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Flower induction and development in saffron: Timing and hormone signalling pathways

Begoña Renau-Morata [a], Sergio G. Nebauer [a], Víctor García-Carpintero [b], Joaquín Cañizares [b], Eugenio Gómez Minguet [a], Marcelino de los Mozos [c], Rosa V. Molina [a,*]

[a] Departamento de Producción Vegetal, Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain
[b] Instituto Universitario de Conservación y Mejora de la Agrodiversidad Valenciana, Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain
[c] Departamento de Gestión y Conservación de Recursos Fitogenéticos, Centro de Investigación Agroforestal de Albaladejito, Cuenca, Spain

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ABSTRACT

The demand for saffron is expected to rise in the coming years due to its nutraceutical and medicinal properties. To cope with this, it will be necessary to develop a mechanised production of saffron. Upgrading the production methods requires accurate control of the flowering time in this species. Nevertheless, little is known about the control of flowering time in *Crocus sativus* L. The aim of this study is to gain insight into the floral induction regulatory networks operating in this species. A transcriptomic analysis was performed from saffron main buds in different stages of development. The identification of putative integrators of flowering time signals, like *FT*, as well as meristem identity genes, such as *LFY* and *TFL1*, permitted the definition of the time of flowering induction of the buds, being able to use them as molecular markers. The identification of the transcripts encoded by a *DROOPING LEAF-like (DL)* gene is of particular relevance because this gene might be a novel factor for carpel specification in saffron.

To elucidate the hormonal signalling networks working during flower induction, transcriptomic data were used, and the content of IAA, ABA and gibberellins was determined in competent and non-competent buds to flower, during the saffron life cycle. Our results suggested that ABA might be negatively regulating corm dormancy release, but its involvement in flower induction cannot be ruled out. *ABI5* and the mediator of ABA regulated dormancy gene *MARD1,* could be key players of this pathway. In addition, a drop in GA_4 levels may also be a necessary, but insufficient, condition for floral induction and development. *DELLA*, *TFL1* and *PIF3* genes might be involved in the gibberellin pathway. Notably, IAA seems to be a positive regulator of the process, involving *MP/ARF5* and *ANT* genes in the pathway. Taken together, these results pave the way to the unveiling of the regulatory networks controlling the vegetative-to-reproductive phase change in saffron.

1. Introduction

In the last few years, increasing interest in food safety has led to a preference for natural colorants and additives instead of synthetic chemicals. It has also led to increased attention being directed towards nutraceutical foods. In this context, saffron obtained from the stigmas of *Crocus sativus* L., is of particular importance, which is enhanced by their potential biomedical applications (Abdullaev and Espinosa-Aguirre, 2004; Schmidt et al., 2007; Bathaie and Mousavi, 2010; Howes and Perry, 2011; Gohari et al., 2013).

However, the widespread use of this spice is hampered by its high price. Saffron is the world's highest-priced spice because the technology for saffron production has not changed since ancient times, and intensive labour is required for flower picking and stigma separation. An alternative is to grow the plants in containers in order to facilitate the mechanisation of flower harvesting and stigma separation (Molina et al., 2004). To achieve this goal, the flowering season must be extended as much as possible for the purposes of maximising the use of harvesting systems and, thus, reduce overall installation and running costs (Molina et al., 2005a).

Within the life cycle of saffron, flowering occurs during autumn (October-November), and it is followed by a vegetative stage throughout winter and the formation of the replacement corms at the base of the shoots. At the beginning of the dry season (April-May), the leaves

* Corresponding author.
E-mail address: rvmolina@bvg.upv.es (R.V. Molina).

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senesce and wither, and the corms go into dormancy. The transition from vegetative to reproductive stage might occur shortly afterwards in the apex of the buds of underground corms (Molina et al., 2005a).

The saffron flowering physiology has been partially characterised and there is extensive knowledge of the influence of temperature on the flower induction (Molina et al., 2004, 2005a, 2005b). Saffron crocus corms have no cold requirement to break dormancy or to complete flower formation, as is often found in geophytes (Dole, 2003; Rees, 1992). According to Molina et al. (2005b), the best temperature range for flower induction in *C. sativus* is from 23 to 27 °C, taking place from the end of spring to mid-summer after the senescence and death of the above-ground organs (Milyaeva and Azizbekova, 1978; Koul and Farooq, 1984; Molina et al., 2005b). However, there is no accurate knowledge of the time at which flower induction takes place in saffron. Unraveling genetic markers for the flower induction process in saffron could be of great interest.

