an average of 249 aa; the molecular mass varied from 16 to 60 kDa, and the isoelectric point was between 4.55 and 10.25 (Table S1).

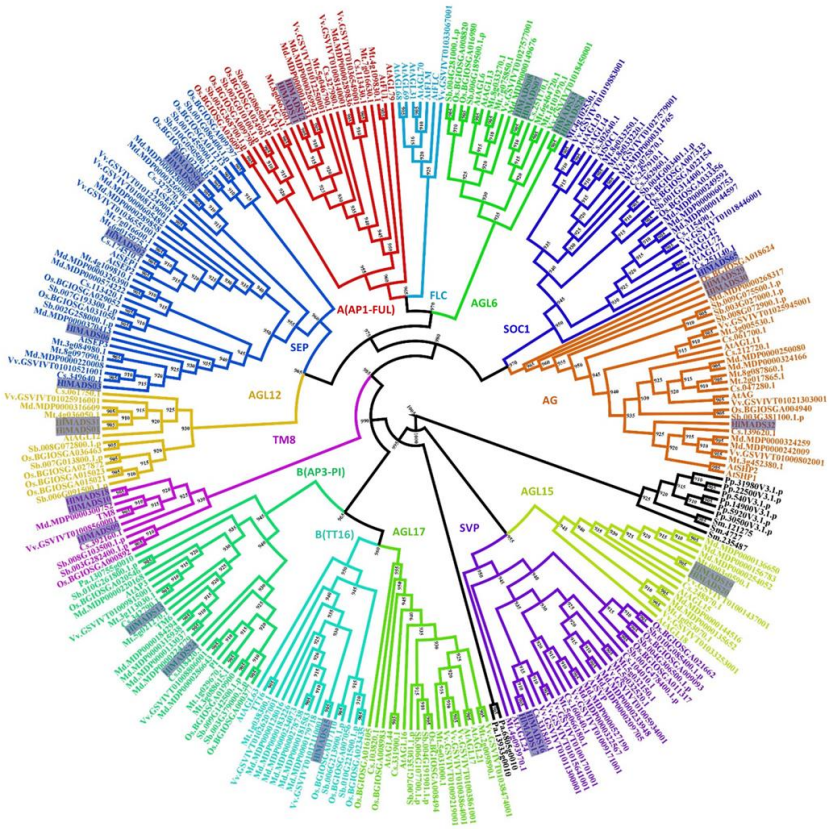
Gene Ontology (GO) analysis was performed on the 65 hop MADS-box proteins with the Blast2GO software (Table S3). All hop MADS-box proteins were classified into the three main categories (cellular component, molecular function, and biological process) and their subcategories (Figure S6). In this analysis, 48 proteins returned for cellular component and further split into six subgroups, being the ‘cell part, cell, and organelle’ subcategory the most over-represented subgroup (74%), which is a share even greater than that of *Arabidopsis* (56%), according to our analyses. In addition, 1.5% of the hop MADS-box proteins were annotated into the membrane subcategory, in contrast with 2.8% in *Arabidopsis.* On the other hand, all 65 hop MADS-box proteins were annotated in the subcategory binding of the category molecular function, a result identical to *Arabidopsis*. The second most represented molecular function subcategory in the hop MADS-box protein set was transcription regulator activity, with 75% of the proteins, in contrast with 87% for *Arabidopsis*. The subcategory catalytic activity did not

contain any hop MADS-box proteins. Finally, the category biological process contained 51 hop MADS-box proteins (79%). The subcategories biological regulation, cellular processes, regulation of biological processes, and metabolic process were the most overrepresented (97.5%) in hop, followed by positive regulation of biological process subcategory (92.5%). All other subcategories were represented by less than 20% of the hop MADS-box protein set.

3.2. Phylogenetic analyses revealed clades and potential functions of hop MADS-box genes

Protein domain analyses identified 29 type-II and 36 type-I MADS-box proteins encoded in the hop genome. Each set was submitted to separate phylogenetic analyses to further classify them into subfamilies (Figures 1, 2, S1). Our results revealed five members in the SEP clade, two in the A clade (AP1-FUL), two in the B clade (AP3-PI), three in the C/D clade (AG), four in the AGL6, two in the AGL12, two in the AGL15, three in the TM8, one in the BS (TT16), two in the SVP, and one in the SOC1 clade (Figure 1, Table S1). Two type-II proteins were classified as MIKC* (Figure 1S). Remarkably, the FLC and AGL17 subfamilies are not represented in the hop genome. The only member of the SOC1 subfamily (*HlMADS65*) was found when a tBLASTn was performed using the *Arabidopsis* SOC1 protein sequence as a query, and AUGUSTUS was run on the genome region identified in the output. *HlMADS65* lies within the region annotated as an intron of *000453F.g47* (Figure S2). The same approach was used for other genes resulting in no new sequences.

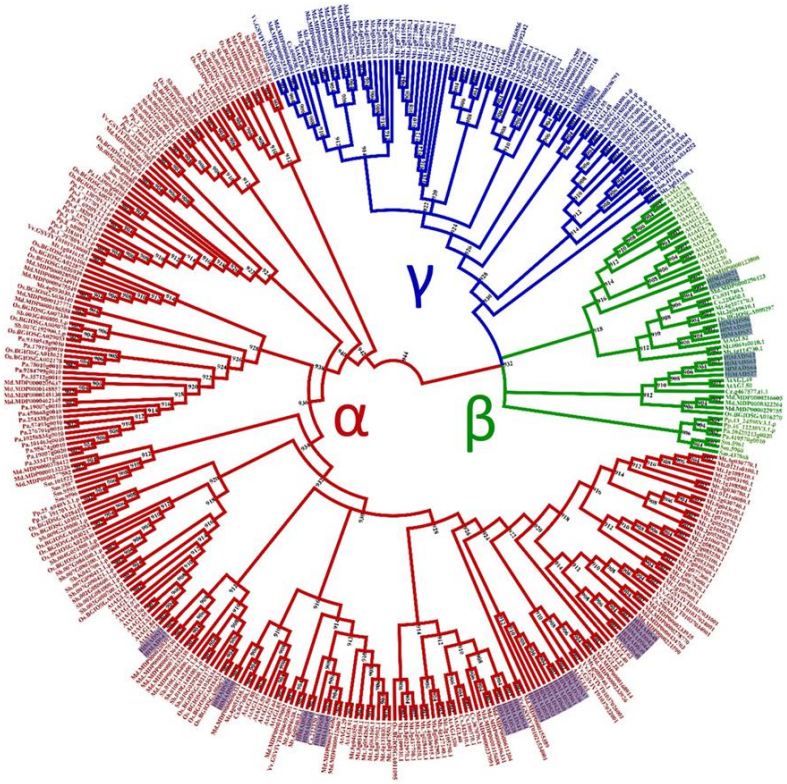
Figure 1- Phylogenetic tree of MIKCc-type MADS-box proteins of Humulus lupulus ((Hl) (27 sequences, highlighted)), Arabidopsis thaliana ((At)(39)), Solanum lycopersicum (TM6 and TM8), Physcomitrella patens ((Pp) (6)), Selaginella moellendorffii ((Sm) (3)), Piceaabies ((Pa) (3)), Sorghum bicolor ((Sb) (32)), Oryza sativa subsp. indica ((Os) (32)), Cucumis sativus ((Cs) (26)), Malus domestica ((Md) (42)), Medi-cago truncatula ((Mt) (35)), and Vitis vinifera ((Vv) (33)). Sequences from Selaginella moellendorffii, Physcomitrella patens, and Piceaabies were used as outgroups (in black font).



Source: From the author (2022)

For the type-I MADS-box proteins, the α-subfamily encompasses the most represented group, with 26 members, followed by the β-subfamily (9 members). In contrast, the γ-subfamily is represented by a single member (Figure 2).

Figure 2. Phylogenetic tree of I-type MADS-box proteins of Humulus lupulus ((Hl) (36 sequences, high-lighted)), Arabidopsis thaliana ((At) (58)), Chlamydomonas reinhardtii ((Cr) (1)), Physcomitrella patens ((Pp) (16)), Selaginella moellendorffii ((Sm)(14)), Piceaabies ((Pa) (18)), Sorghum bicolor ((Sb) (38)), Oryza sativa subsp. indica ((Os) (36)), Cucumis sativus ((Cs) (12)), Malus domestica ((Md) (56)), Medicago truncatula ((Mt) (79)), and Vitis vinifera ((Vv) (10)).

