IN SILICO ANALYSIS OF THE LETTUCE (*Lactuca sativa* L.) MADS-BOX GENE FAMILY

ANÁLISE IN SILICO DOS GENES DA FAMÍLIA MADS-BOX EM ALFACE (Lactuca sativa L.*)*

ANÁLISIS IN SILICO DE LOS GENES DE LA FAMILIA MADS-BOX EN LECHUGA (Lactuca sativa L.*)*

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ABSTRACT

High temperatures can negatively affect lettuce production by promoting early bolting, which leads to increased levels of latex accumulation in the leaves, causing them to become bitter. The lack of adaptation of this culture to such conditions made it an object of study for plant breeding programs, resulting in well succeeded studies. However, little is known about the genes that regulate lettuce flowering. A better understanding of the complex genic interactions involved in the process of lettuce floral initiation is of great importance, since it can enable the development of late-bolting cultivars through plant genetic transformation. *MADS-box* transcriptional factors are key flowering regulators and have been extensively studied during the flowering process in several species. Thus, this study aimed to identify and characterize the Lettuce *MADS-box* gene family through the use of bioinformatics tools. The computational analysis consisted in gene prediction, alignment, and phylogenetic analysis. 91 sequences of putative *MADS-box* genes were identified and characterized by a phylogenetic study of 20 *MADS-box* genes. Future studies comprising mutants for these genes in plant model species and in lettuce will enable a better understanding of the functions performed by these genes during lettuce flowering, as well as, a better comprehension of this process.

Keywords: Bioinformatics. Bolting. Flowering.

RESUMO

Altas temperaturas podem afetar negativamente a cultura da alface, promovendo o pendoamento precoce, o qual leva ao aumento na concentração de látex nas folhas, tornando-as amargas. A falta de tolerância da cultura a tais condições tornou-se objeto de programas de melhoramento genético. Porém pouco se sabe sobre os genes que regulam o florescimento da alface. O entendimento das complexas interações gênicas envolvidas, no processo de iniciação floral da alface, é de grande importância, pois pode possibilitar o desenvolvimento de cultivares com pendoamento tardio, por meio da transformação genética. Os fatores de transcrição MADS-box são reguladores-chave do florescimento e já foram extensivamente estudados durante o processo de florescimento de várias

espécies. Assim, o presente trabalho teve por objetivo identificar e caracterizar os genes da família MADS-box de Lactuca sativa por meio do uso de ferramentas de bioinformática. As análises consistiram na predição dos genes, alinhamento e análise filogenética. 91 sequências de prováveis genes MADS-box foram obtidos, e 20 genes caracterizados por meio de um estudo filogenético. Estudos futuros que envolvam mutantes, para esses genes, em plantas-modelo e em alface, possibilitarão melhor entendimento das funções desempenhadas por esses genes no florescimento da alface, assim como melhor compreensão desse processo.
***Palavras-chave**: Bioinformática. Pendoamento. Florescimento.*

RESUMEN
Las altas temperaturas pueden afectar negativamente el cultivo de lechuga promoviendo espigamiento precoz, lo cual eleva la concentración de latex en las hojas tornandolas más amargas. La falta de adaptación de este cultivoa esas condiciones se ha vuelto objetivo para mejoramiento genético. Sin embargo, se tiene poco conocimiento sobre los genes que regulan el florecimiento en lechuga. Un mejor entendimiento de las complejas interacciones génicas envueltas en el proceso de iniciación floral de la lechuga es de gran importancia devido a que posibilita el desarrollo de cultivares con espigamiento tardío por medio de transformación genética. Los factores de transcripción MADS-box son reguladores claves del florecimiento y ya fueron ampliamente estudiados durante el proceso de florecimiento de varias especies. El presente trabajo tuvo como objetivo identificar y caracterizar los genes de la familia MADS-box de Lactuca sativa con el uso de herramientas de bioinformatica. Los análisis consistieron en la predicción de los genes, aliniamiento y análisis filogenética. Se obtuvieron 91 secuencias de probables genes de la caja MADS y 20 genes caracterizados a través de un estudio filogenético. Estudios futuros utilizando mutantes para esos genes en plantas modelos y en lechuga posibilitaran un mejor entendimiento de las funciones desempeñadas por estos genes en el florecimiento de lechuga, asi como una mejor comprensión de ese proceso como un todo.
***Descriptores**: Bioinformática. Pendoamento. Floración.*