Similar to Tulipa (De Hertogh et al., 1983), Freesia (Imanishi, 1993), Hyacinthus, Iris, Muscari (Le Nard and De Hertogh, 1993), Narcissus (Li et al., 2015) and most geophytes, saffron is thermo-periodic. After flower initiation in summer, a drop in temperatures (until reaching values of between 10–17 °C) is required to promote flower bud development and scape elongation (Plessner et al., 1989; Molina et al., 2005b).

The research into saffron carried out so far has allowed a comprehensive understanding of the influence of temperature on flower induction, but there are few studies into other factors influencing flowering in saffron, such as sugars or hormones (Azizbekova et al., 1978; Farooq and Koul, 1983; Jirage et al., 1994; Bagri et al., 2017) and nor are the molecular mechanisms controlling flowering induction processes well known. Based on transcriptomic profiles, Qian et al. (2019) proposed the involment of the photoperiodic and vernalization pathways in saffron floral induction. However, taking into account that the analysed corms were in a leafless state both during the floral induction period and two months before, the physiological meaning of this statement is not so straightforward. In addition, the corms that developed flowers were maintained at 20−25 °C in this study, so the involvement of a vernalization pathway on these materials is difficult to explain. Hu et al. (2020) suggests that sugar signalling may participate in flower induction.

Plant hormones constitute a major signalling network that relay external or internal variations and contribute to the extraordinary plasticity of the flowering process (Conti, 2017). Along with other signalling pathways (photoperiod, vernalization, ambient temperature, autonomous and age), phytohormone signalling pathways converge to regulate a small number of floral integrator genes like *FLOWERING LOCUS T (FT)* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANTS 1 (SOC1)*, which activate the flower meristem identity genes *LEAFY (LFY), APETALA 1 (AP1)* and *FRUITFULL* (Amasino, 2010; Fornara et al., 2010; Andres and Coupland, 2012). Most of the studies into the hormonal control of flowering have been performed on model plant species, especially on *Arabidopsis thaliana*, and GA is probably the most widely- studied hormone in flowering. However, the involvement of other hormones, including auxins (IAA), abscisic acid (ABA), jasmonate (JA), salicylic acid (SA), brassinosteroids (BRs), cytokinin (CKs) ethylene (ET) and nitric oxide (NO), has also been considered (Davis, 2009; Kazan and Lyons, 2016; Park et al., 2016; Yamaguchi et al., 2013, 2016; Conti, 2017).

In recent years, molecular studies have defined some hormone signalling pathways leading to the transcriptional activation of a small number of floral integrator genes. The role of GAs in the promotion of flower induction is probably the most commonly studied (see Mutasa-Gottgens and Hedden, 2009; Conti, 2017 for a review). Changes in GA level in the shoot apical meristem (SAM) have an effect on GA-signalling DELLA protein accumulation which orchestrates different pathways that collectively contribute to the switch to flowering, as reviewed by Conti (2017). GAs, through a DELLA-dependent mechanism, activate the expression of microRNA159 (miR159), which targets MYB33, a direct activator of the floral meristem identity gene *LFY* (Achard et al., 2004; Blazquez et al., 1998; Blazquez and Weigel, 2000; Gocal et al., 2001). *LFY* can also be activated by GAs, through an alternative pathway, by SOC1 (Achard et al., 2004; Moon et al., 2003; Lee et al., 2000; Lee and Lee, 2010). In addition, the shoot meristem component SPL15, an Arabidopsis transcription factor, coordinates floral initiation in non-inductive environments by integrating cues derived from plant age and the phytohormone gibberellin (Hyun et al., 2016). SPL15 is the key target of DELLA and the role of GAs is to remove the DELLA-imposed block on the SPL transcription factors. However, GAs inhibit flowering in many perennials, and in other species they have little influence on flower initiation (Mutasa-Gottgens and Hedden, 2009). A model for the GA inhibition of flowering in different perennial species, like rose or apple, mediated by the induction of the *TFL1* homolog, has been proposed (Randoux et al., 2012; Elsysy and Hirst, 2019). Interestingly, a recent study into grapevine showed a suppressed expression of *VvTFL1*, an antagonistic flowering gene, in the shoot of GA-insensitive mutants (Arro et al., 2019).