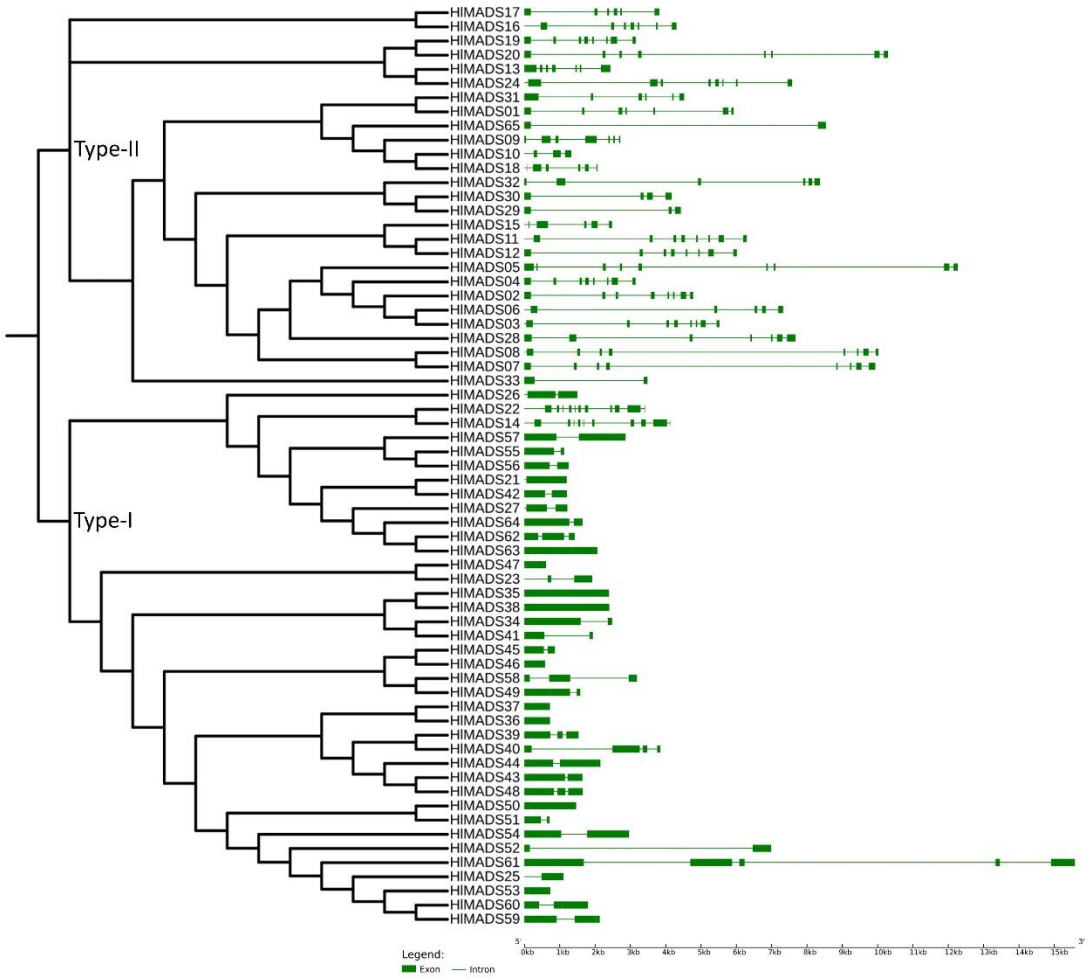


Source: From the author (2022)

3.3. Structural and motifs analyses of hop MADS-box genes

To confirm our phylogenetic relationships and gain further insights into gene functions, we explored the exon-intron architecture of MADS-box genes. The number of exons among the 29 type-II MADS-box genes varied from 2 (*HlMADS33* and *HLMADS65*) to 13 (*HlMADS22*, the sole member of the MIKC* group). In hop, *HlMADS05* is the longest type-II MADS-box gene (12.5 kb), with 9 exons and 8 introns. In the type-I group, the exon number varied from 1 to 5. Overall, *HlMADS61* was the longest gene (16 kb), whereas the shortest ones were *HlMADS46* (584 bp) and *HlMADS47* (613 bp), both with only one exon each (Figure 3), all falling in the type-I clade.

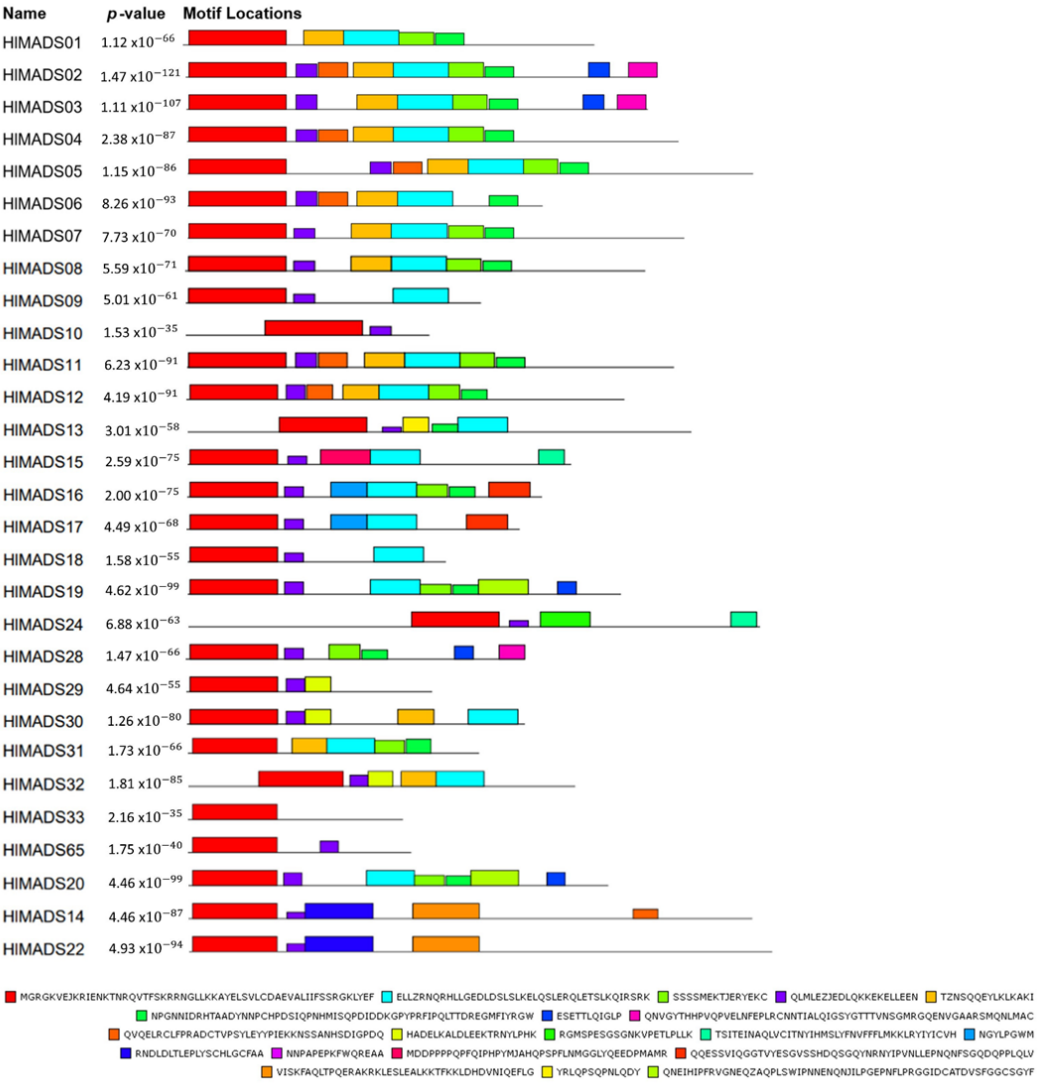
Figure 3- Phylogenetic tree and structure of 65 hop MADS-box genes. Exons are represented by green solid boxes, and introns by green lines.