INTRODUÇÃO

Lettuce (*Lactuca sativa* L.) is a leafy vegetable harvested and consumed during its vegetative stage, being one of the most cultivated vegetables in the world (FUKUDA et al., 2017; DE ALMEIDA et al., 2019). Originated in temperate climate regions, ideal temperatures for lettuce development range from 20 °C to 25 °C (HENZ and SUINAGA, 2009; MALDONADE et al., 2014). In 2016, the total cultivation area of lettuce and chicory worldwide was represented by 1,223,407 million ha, which yielded 26,799,564 million tons of these vegetables (FAO, 2016).

The lettuce production cycle is short, from 45 to 60 days, allowing its production throughout the year (MALDONADE et al., 2014). However, although the lettuce production system is well established, problems related to its cultivation are still present. These problems are directly linked to environmental factors, such as temperature and photoperiod (ZHOU et al., 2019), with temperature posing as the main drawback for lettuce cultivation in hot climate regions (DE ALMEIDA et al., 2019).

Lettuce production under high temperatures often leads to losses due heat stress, which accelerates plant metabolism, and

consequently decreases photosynthesis, resulting in smaller plants due to early bolting. Meanwhile, besides affecting lettuce head formation, high temperatures increases leaf latex accumulation, which gives them a bitter taste (CÁSSERES, 1980; HENZ and SUINAGA, 2009; SALA, 2011).

The lack of tolerance to these conditions stimulated plant breeding programs to initiate studies aiming the development of summer lettuce cultivars, which resulted in the generation of late-bolting cultivars (SILVEIRA et al., 2002). However, little is known about the molecular mechanisms that control lettuce flowering. Thus, the search for genes involved in the regulation of this process is of paramount importance for elucidating the mechanism controlling this developmental phase, enabling the generation, through biotechnological approaches, of lettuce cultivars showing late bolting patterns, which is important for a better formation of lettuce heads, a feature desired by every lettuce producer.

The regulation of flowering time and its comprehension is a complex process, considering that the transition from the vegetative to the reproductive phase integrates several endogenous and environmental signals. Thus, the direct regulation of the genes should be analyzed in conjunction with their interaction for a better understanding of the flowering process (VALENTIM et al., 2015).

Flowering time regulation have been extensively studied in the plant model species Arabidopsis (*Arabidopsis thaliana*) (BALANZA et al., 2019; KIM, 2020; PETRELLA et al., 2020; QUESTA et al., 2020; ŠKILJAICA et al, 2020; XIE et al., 2020). Genetic and molecular studies have established the presence of five different pathways that control flower development: the Photoperiod, the Vernalization, the Gibberellic acid, the Aging, and the Autonomous pathways (SRIKANTH and SCHMID, 2011). The input from these pathways converge in the regulation of a small group of genes, named flowering time integration genes (FORNARA et al., 2010).

Among the flowering time integrator genes, the *MADS-box* transcriptional factors act as key-regulators of many processes, not only in plants but also in cellular development of different organisms, such as fungi and animals (BECKER and THEIßEN, 2003; AIROLDI and DAVIES, 2012; GRAMZOW et al., 2010). Member from the *MADS-box* gene family are characterized by the presence of a conserved N-terminal domain, which encodes for a DNA biding domain, named MADS. The MADS domain has approximately 60 amino acids and it recognizes a specific sequence [CC(A/T)6GG], called CArG-box, which is located in the regulatory region of their target genes (HAYES et al., 1988; SHORE and SHARROCKS, 1995; RIECHMANN et al., 1996; KHAN et al., 2012).