ABA is another important phytormone, which has typically been antagonistic towards gibberellins in many different processes, such as seed dormancy and germination (Cutler et al., 2010; Shu et al., 2016a, 2018, Nonogaki, 2017). However, the contribution of ABA to floral transition is controversial. Both negative and positive effects have been reported (Riboni et al., 2013, 2016, Wang et al., 2013; Shu et al., 2016b, 2018, Conti, 2017). ABA appears as a positive regulator of flower induction under long days. ABA might facilitate *FT* upregulation by *CO*, in part through ABI3 degradation (Kurup et al., 2000; Zhang et al., 2005; Riboni et al., 2016). By contrast, it has been proved that the transcription factors, ABI4 and ABI5, negatively mediate flowering by promoting the transcription of the *FLC* gene, a key repressor of flowering initiation (Wang et al., 2013; Shu et al., 2016b, 2018).

Recently, auxins have also been implicated in the upregulation of the floral meristem identity transcription factor *LFY*. The AUXIN RESPONSE FACTOR5/ MONOPTEROS (ARF5/MP) directly induces *LFY* expression upon auxin sensing (Yamaguchi et al., 2013). In addition, two transcription factors belonging to the AINTEGUMENTA-LIKE/PLETHORA family, ANT and AIL6/PLT3, act in parallel with MP to upregulate *LFY* in response to auxin (Yamaguchi et al., 2016).

As regards the influence of the hormones on saffron flowering, studies carried out in the 70 s and 80 s showed both an effect of the application of GAs on flower formation (Azizbekova et al., 1978), as well as changes in their activity levels during the annual cycle (Farooq and Koul, 1983). However, there was no analysis of either the identification or quantification of the GAs present in the plant's organs during its life cycle or of its relationship with floral transition. The transcriptomic analysis carried out by Qian et al. (2019) identified changes in gene expression between buds that differed in their ability to flower. Some of them are involved in the gibberellins' synthesis pathway, like putative gibberellin 3-betadioxygenase1-like (*CsG3OX*), GAST-type proteins (*CsGASA6*) and gibberellin-regulated protein14 (*CsGASAE*). However, their physiological role in flower induction is not clear. KEGG enrichment analyses of DEGs between flowering and non-flowering saffron crocuses also indicate that DEGs could be assigned to plant hormone signal transduction pathways including, for example, auxin-responsive protein IAA10-like or abscisic acid receptor PYL8-like. Although a possible involvement of these hormones in the saffron floral induction process could be hypothesized, changes in endogenous levels during vegetative development and floral transition period have not been measured. Likewise, phytohormone signalling pathways related to the regulatory network controlling flowering during the vegetative-to-reproductive phase change has not been clearly stated.

GA_3 content in saffron buds during flower development were measured by Hu et a. (2020) and they conclude that GA_S other than GA_3 might mediate the saffron flower transition. However, taking into account auxin levels and DEGs in auxin related genes, they suggested the

involvement of auxins in flowering. However, regulatory pathways are not proposed.

The aim of this study is to gain knowledge about floral induction regulatory networks operating in saffron by answering the following questions: *1) Is it possible to determine in an accurate way the flower induction period by identifying molecular markers related to this process? 2) Could phytohormones be mediating flower induction in saffron? 3) Which working models dealing with the effect of hormones on saffron flower induction could be suggested?* In relation to the development of the floral primordia, we also ask *4) Are there new players?*

In order to identify genetic markers related to the floral transition process, a transcriptomic analysis of saffron buds was performed at different developmental stages. To unveil what hormones could be mediating flower induction in saffron, we measured the changes in the level of different hormones during the saffron life cycle, and most especially during the time around floral induction, in the main bud, as well as in axillary buds (without the ability to flower). Using the transcriptomic results, we additionally proposed working models dealing with the role of hormones in saffron flower induction.