Source: From the author (2022)

The MEME tool was used to visualize conserved protein domains. While all type-II proteins displayed the MADS-box domain in the tool output, only 21 proteins have the K-box domain, even though this motif is a characteristic feature of this group (Figure 4). However, the NCBI's Conserved Domain Search tool resulted in 23 proteins with the K-box domain, consequently adding HlMADS28 and HlMADS29 to the list. Even though the MADS domain did not appear for HlMADS55, HlMADS56 and HlMADS57, in the MEME analyses, the domain presence was confirmed by NCBI and PFAM conserved domain inference tools. All I-type proteins displayed the MADS domain in MEME but at diverse locations of the peptide sequence (Figures 3S and S4).

Figure 4- Motif distribution of hop type-I MADS-box proteins. Protein sequences are represented by black lines, and the conserved motifs are represented by colored boxes with the MADS-box domain in red.



Source: From the author (2022)

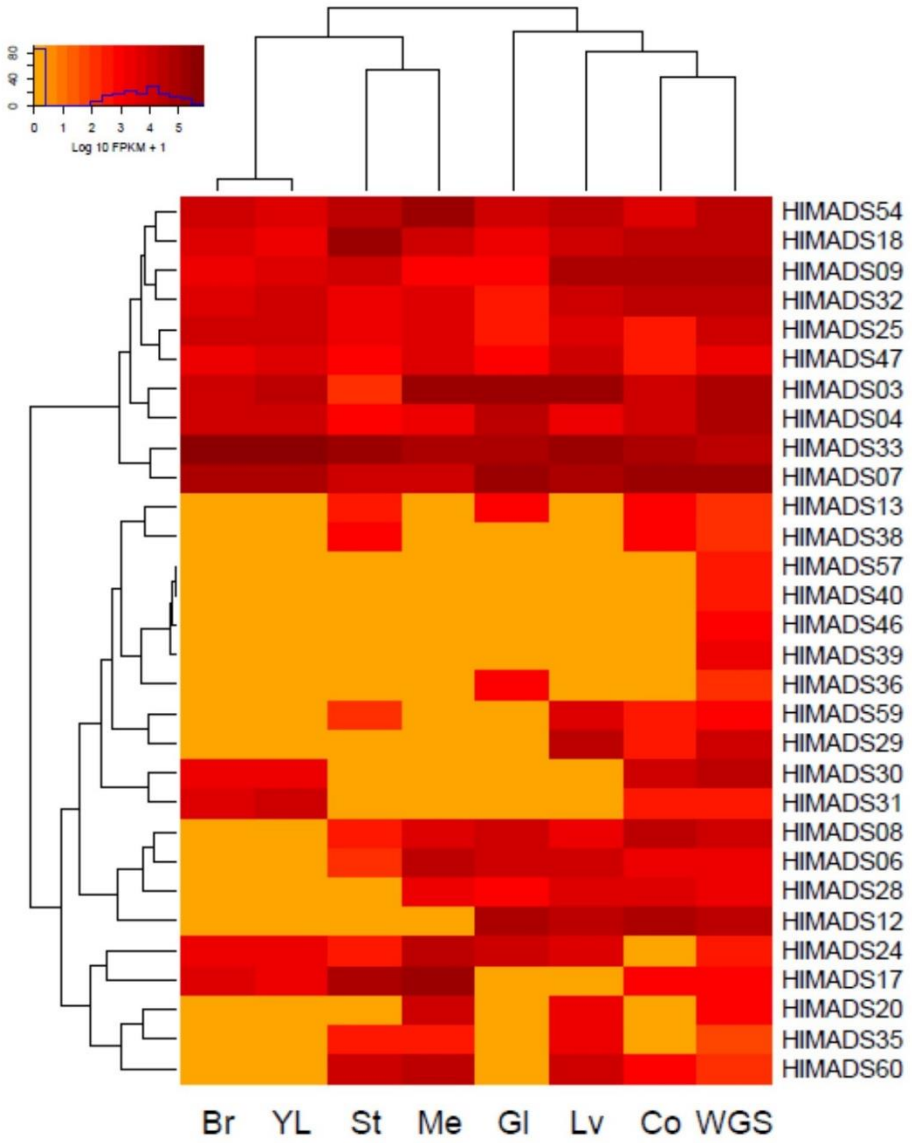
3.4. Transcriptional profile of hop MADS-box genes in different tissues

The expression profiling of MADS-box genes in different hop tissues was determined through the analysis of publicly available RNA-Seq data deposited in the NCBI-SRA database. Thirty genes were expressed in different samples (Figure 5), whereas 35 were not expressed. Of the expressed genes, 18 are of the MIKC^{c} type, and 12 are type I. Some genes belonging to the same subfamily showed distinct expression patterns. For example, in the SEP subfamily,

HlMADS03 and *HlMADS04* were expressed in all samples analyzed, whereas *HlMADS06* was expressed only in the stem, meristem, glands, mature leaves, and cones without glands. Interestingly, young leaves and bracts showed an identical expression pattern for the MADS-box genes. Moreover, *HlMADS17*, *HlMADS30*, and *HlMADS31* (respective members of the SVP, C/D(AG), and AGL12 clades) were expressed in young leaves compared to the mature organ, indicating a possible function during leaf development and expansion.

We identified two gene groups regarding the expression profile (Figure 5). The first encompassed constitutive genes: two SEP (*HlMADS03* and *HlMADS04*), two TM8 (*HlMADS09* and *HlMADS18*), two AGL6-like (*HlMADS07* and *HlMADS33*), one of the C/D (AG) subfamily (*HlMADS32*), and three type-I α (*HlMADS25*, *HlMADS47*, and *HlMADS54*). The second group included 20 genes (9 type-I and 11 MIKC^{c}) that showed variable expression among the samples. The B(AP3-PI) subfamily member, *HlMADS13*, was expressed in the stem, glands, and cones, whereas four type-I genes (*HlMADS57*, *HlMADS40*, *HlMADS46*, and *HlMADS39*) were expressed only in the whole plant sample. Interestingly, a type-I α gene, *HlMADS36*, was expressed only in the glands. On the other hand, two C/D(AG) subfamily genes (*HlMADS29* and *HlMADS30*) are in this group, with *HlMADS29* being expressed in mature leaves and cones, while *HlMADS30* expressed in the bracts and young leaves, similar to *HlMADS31*. Meanwhile, *HlMADS06* (SEP) and *HlMADS08* (AGL6) did not express in bracts or young leaves; *HlMADS28* (AGL6) expressed in the meristem, glands, mature leaf, and cones; *HlMADS12* [A(AP1-FUL)] expressed in glands, leaf, and cones; *HlMADS24* [B(AP3-PI)] expressed in all samples but cones without glands. In turn, *HlMADS17* (SVP) was expressed in all samples but glands and mature leaves, whereas *HlMADS20* was expressed only in the meristem and mature leaves. Lastly, *HlMADS35* and *HlMADS60* (type-I α-subfamily) expressed in the stem, meristem, and mature leaves, while *HlMADS60* expressed in cones, and *HlMADS35* did not.

Figure 5- Expression profiling of hop MADS-box genes in RNA-Seq libraries: bracts (Br), young leaves (YL), stem (St), vegetative meristem (Me), glands (Gl), mature leaves (Lv), cones without glands (Co), and a mélange of plant tissues collected throughout the whole growing season (WGS).



Source: From the author (2022)

4. Discussion

Many MADS-box proteins function as master regulatory transcription factors controlling critical aspects of plant development and agricultural traits. The genomic characterization of this gene family has been carried out for several plant species, such as *Arabidopsis* (107 genes: (Pařenicová et al., 2003)), rice (75 genes: (Arora et al., 2007)), grapevine (74 genes: (Grimplet et al., 2016)), *Pyrus* (75 genes: (Meng et al., 2019)), to name a

few. This work identified and provided the transcriptional profiling of 65 MADS-box genes in the hop (*H. lupulus* L.) genome. First, we carried out a *de novo* gene annotation of the hop genome and combined it with the official one available on the HopBase platform (Hill et al., 2017). This pipeline identified 7 novel MADS-box genes (*HlMADS28-33* and *HlMADS65*) in the hop genome with reads from RNAseq libraries aligned on these genes (Figure S2 and S7). Also, it was possible to find some genes (i.e., *HlMADS01* and *HLMADS02*) which were only identified when AUGUSTUS was run with the UTR parameters turned off. This finding shows that *de novo* gene prediction outputs, as well as the official functional genome annotation, must be interpreted with caution.