The plant *MADS-box* gene family has been mainly studied in Arabidopsis, where more than 100 genes belonging to this family classified into type I and type II *MADS-box* genes, were already identified (BECKER and THEIßEN, 2003). Type II *MADS-box*, characterized by the MICK domain, have been more extensively studied since they directly

participates in many developmental process, including root, flower, seed and embryo development (ALVAREZ-BUYLLA et al., 2000; GRAMZOW and THEIßEN, 2015)

Among the flowering integrator genes, FLOWERING *LOCUS T* (*FT*), *SUPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), a *MADS-box* gene, and *LEAFY* (*LFY*), are the main regulators of flower development (FORNARA et al., 2010; PARCY, 2004). These genes are under control of the *MADS-box* gene *FLOWERING LOCUS C* (*FLC*), which is the main repressor of flowering. *FLC* is positively regulated by the *FRIGIDA* (*FRI*) gene, and these genes act simultaneously to repress flowering. Therefore, considering the different pathways involved in flowering regulation, the control of this developmental process depends on the interaction between *FLC* and *FRI*, as well as, on the *FLC* action over the integrator genes (MICHAELS and AMASINO, 1999; FORNARA et al. 2010).

The sequencing of the lettuce genome and the generation of a sequence database (*Lettuce Genome Resource* - https://lgr.genomecenter.ucdavis.edu), has facilitated the search for lettuce genes involved in different aspects of plant growth and development, and through the use of bioinformatics tools and molecular biology approaches, these genes can be identified and characterized in a relatively short time (AGRAWAL and SILAKARI, 2015).

Thus, considering the worldwide importance of lettuce culture and the availability of tools to study the genome of this species, this study aimed to investigate the molecular aspects of lettuce flowering through the *in silico* characterization of the lettuce *MADS-box* gene family.

MATERIALS AND METHODS

In silico analysis

Putative lettuce MADS-box genes were obtained from keyword searches in the Phytozome database. In order to identify grouping motifs among the sequences obtained, the MEME (*Multiple Expectation Minimization for Motif Elucidation*; http://meme-suite.org/) version 5.1.1 (BAYLEY and ELKAN 1994) was employed, using the following parameters: maximum number of motifs 20 and optimum range between 6 and 70 (PARENICOVÁ et al., 2003).