2. Material and methods

2.1. Plant material and growth conditions

The saffron corms used in this study were grown in the experimental fields of an agricultural cooperative in eastern Spain (ANECOOP, Valencia). Corms were planted in furrows, at a depth of 15 cm, with 15 cm between plants and 50 cm between furrows. The cultivation practices employed were those commonly used for this crop. Rainfield conditions met the water requirements at the start of growth (October to November), and plants were drip irrigated from December to April.

2.2. Transcriptomic analysis

2.2.1. Bud sampling

Buds were sampled at different time points after leaf senescence: from May, when vegetative meristems enter dormancy, until September, when floral whorls are being developed (Supplemental Fig. S1 and Fig. 1). During this period the corms were kept under two different conditions in which floral induction can take place. While one subset of corms was maintained in the field [F], the other was lifted in mid-May and stored in chambers [C] under controlled conditions (in the dark, at 25 °C and at a relative humidity of 80 %). The latter conditions resemble those that would be followed in the mechanised production of saffron (Molina et al., 2005b).

Corms from both field and chambers were sampled on 5 different dates, corresponding to different developmental stages (Fig. 1): S1) 24–31 May; S2) 16–21 June; S3) 11–18 July; S4) 26 July-1 August; S5) 8–11 September. For each date and each environmental condition, the main buds from 70 to 80 corms were dissected with a scalpel and pooled together to form one biological replicate. Each pool of buds was homogenized by using liquid nitrogen, mortar, and pestle and stored at −80 °C until use. Two replicates were used for each date. Any similarity in the expression patterns of both induction conditions would suggest the involvement of those genes in the physiological processes that are taking place.

2.2.2. Total RNA extraction, library construction and sequencing

Total RNA was extracted from the buds as described by Landolino et al. (2004). Genomic DNA was removed using Turbo DNA-Free Kit (Invitrogen) following the manufacturer's protocol. The RNA quality, mRNA library preparation and the sequencing were performed by Centre nacional d'anàlisi genòmica (CNAG-CRG, Barcelona, Spain). An Illumina HiSeq 4000 sequencer was used to generate inward paired-end reads of 100 bp. The reads are deposited on the NCBI in the bioproject PRJNA 638232.