In our phylogenetic analyses, 27 MADS-box proteins were classified into 11 clades based on their relationships with the *Arabidopsis* MIKC^{c}-type and TM8 sub-families. We discovered that the AGL17 subfamily is not represented in the hop genome, similarly to pears (Meng et al., 2019). Neither the FLC subfamily is represented in the hop genome, suggesting that the species lacks the vernalization route completely. Accordingly, it has been demonstrated that hop does not require vernalization to trigger flowering (Bauerle, 2019), but instead, the process must involve other induction routes, such as photoperiod and age (Thomas and Schwabe, 1969).

According to the photoperiod route in *Arabidopsis*, SOC1 integrates photoperiod signals to promote flowering under long-day conditions (Jung et al., 2012). It was possible that in hop, a functional homolog was similarly involved in flowering induction. However, no SOC1 ortholog was identified in our gene prediction pipelines. Notwithstanding, the HopBase genome annotation includes a gene (000453F.g47) that contains only the K-box domain characteristic of the SOC subfamily. That may be the reason our BlastP analysis using the MADS-box consensus sequence as the query did not identify this gene in the hop proteome. However, the MADS domain for this gene was recognized by AUGUSTUS in the first annotated intron of *000453F.g47,* where RNA-Seq reads aligned (Figure S2). This gene displays two exons and encodes a protein with the MADS domain but without the K-box, suggesting that it was incorrectly annotated or may undergo intron retention, which is the most predominant mechanism of alternative splicing in plants (Ullah et al., 2018). Moreover, two SVP subfamily genes were identified in the hop genome, with *HlMADS17* expressed in young leaves only, indicating potential participation in leaf development and expansion. Finally, two AGL15 subfamily genes (*HlMADS19* and *HlMADS20*) were identified, with the latter expressed in mature leaves and the meristem. In hop, these genes could act as repressors of flowering

transition since members of this subfamily act as flowering repressors in *Arabidopsis* (Adamczyk et al., 2007) and promote the expression of miR156, a *bona fide* marker of plant juvenility (Serivichyaswat et al., 2015).

Our phylogenetic study also identified two AP1-FUL subfamily genes (*HlMADS11* and *HlMADS12*) in the hop genome. In *Arabidopsis*, AP1 determines floral meristem identity and, later, also in petal and sepal development (Gustafson-Brown et al., 1994; Irish and Sussex, 1990). The expression of *HlMADS12* in hop cones suggests a possible participation in determining floral organ identity (Figure 5). Even though female flowers of hop (cones or strobiles) entirely lack the perianth (sepals and petals), male flowers have sepals (Shephard et al., 2000), thus showing that the A function of the ABC model of flower development is present in hop. Another hypothesis for the lack of perianth structures in female hop flowers could be a lack of the E-class (SEP subfamily) function. However, this subfamily is the most represented within the MIKC^c clade in this species, with 5 genes expressed in different tissues (Figure 5). The repression mechanism of the A- and B-functions in the first two whorls during cone development remains to be ascertained. Figure 1 shows the B-class *HlMADS13* is closest to AP3 while *HlMADS24* is closest to PI. These genes show distinct gene structures, with *HlMADS24* being longer than *HlMADS13*, containing an extra exon (Figure 3), and lacking the K-box (Figure 4). When comparing GO terms between *HlMADS13* and *HlMADS24* (Table S3), both display the same terms, which results are comparable for the *Arabidopsis* AP3 and PI. Therefore, it is plausible that the B-function in hop is conserved during flower development.

Dioecious reproduction is a common feature found in the Cannabaceae, including *Cannabis* and *Humulus*. The organ positions in male (staminate) and female (pistillate) hop flowers deviate from the prevalent four-whorl scheme (sepals-petals-stamens-carpels) observed in many angiosperm groups (Shephard et al., 2000). After the shoot meristem undergoes flowering transition, the formation of floral organs is initiated. Male and female inflorescence meristems are distinguishable at the anatomical level very early on (Shephard et al., 2000), when the program of floral organ developmental fate has already been decided. According to the classic ABC model, also known as the ABCDE model, the expression of class A and C genes are mutually exclusive in the floral meristem (Coen and Meyerowitz, 1991). Three C/D-class genes were identified in the hop genome (Figure 1). The presence of sepals in the first of the two whorls of the male flower specifies the presence of the A-class function. In contrast, the development of stamens in the second whorl reveals the expression of B and C class genes and repression of the class A gene function. On the other hand, in female flowers (cones), the

formation of a rudimentary perianth (without the development of sepals or petals) indicates an absence of ABC gene expression. In contrast, the development of two carpels in its second whorl indicates the exclusive expression of the C function. Finally, *HlMADS29* probably carries out the D-class function because it is expressed in the cones (Figure 5) and its gene annotation is associated with ovule development (GO:0048481). A more refined definition of gene expression within the developing flower and functional analyses to define the role of ABCDE genes are warranted to better understand the genetics of floral organ development in this species.

The 36 type-I MADS-box genes identified in the hop genome were classified into three subfamilies, α (26 genes), β (9), and γ (1). These genes were more structurally diverse but contained fewer exons than the type-II genes (Figure 3). Previous research reported similar results in other species (Meng et al., 2019; Pařenicová et al., 2003; Zhang et al., 2017). The amino acid sequence in this group was also more diverse than in the type-II group, and the MADS-box was not detected in some analyses for three members of the β-subfamily (*HlMADS55, HlMADS56,* and *HlMADS57*), although they were present when manually inspected (Figure S4). The MADS motif is somewhat divergent in these three proteins and required a higher sensitivity from the sequence analysis tool. According to the gene expression profile, 12 type-I *MADS-box* were expressed in the RNA-Seq libraries analyzed, with *HlMADS57* the only member of the β-subfamily to be expressed. Interestingly, *HlMADS36* was exclusively expressed in all three gland samples analyzed (Figure S5), which allowed us to hypothesize that it may coordinate the biosynthesis of resin and specialized metabolites (Okada and Ito, 2001) or participate in gland development. Therefore, it is important to further verify the exact timing expression domain and function of *HlMADS36*. Interestingly, *HlMADS36* is associated with a GO term (0045944) involved in multiple processes related to transcriptional induction of genes related to the metabolism of organic compounds. Overall, our findings provide perspectives on functional analyses and breeding of hop.

5. Conclusion

In this work, we identified 65 MADS-box genes in the hop genome, with 36 being of type I and 29 genes of type II. Phylogenetic analyses showed that 27 type-II MADS-box genes belonged to 12 subfamilies, while 2 genes were of type MIKC*. Meanwhile, type-I MADS-box genes were classified in α-subfamily (26 members), β-subfamily (9 members), and γ-subfamily (a single member). The gene structure of type-I genes was less complex than that of type II

genes, with fewer exons, even though the longest MADS-box gene was of type I. Some MIKC^{C}-type MADS-box proteins did not display the K-box domain. Members of the FLC subfamily were not found in the hop genome. The only SOC1 subfamily member in the hop genome may undergo alternative splicing with intron retention. Genes of the ABCDE model of flower development were expressed in cones. One gene, a member of the α-subfamily, was found exclusively expressed in lupulin glands, with potential implications for specialized metabolism. Thus, this work contributes to understanding the evolutionary history of MADS-box in hop and provides perspectives on functional analysis and crop breeding.

6. References

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7. Supplementary Material

Table S1- *MADS-box* gene identified in the hop genome.