Phylogenetic analysis

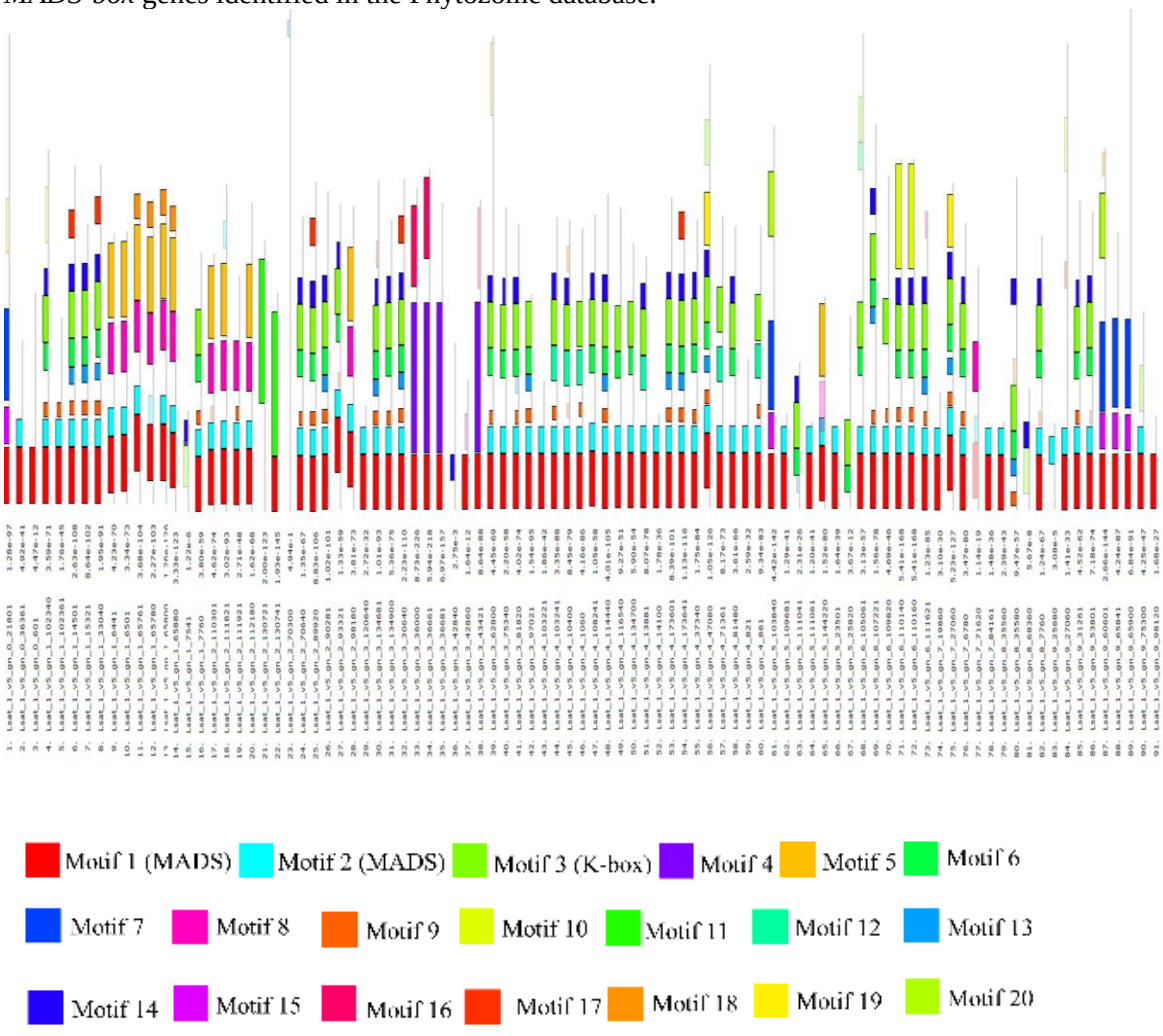
Protein sequence alignments, comprising the putative lettuce *MADS-box* genes found in the Phytozome database and Type II *MADS-box* genes from Arabidopsis, obtained from Arabidopsis Gene Regulatory Information Server-AGRIS database (https://agris-knowledgebase.org), were performed by the ClustalW program (THOMPSON et al., 1994) using default parameters. The phylogenetic tree was generated with MEGA software, version 4.0 (TAMURA et al., 2007), with neighbor-joining comparison model (SAITOU and NEI, 1987), p- distance method and pair-wise suppression. Bootstrap values from 10,000 replicates were used to assess the robustness of the trees (SITNIKOVA et al., 1995).

RESULTS AND DISCUSSION

In silico analysis

The search for lettuce *MADS-box* genes in the Phytozome database resulted in 91 sequences, which were analyzed for grouped motifs using the MEME program (Figure 1). The *MADS-box* gene family is divided into two lineages, named Type I and Type II lineages. Type II lineage contain genes that present a common domain, the MICK domain, which is formed by a group of four domains, MADS (M), Intervening (I), Keratin-like (K), and the (C) terminal domain, and have been extensively studied in different species due to their direct involvement in many plant developmental processes (ALVAREZBUYLLA et al., 2000; GRAMZOW et al., 2010; GRAMZOW and THEIßEN, 2015). The MADS (*motifs 1 and 2*) or K-box (*motif 3)* domains, which had their identity confirmed through analysis in the *Conserved Domain* database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), were found in all putative lettuce *MADS-box* genes, except for three sequences (Lsat_1_v5_gn_2_130721,Lsat_1_v5_gn_2_70300 e Lsat_1_v5_gn_3_42840) (Figure 1).

Figure 1. Grouped motif analysis of the amino acid sequences encoded by all the putative lettuce *MADS-box* genes identified in the Phytozome database.



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Phylogenetic analysis

The relationship among the putative lettuce *MADS-box* genes identified in this study and the Type II *MADS-box* genes from Arabidopsis was assessed through the generation of a phylogenetic tree, which was only possible to be constructed after the exclusion of 13 lettuce incomplete sequences. The analysis of the phylogenetic tree permitted the identification of twenty clades, from which five of them were formed exclusively by lettuce sequences identified in this study, preventing their identification as putative Type II *MADS-box* genes (Figure 2). The nomenclature proposed by the Phytozome database was maintained.

None of the lettuce sequences identified here were found to group in the clade presenting the *FLOWERING LOCUS C (FLC)/ MADS AFFECTING FLOWERING (MAF)* and *AGAMOUS LIKE 63(AGL63)* genes (Figure 2). However, a previous study indicates the presence of several putative lettuce *FLC* orthologues (NING et al., 2019). Within the 13 remaining clades, it could be identified twenty putative lettuce Type II *MADS-box* genes, with most of the booststrap values being higher than 90%, indicating the robustness of these clades (Figure 2). Bootstrap value higher than 70% are considered acceptable (HILLS and BULL, 1993). In addition, the identified lettuce sequences showed identity levels of at least 51%, when compared *MADS-box* genes from other species (Table 1).

Table 1. Comparison of the lettuce putative *MADS-box* genes found in the Phytozome database and their best hit of the blastp analysis on the NCBI database.

Gene	ID	Gene/Specie/Size	E-value	Identity	Positive
Lsat_1_v5_gn_3_134900 (*SOC1*)	XP_023732937.1	*SOC1*/*Lactuca sativa*/218aa	1e-76	218/218(100%)	218/218(100%)
Lsat_1_v5_gn_8_7760	XP_023755627.1	*AGL42*/ *Lactuca sativa* /207aa	2e-69	207/207(100%)	207/207(100%)
Lsat_1_v5_gn_9_53801	XP_023753340.1	*AGL42*/ *Lactuca sativa* /225	8e-161	225/225(100%)	225/225(100%)
Lsat_1_v5_gn_9_31261 (*AGL18*)	XP_027364327.1	*AGL18*/ *Abrus precatorius*/233aa	3e-67	120/235(51%)	167/235(71%)
Lsat_1_v5_gn_6_107221 (*AGL16*)	XP_023750511.1	*AGL16*/ *Lactuca sativa*/291aa	0.0	291/291(100%)	291/291(100%)
Lsat_1_v5_gn_6_110140 (*TESTA 16*)	XP_022762460.1	*TESTA 16*/ *Durio zibethinus*/264aa	1e-85	142/240 (59%)	179/240 (74%)
Lsat_1_v5_gn_6_110160 (*TESTA 16*)	XP_022762460.1	*TESTA 16*/ *Durio zibethinus*/264aa	1e-85	142/240 (59%)	179/240 (74%)