Gene ID	Gene name	Contig Location	CDS (bp)	Exon No.	Strand	Protein size (residues)	MW (Da)	pI	Subfamily
g126639.t1	HlMADS01	005050F:261365:267689	648	7	-	215	24191.15	8.66	AGL12
g4287.t1	HlMADS02	000025F:2663950:2669377	747	9	-	248	28673.47	8.96	E(SEP)
000727F.g16.t1	HlMADS03	000727F:300503:306308	732	8	+	243	28010.83	8.78	E(SEP)
000079F.g81.t1	HlMADS04	000079F:2009261:2019723	780	8	+	259	29495.61	7.57	E(SEP)
001022F.g25.t2	HlMADS05	001022F:835353:848110	897	9	-	298	34337.21	7.57	E(SEP)
006765F.g2.t1	HlMADS06	006765F:49016:57362	567	7	+	188	22130.51	9.68	E(SEP)
000139F.g16.t1	HlMADS07	000139F:691180:701232	843	8	-	262	30480.39	8.73	AGL6
000261F.g24.t1	HlMADS08	000261F:1206883:1217085	729	8	+	242	28455.11	8.87	AGL6
000155F.g57.t2	HlMADS09	000155F:1174916:1179017	828	9	-	156	17770.36	9.45	TM8
004947F.g24.t1	HlMADS10	004947F:296392:297739	477	4	+	129	14846.84	9.37	TM8
006412F.g5.t1	HlMADS11	006412F:101506:107824	774	8	+	257	29984.02	8.2	A(AP1-FUL)
005516F.g3.t1	HlMADS12	005516F:100575:106871	771	8	-	256	29778.06	8.97	A(AP1-FUL)
000562F.g29.t1	HlMADS13	000562F:405065:407961	930	7	-	295	33152.62	6.55	B(AP3-PI)
000267F.g44.t1	HlMADS14	000267F:1322662:1327001	1026	11	+	341	38315.62	6.26	MIKC*
000006F.g39.t2	HlMADS15	000006F:1630741:1633244	654	5	+	225	25689.19	7.07	BS(TT16)
008535F.g2.t2	HlMADS16	008535F:35736:40789	628	9	+	208	23976.43	6.55	SVP
003895F.g10.t1	HlMADS17	003895F:119273:124319	588	7	-	195	22415.69	6.86	SVP
004012F.g22.t2	HlMADS18	004012F:362046:365098	519	8	+	152	17407.84	9.3	TM8
002163F.g38.t1	HlMADS19	002163F:484371:488562	765	9	-	254	28867.72	8.23	AGL15

g128133.t1	HlMADS20	005238F:252962:256110	597	8	-	254	28872.72	8.24	AGL15
004180F.g12.t1	HlMADS21	004180F:187393:188704	1140	1	+	379	42912.16	6.83	Beta
000351F.g15.t1	HlMADS22	000351F:597580:601145	1062	13	+	353	40110.17	6.61	MIKC*
000962F.g16.t1	HlMADS23	000962F:609956:612029	603	3	+	200	22140.06	5.75	Alpha
000586F.g19.t1	HlMADS24	000586F:511047:518658	1008	8	+	335	38534.19	8.84	B(AP3-PI)
000238F.g55.t1	HlMADS25	000238F:849739:851157	621	2	+	206	23333.47	6.08	Alpha
001957F.g8.t1	HlMADS26	001957F:323596:325350	1338	2	+	445	48673.45	9.02	Gamma
000887F.g29.t1	HlMADS27	000887F:890609:891924	915	2	+	304	35110.43	5.25	Beta
g39417.t1	HlMADS28	000628F:1010162:1017835	597	7	-	198	22484.65	8.94	AGL6
g90065.t1	HlMADS29	002424F:490038:494465	435	3	-	144	17011.7	9.6	C/D(AG)
g21585.t1	HlMADS30	000253F:1451643:1455810	597	4	-	198	23006.82	9.7	C/D(AG)
g98509.t1	HlMADS31	002879F:359907:364430	531	6	-	176	20176.59	8.77	AGL12
g57516.t1	HlMADS32	001131F:491536:499904	705	6	-	234	26967.13	9.91	C/D(AG)
g120931.t1	HlMADS33	004517F:301077:304561	393	2	+	130	15020.97	10.25	AGL6
g105746.t1	HlMADS34	003321F:169727:172210	630	2	-	209	22689.95	8.91	Alpha
g126627.t1	HlMADS35	005050F:116796:119190	594	1	+	197	21541.38	7.71	Alpha
g145841.t1	HlMADS36	008392F:89967:90690	585	1	-	194	22721.07	8.63	Alpha
g115481.t1	HlMADS37	004059F:231977:232700	585	1	-	194	22663.03	8.84	Alpha
g98489.t1	HlMADS38	002879F:155406:157810	627	1	+	208	22639.82	9.18	Alpha
g70848.t1	HlMADS39	001574F:102007:103540	990	3	-	329	36408.27	9.79	Alpha
g148256.t1	HlMADS40	009280F:58747:62590	867	4	-	288	31504.4	9.49	Alpha
g63684.t1	HlMADS41	001329F:293116:295050	573	2	+	190	20693.75	9.61	Alpha
g116966.t1	HlMADS42	004180F:181006:182210	960	2	+	319	35943.8	8.58	Beta
g115485.t1	HlMADS43	004059F:341647:343290	1297	2	-	431	46701.58	9.36	Alpha
g115484.t1	HlMADS44	004059F::322287:324440	1644	2	-	547	60026.5	9.46	Alpha
g143617.t1	HlMADS45	007779F:47307:48170	459	2	-	152	17620.07	9.69	Alpha
g93707.t1	HlMADS46	002616F:159796:160380	546	1	+	181	20710.51	9.26	Alpha
g80284.t1	HlMADS47	001966F:73257:73870	501	1	-	166	18423.71	5.68	Alpha
g76680.t1	HlMADS48	001803F:4107:5760	1266	3	-	421	45520.49	9.34	Alpha
g149874.t1	HlMADS49	010116F:52407:53990	597	2	-	198	22248.4	8.78	Alpha
g93699.t1	HlMADS50	002616F:17167:18630	672	1	-	223	25007.1	5.87	Alpha
g149872.t1	HlMADS51	010116F:46656:47370	498	2	+	165	18540.17	5.11	Alpha
g17586.t1	HlMADS52	000193F:209997:216980	474	2	-	157	18031.71	6.52	Alpha
g140155.t1	HlMADS53	007017F:101746:102480	546	1	+	181	20852.1	6.32	Alpha
g69465.t1	HlMADS54	001521F:226037:229000	555	2	-	184	20509.14	5.82	Alpha
g132514.t1	HlMADS55	005766F:205237:206360	723	2	-	240	27195.68	8.72	Beta
g123814.t1	HlMADS56	004793F:82436:83690	987	2	+	328	36872.64	8.84	Beta
g37290.t1	HlMADS57	000578F:1005602:1008466	1256	2	+	395	44459.12	9.34	Beta
g93696.t1	HlMADS58	002616F:866:4050	708	3	+	235	26027.62	9.24	Alpha
g20630.t1	HlMADS59	000238F:870733:872867	759	2	+	252	28642.71	5.71	Alpha
g71695.t1	HlMADS60	001607F:395446:397250	591	2	+	196	22028.87	5.2	Alpha
g34447.t1	HlMADS61	000516F:40106:55690	1148	5	+	359	40443.46	8.84	Alpha

g15081.t1	HlMADS62	000156F:497105:498528	1100	3	-	343	39980.1	4.56	Beta
g110780.t1	HlMADS63	003683F:338687:340750	664	1	-	220	25301.54	4.76	Beta
g32470.t1	HlMADS64	000472F:1676:3320	1247	2	+	392	45421.25	4.55	Beta
000453F.g47.t2	HlMADS65	000453F:1097840:1106375	408	2	-	135	15911.6	9.69	SOC

Source: From the author (2022)

Table S2- RNA-seq libraries used to hints AUGUSTUS

RNA-seq library	**Tissues**
SRR10589377	Leaf, bract and lupulin glands
SRR10549511	Leaf, bract and lupulin glands
SRR10541757	Leaf, bract and lupulin glands
SRR10320795	Non-floral Cascade RNA-seq (stem)
SRR10320793	Meristem-Cascade
SRR10320791	Leaf-Cascade
SRR4242068	Roots, sprouts, leaves, stems, flowers, cones
ERR2040411	Young leaves

Source: From the author (2022)