Lsat_1_v5_gn_3_75340 (*AP3*)	ADU15473.1	*AP3/ Actinidia chinensis*/228aa	7e-98	143/209(68%%)	176/209(84%)
Lsat_1_v5_gn_4_116540 (*AP3*)	AIC33047.1	*AP3/Chrysanthemum lavandulifolium*/227aa	2e-152	209/229(91%)	218/229(95%)
Lsat_1_v5_gn_1_7760 (*PI*)	AOD74998.1	*PI/ Tagetes erecta* /196aa	1e-138	191/196(97%)	193/196(98%)
Lsat_1_v5_gn_2_70640 (*SVP*)	PWA44102.1	*SVP/ Artemisia annua*/223aa	2e-128	189/223(85%)	202/223(90%)
Lsat_1_v5_gn_3_62800	XP_022001247.1	*SVP/ Helianthus annuus*/358aa	2e-131	191/223(86%)	206/223(92%)
Lsat_1_v5_gn_6_105061	XP_023750479.1	*AGL24/ Lactuca sativa*/359aa	1e-153	217/218(99%)	218/218(100%)
Lsat_1_v5_gn_9_75300	PWA72742.1	*AGL24/ Artemisia annua* /149aa	6e-62	124/228(54%)	138/228(60%)
Lsat_1_v5_gn_4_134700 (*AGL12*)	XP_023729353.1	*AGL12/ Lactuca sativa*/200aa	3e-145	200/200(100%)	200/200(100%)
Lsat_1_v5_gn_6_111621	XP_023760420.1	*AGL11/ Lactuca sativa*/226aa	3e-166	226/226(100%)	226/226(100%)
Lsat_1_v5_gn_4_47080 (*AGL1*)	XP_023738865.1	*AG/ Lactuca sativa*/335aa	0.0	247/247(100%)	247/247(100%)
Lsat_1_v5_gn_7_27060 (*AGL1*)	XP_023732806.1	*AG/ Lactuca sativa*/247aa	0.0	247/247(100%)	247/247(100%)
Lsat_1_v5_gn_4_173601 (*AGL8*)	QCQ83097.1	*FUL/Ambrosia artemisiifolia*/235aa	3e-105	170/244(70%)	184/244(76%)
Lsat_1_v5_gn_2_90281 (*AGL8*)	QCQ83097.1	*FUL/Ambrosia artemisiifolia* /241aa	1e-123	184/243(76%)	206/243(84%)
Lsat_1_v5_gn_3_97021 (*AGL8*)	QCQ83097.1	*FUL/Ambrosia artemisiifolia* /162aa	3e-104	151/157(96%)	153/157(97%)
Lsat_1_v5_gn_3_134681 (*AGL13*)	XP_023732929.1	*6-like/Lactuca sativa*/249aa	0.0	249/249(100%)	249/249(100%)
Lsat_1_v5_gn_1_14501 (*AGL3*)	ADU15479.1	*SEP4/ Actinidia chinensis*/249aa	2e-124	180/250(72%)	208/250(83%)
Lsat_1_v5_gn_3_30640	XP_023770719.1	*SEP3/ Lactuca sativa*/250aa	0.0	250/250(100%)	250/250(100%)
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Lsat_1_v5_gn_2_89920	QCQ83112.1	*SEP2/ Ambrosia artemisiifolia* /250aa	129	198/255(78%)	211/255(82%)
Lsat_1_v5_gn_4_173641	XP_023737701.1	*SEP1/ Lactuca sativa*/225aa	1e-166	225/225(100%)	225/225(100%)
Lsat_1_v5_gn_6_109820	XP_022038683.1	*AGL104/ Helianthus annuus*/252aa	0.0	197/300(66%)	224/300(74%)
Lsat_1_v5_gn_9_27060	XP_023761277.1	*AGL65/ Lactuca sativa*/353aa	0.0	353/353(100%)	353/353(100%)

Among the twenty genes identified (Figure 2), eight (*SEPALLATA-SEP1, SEP2, SEP3, SEP4; AGAMOUS –AG; PISTILLATA-PI; APETALA 3-AP3 e SEEDSTICK -STK)* belong to the ABCDE model, the most used model to describe the development of floral whorls, where each letter corresponds to a class of genes (WIEGEL and MEYEROWITZ, 1994; DITTA et al., 2004; CAUSIER et al., 2010; *THEIßEN et al.*, 2016). The remaining 12 genes do not belong to the ABCDE model, but they have been shown to be involved in the transition to the reproductive phase, in the development of plant reproductive structures, such as the pollen grains and the ovule, and in the control of seed dormancy.

The *SOC1* gene is included in the first clade of the phylogenetic tree (Figure 2). In the literature, *SOC1* is described as a gene that displays an essential role during lettuce bolting, being considered as marker gene of the lettuce floral transition process. Chen et al., (2018) observed high expression levels of *SOC1* when plants were shifted, after 24 days under temperatures between 15 °C / 25 °C, to temperatures ranging from 25 °C to 35 °C, which led to the early bolting of the plants.

The second clade of the phylogenetic tree contains the *AGAMOUS LIKE 42 (AGL42)* gene (Figure 2). In Arabidopsis, this gene is expressed in the shoot apical meristem (SAM) during the vegetative phase, as well as, during the transition to the reproductive phase. In addition, *AGL42* expression is directly regulated by *SOC1* (DORCA-FORNEL et al., 2011).

The *AGAMOUS LIKE 18 (AGL18)* and *AGAMOUS LIKE 16 (AGL16)* genes were found to belong to the third clade (Figure 2). In Arabidopsis, Fernandez et al. (2014) showed that *AGL18* acts as a flowering repressor, similarly to *AGL16* (HU et al., 2014), *AGAMOUS LIKE 12 (AGL12)* (TAPIA-LOPES et al., 2008), and *SHORT VEGETATIVE PHASE (SVP)*, also identified in this study (Figure 2). The *SVP* gene from *Lilium longiflorum*, when inserted in Arabidopsis plants, have been shown promote a significant delay in the flowering of these plants (TANG et al., 2020). On the other hand, *AGL24*, which has been grouped in the same clade as the *SVP* gene (Figure 2), promotes flowering by positively regulating *SOC1* expression in Arabidopsis (LIU et al., 2008).

The *FRUITFULL* (*FUL)* gene, also identified in this study (Figure 2), displays an

important role in the formation of the terminal floral structure, positively regulating the *AG* gene through the repression of *APETALA 2 (AP2)* genes, which down-regulates *AG* at the SAM (BALANZA et al., 2019).

The *ARABIDOPSIS BSISTER* (*ABS*) gene, identified in this phylogenetic analysis, closely related to the ap3/pi clade (Figure 2), constitutes one of the smaller clades. *ABS* displays an important role during ovule development (EHLERS et al., 2016), similar to *AGAMOUS LIKE 13 (AGL13)*, located in the clade next to the *FUL* gene clade (Figure 2). *AGL13*, besides regulating ovule development, contributes to pollen grain formation (ROUNSLEY etal., 1995; HSU et al., 2014). *AGAMOUS LIKE 65 (AGL65)* and *AGAMOUS LIKE 104 (AGL104)* are members of the last clade containing lettuce putative *MADS-box* genes (figure 2). Both genes are expressed during pollen grain formation, regulating their growth and maturation (ADAMCZYK and FERNANDEZ, 2009).