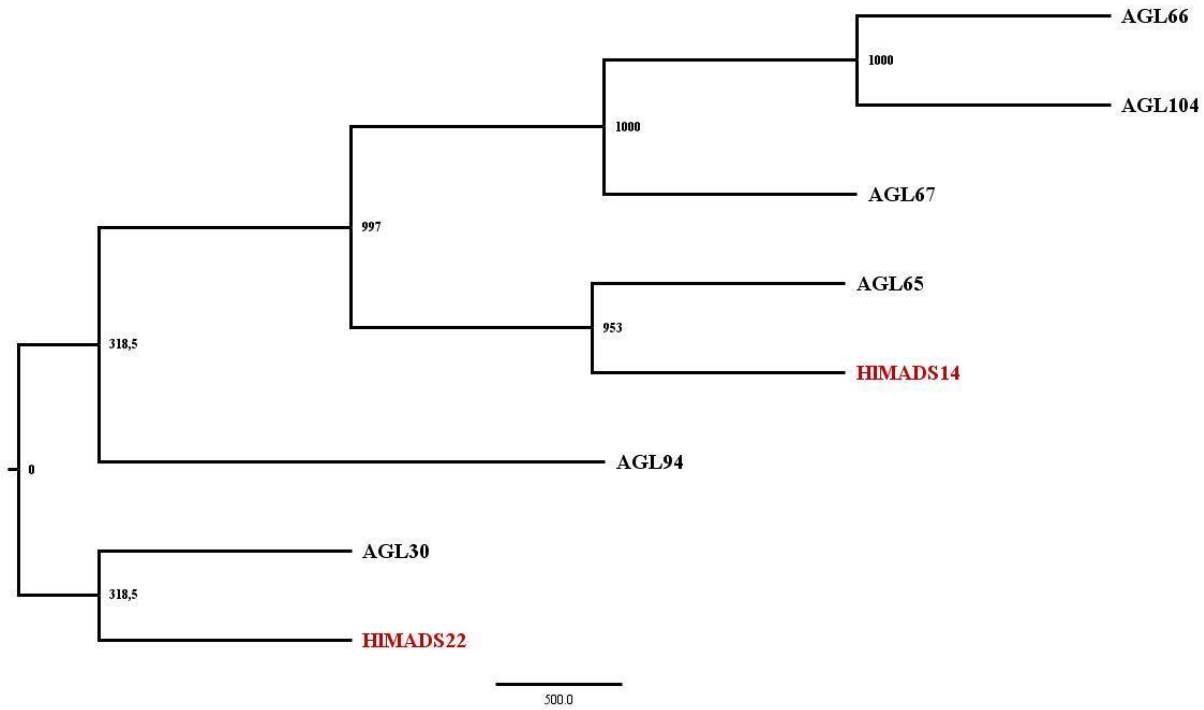
Table S3- Gene Ontology terms of hop MADS-box genes

Genes	**GO terms**
HlMADS02	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS03	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS04	GO:0000977 GO:0003700 GO:0005634 GO:0045944 GO:0046983
HlMADS05	GO:0000977 GO:0003700 GO:0005634 GO:0045944 GO:0046983
HlMADS06	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS07	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS08	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS28	GO:0000977 GO:0045944 GO:0046983
HlMADS33	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0010582 GO:0045944 GO:0046983
HlMADS01	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0010228 GO:0045944 GO:0046983 GO:0048364
HlMADS31	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0010228 GO:0045944 GO:0046983 GO:0048364
HlMADS11	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0010582 GO:0045944 GO:0046983
HlMADS12	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0010582 GO:0045944 GO:0046983
HlMADS13	GO:0000977 GO:0003700 GO:0005634 GO:0045944 GO:0046983
HlMADS24	GO:0000977 GO:0003700 GO:0005634 GO:0045944 GO:0046983
HlMADS29	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0016020 GO:0045944 GO:0046983 GO:0048481 GO:0090376
HlMADS30	GO:0000977 GO:0003700 GO:0005634 GO:0045944 GO:0046983 GO:0090376
HlMADS32	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983 GO:0090376
HlMADS19	GO:0000977 GO:0003700 GO:0005634 GO:0045944 GO:0046983
HlMADS20	GO:0000977 GO:0003700 GO:0005634 GO:0045944 GO:0046983
HlMADS09	GO:0000977 GO:0003700 GO:0005634 GO:0045944 GO:0046983
HlMADS10	GO:0000977 GO:0003700 GO:0005634 GO:0045944 GO:0046983
HlMADS18	GO:0000977 GO:0003700 GO:0005634 GO:0045944 GO:0046983
HlMADS15	GO:0000977 GO:0003700 GO:0005634 GO:0008360 GO:0045595 GO:0045944 GO:0046983 GO:0048316 GO:0051302 GO:0080060 GO:0080155 GO:2000029
HlMADS16	GO:0000977 GO:0003700 GO:0005634 GO:0045944 GO:0046983
HlMADS17	GO:0000977 GO:0003700 GO:0005634 GO:0045944 GO:0046983
HlMADS65	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0010048 GO:0032501 GO:0045944 GO:0046983 GO:0048510
HlMADS14	GO:0003677 GO:0046983
HlMADS22	GO:0000981 GO:0000987 GO:0045944 GO:0046983
HlMADS23	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983 GO:0048481
HlMADS25	GO:0000977 GO:0000981 GO:0005634 GO:0006357 GO:0008134 GO:0046983

HlMADS34	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS35	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS36	GO:0000977 GO:0045944 GO:0046983
HlMADS37	GO:0000977 GO:0045944 GO:0046983
HlMADS38	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0046983
HlMADS39	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS40	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS41	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS43	GO:0000977 GO:0000981 GO:0005634 GO:0006357 GO:0008134 GO:0046983
HlMADS44	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS45	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS46	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS47	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS48	GO:0000977 GO:0000981 GO:0005634 GO:0006357 GO:0008134 GO:0046983
HlMADS49	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS50	GO:0000976 GO:0046983
HlMADS51	GO:0000976 GO:0046983
HlMADS52	GO:0000976 GO:0046983
HlMADS53	GO:0000977 GO:0000981 GO:0005634 GO:0006357 GO:0008134 GO:0046983
HlMADS54	GO:0000977 GO:0000981 GO:0005634 GO:0006357 GO:0008134 GO:0046983
HlMADS58	GO:0000977 GO:0005634 GO:0045944 GO:0046983
HlMADS59	GO:0000976 GO:0046983
HlMADS60	GO:0000977 GO:0000981 GO:0005634 GO:0008134 GO:0046983
HlMADS61	GO:0000977 GO:0000981 GO:0005634 GO:0006357 GO:0008134 GO:0046983
HlMADS26	GO:0000977 GO:0000981 GO:0000987 GO:0005634 GO:0008134 GO:0045944 GO:0046983
HlMADS21	GO:0000981 GO:0000987 GO:0045944 GO:0046983
HlMADS27	GO:0003677 GO:0046983
HlMADS42	GO:0000977 GO:0000981 GO:0000987 GO:0005634 GO:0008134 GO:0008360 GO:0045595 GO:0045944 GO:0046983 GO:0048316 GO:0051302 GO:0080060 GO:0080155 GO:2000029
HlMADS55	GO:0003677 GO:0046983
HlMADS56	GO:0003677 GO:0046983
HlMADS57	GO:0003677 GO:0046983
HlMADS62	GO:0003677 GO:0046983
HlMADS63	GO:0003677 GO:0046983
HlMADS64	GO:0003677 GO:0046983

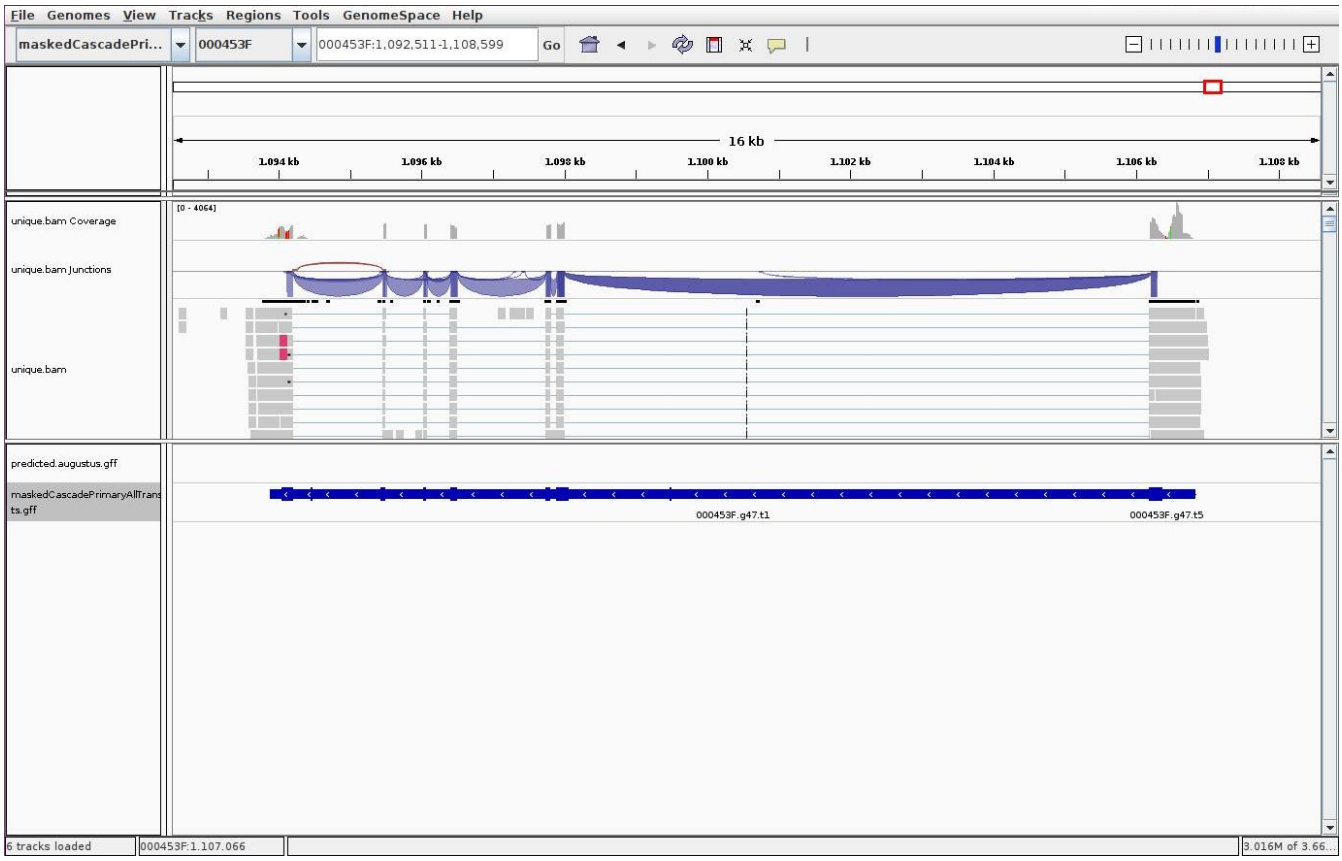
Source: From the author (2022)

Figure S1- Phylogenetic tree of MIKC*-type MADS-box proteins of *H. lupulus* (2, red), Arabidopsis (6).



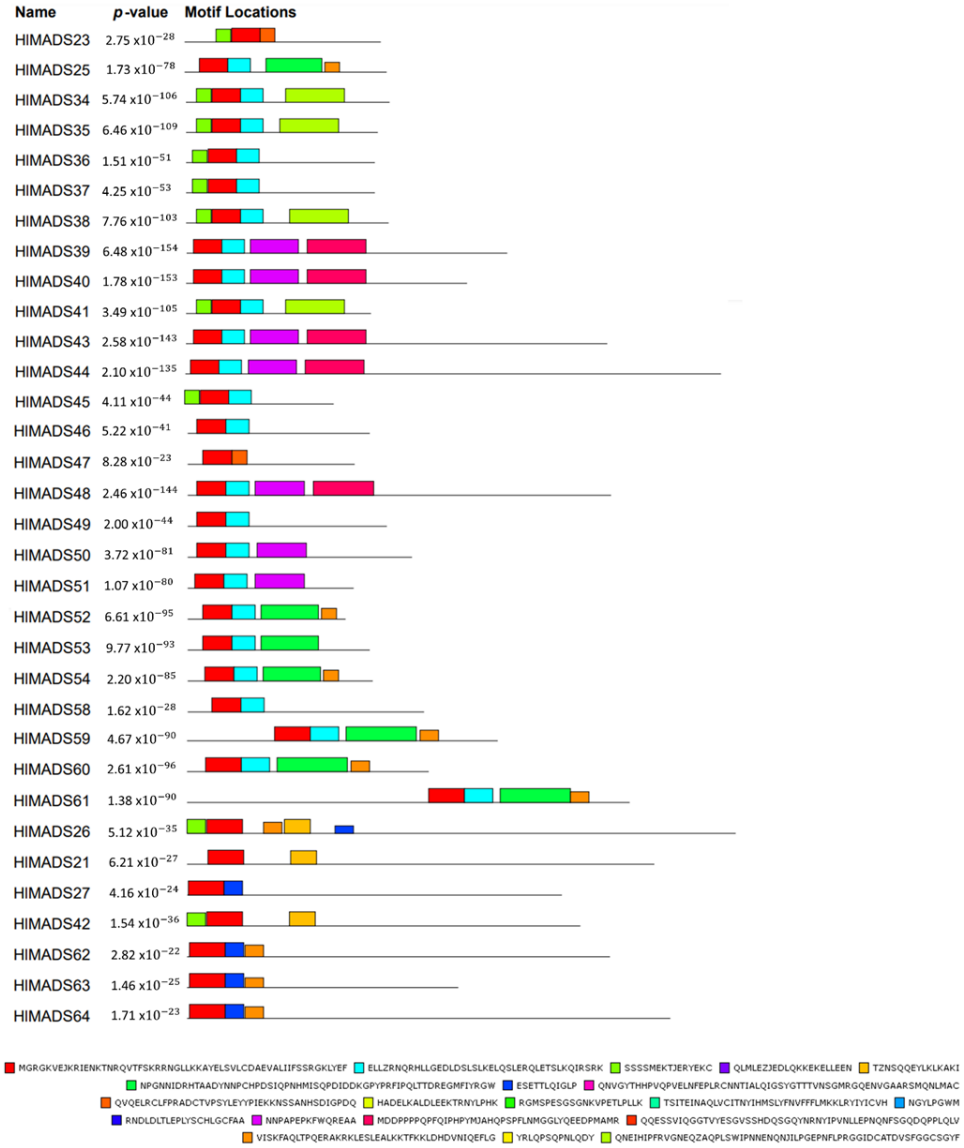
Source: From the author (2022)

Figure S2- RNAseq reads on the gene *000453F.g47*.



Source: From the author (2022)

Figure S3- Motifs distribution of the hop MADS-box proteins. Protein sequences are represented by black lines, and the conserved motifs are represented by colored boxes. The motif consensus sequences are shown in the legend.

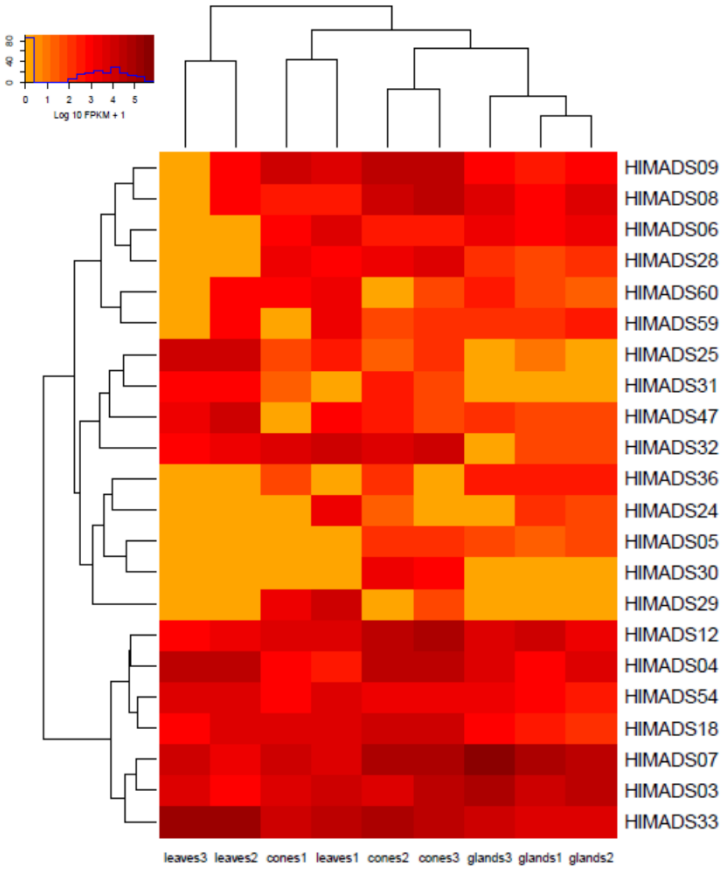


Source: From the author (2022)

Figure S4. Motifs distribution of the hop MADS-box proteins of β-subfamily members. Protein sequences are represented by black lines, and the conserved motifs are represented by colored boxes. **A:** Logo of MADS-box domain. **B:** Motifs distribution of the hop MADS-box proteins of β-subfamily members. **C:** Amino acid residue sequences of selected MADS-box domains.