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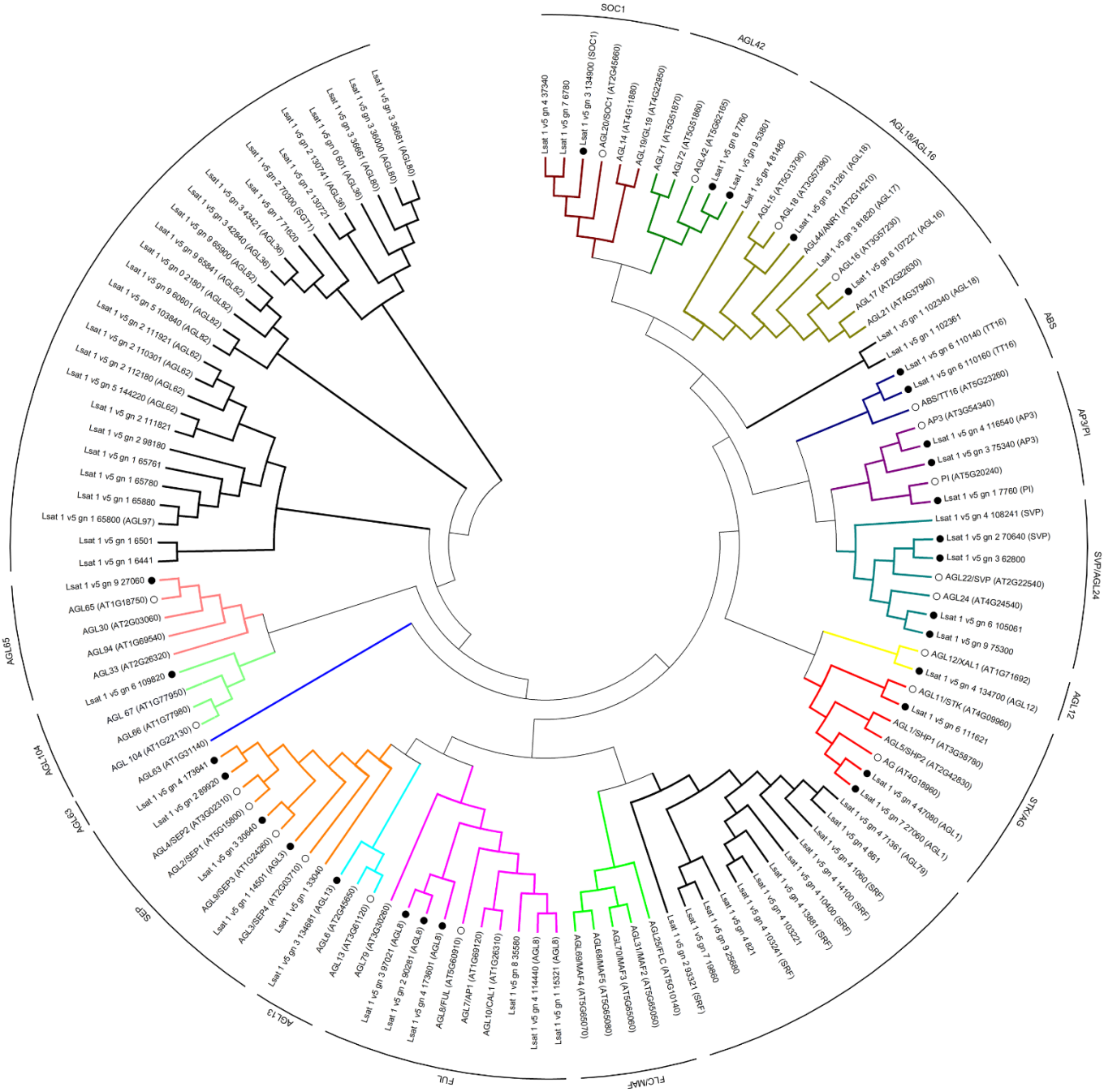
Figure 2. Phylogenetic analysis of the putative lettuce *MADS-box* genes (•) identified in the Phytozome database and the Type II *MADS-box* genes from Arabidopsis (o) obtained from Arabidopsis Gene Regulatory Information Server-AGRIS database. Neighbor-joining comparison model, p-distance method and pair-wise suppression were used for the construction of the phylogenetic tree. Bootstrap values from 10,000 replicates were used to assess the robustness of the tree.



Ning et al. (2019) proposed a model proving that high temperatures cause modifications in the expression of *MADS-box* genes during flower development, which alters the lettuce flowering process under these conditions.

CONCLUSION

Based on the results obtained from the Phytozome database, 20 putative lettuce *MADS-box* genes could be identified in this study. Most of these genes showed high similarity levels with Type II *MADS-box* genes from Arabidopsis, as observed by the bootstrap values. Future studies comprising mutants for the genes identified here in model-species and in Lettuce will enable a better understanding of their function, as well as, a better comprehension of the lettuce flowering process as whole.

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The authors declare that they have no conflict of interest.

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