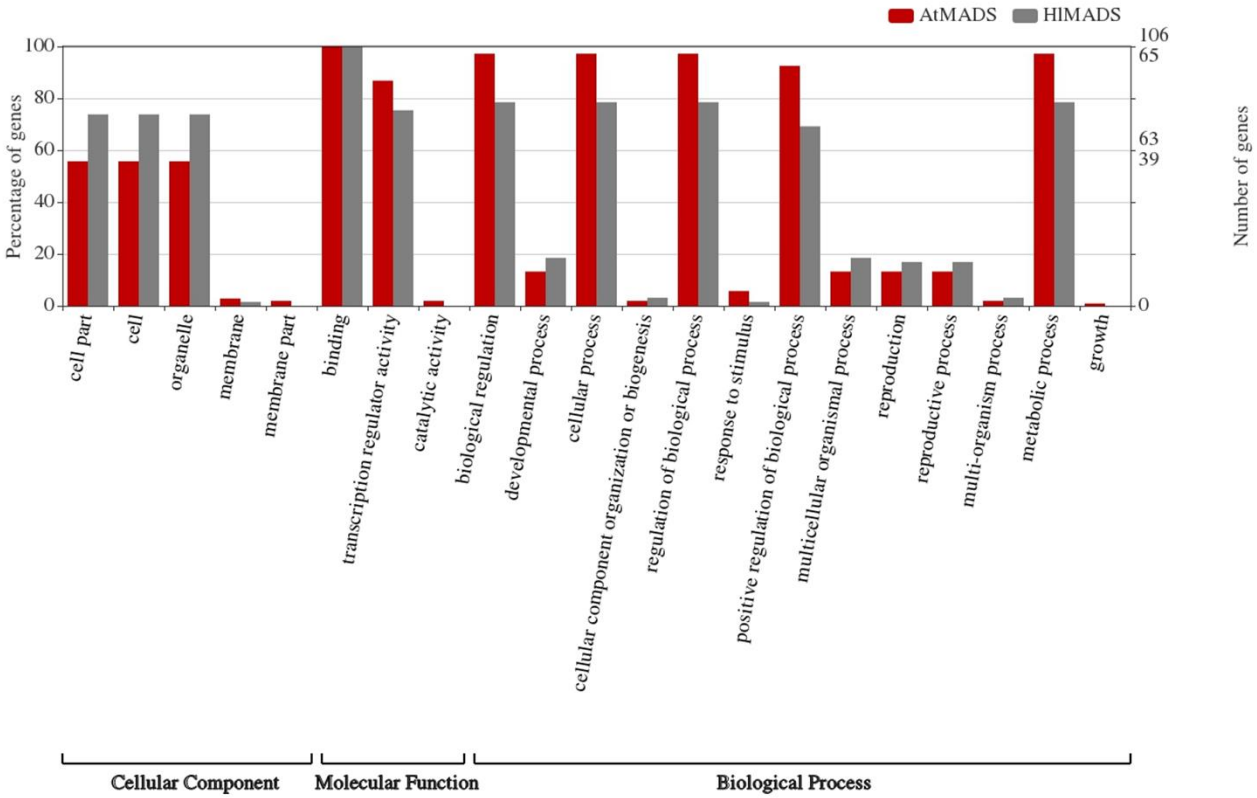
Source: From the author (2022)

Figure S5- Expression profile of the hop MADS-box genes in three RNAseq libraries from cones, leaves, and glands



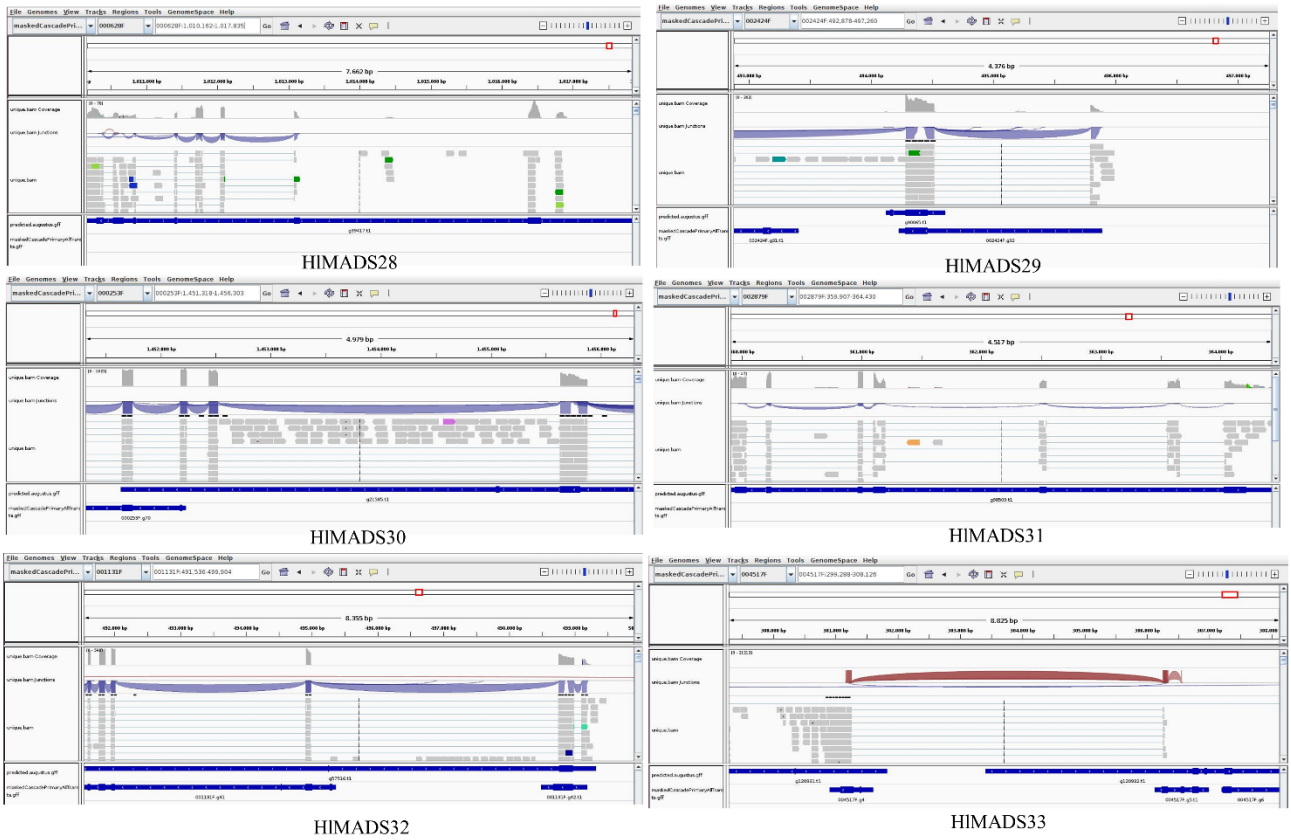
Source: From the author (2022)

Figure S6- Gene Ontology classification of MADS-box genes in hop and *Arabidopsis*.



Source: From the author (2022)

Figure S7. RNAseq reads that are uniquely mapped on 6 novel MADS-box genes (*HlMADS28-33*).



Source: From the author (2022)

CHAPTER IV

General conclusions

In this work, we observed that the development of hop in subtropical Brazilian conditions does not depend on the season of year, but depends on the plant age. This was demonstrated when plants bloomed in any time of the year, being the only parameters to this occurred the number of nodes. We conducted a experiment with the aim to know the pattern of development accompanied with the expression of miR156/172, and found that plants that achieved a determined number of nodes (cultivar dependent) flowered and while plants grew the levels of miR156 decreased, an the levels of miR172 increased in plants with 25 nodes. The fact that plants were growing in the period of October to March was due to the water disponibility by rain. Maybe this parameter could be determinant in another time of year. The identification and *in silico* analyses of MADS-box genes also permitted us to understand the development of hop plants. For example, the absence of members of the FLC subfamily suggest that the vernalization pathway is not required for floral transition in hop plants. Members of AGL15 and SVP also permitted us to know the implications of the MADS-box transcription factors during flowering in the age pathway. The expression profile demonstrated that some MADS-box genes are expressed in different tissues, and this could explain the floral architecture of this plant. Also, the expression profile demonstrated one *MADS-box* expressed exclusively in lupulin glands, with a GO term related to positive regulation of genes related with the secondary metabolism in lupulin gland. Therefore, this gene and other genes described in this work could be used in breeding programs, for example increasing the compounds related to lupulin glands.