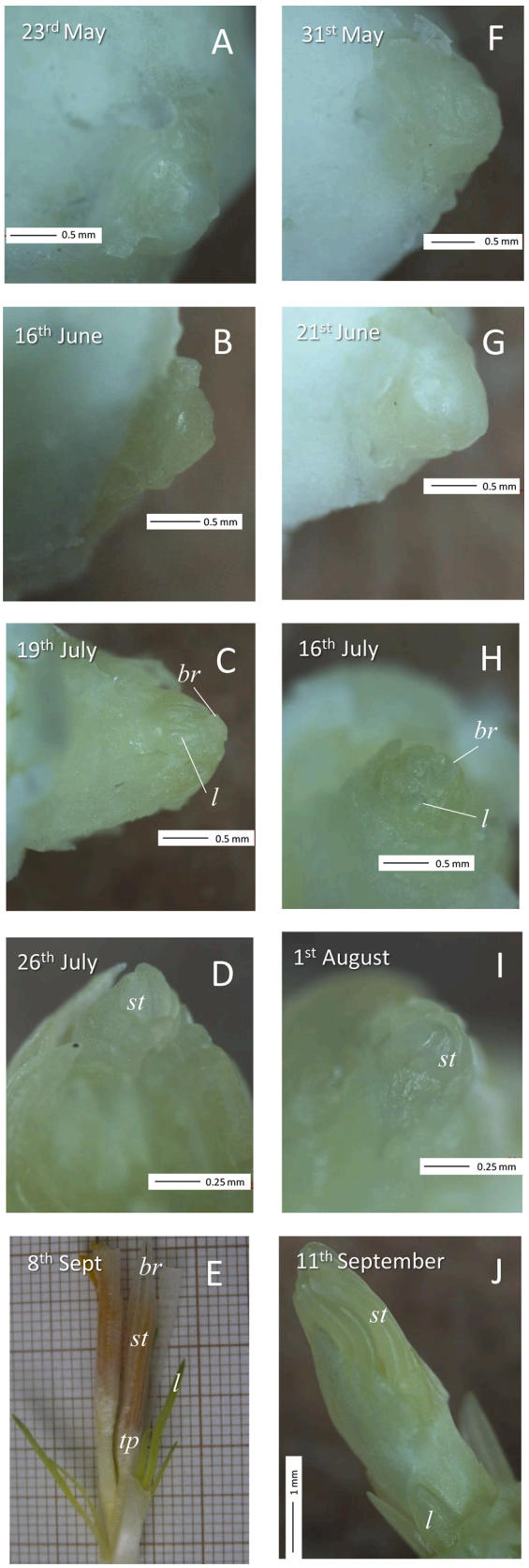

Fig. 1. Morphological changes in the main bud of saffron corms during development. Representative images of buds sampled, from the end of May until early September, in corms coming from the field (A-E), as well as stored at 25 °C (F-J) were shown. *br*, bracts; *l*, leaves; *st*, stamens; *tp*, tepals.

2.2.3. Transcriptome assembly and annotation

Adaptors and bases with a Phred33 quality below 25 were trimmed from reads using Trimmomatic (Bolger et al., 2014), keeping reads whose length was 50 bp or greater. These reads were used to perform a transcriptome assembly with Trinity (Grabherr et al., 2011). This assembly was further processed, removing transcripts of low complexity calculated as DUST score or a length inferior to 500bp with in-house software. Transcripts were organized into gene clusters by transitional blast analysis made with an in-house script. An expression quantification for the gene clusters was made with Salmon (Patro et al., 2017), removing clusters with a Transcripts Per Kilobase Million (TPM) of less than 1. The assembly's final version was created selecting the most expressed transcript for each of the remaining clusters as the representative one. For each of the representative transcripts, a prediction of CDS and proteins was made with Transdecoder (Brian and Papanicolaou, n. d.). These sequences were used to annotate transcripts using Trinnotate pipeline (Bryant et al., 2017). A BUSCO analysis was performed against the lineage dataset embryophyta_odb9 (Creation date: 2016-11-01, number of species: 30, number of BUSCOs: 1440) (Seppey et al., 2019).

2.2.4. Differential expression analysis

An expression quantification analysis was run with RSEM (Li and Dewey, 2011) using Bowtie2 (Langmead and Salzberg, 2012) as read aligner. A Fragments Per Kilobase Million (FPKM) table from the data produced in this analysis was used to perform a principal components analysis (PCA) in order to assess whether experimental groups could be differentiated by their expression profiles using MeV(http://mev.tm4.org). Expected counts were used to run differential expression analyses with EdgeR (Robinson et al., 2010) at gene level with a False Discovery Rate (FDR) < 0.05 and a fold change of absolute value > 2.

2.2.5. Clustering analysis and GO enrichment analysis

In order to elucidate the patterns of expression in differential expressed genes for each experimental comparison these genes were organized into clusters by their expression profiles in FPKM units using MeV (http://mev.tm4.org) with Pearson correlation as metric. The GO enrichment analysis was run with Blast2GO (Gotz et al., 2008) and OmicsBox (https://www.biobam.com/omicsbox/).

2.2.6. Identification of Potential flower induction and flower development regulators (Blast)

Protein sequences of *CsatFT3*, *CsatCEN/TFL1*, *CsatAP3/DEF*, *CsatAG1*, *CsatSEP3 CsSVP*, *and AtPIF3* were used for BLASTP (cutoff of 1e-05) to identify the similarity of the saffron transcripts with these previously isolated genes.

2.3. Real-Time qPCR analysis

Total RNAs were isolated as described above and 1 μg of total RNA was employed for first-strand cDNA synthesis using the PrimeScript RT reagent kit (Takara, Kyoto, Japan). Real-time qPCR was performed using SYBR Premix EX Taq II Kit (Takara) on a CFX Connect real time PCR detection system (Bio-Rad). The total reaction volume was 20 μl, and the primers used are listed in Supplementary Table 1. Three independent biological and technical replicates of each sample were used for RT-PCR analysis. The relative expression levels of the analysed target genes were normalized to that of the reference *tubulin* (Wafai et al., 2015) and calculated using the $2-\Delta\Delta CT$ method (Livak and Schmittgen, 2001). Statistical analyses were performed using the Statgraphics Centurion XVII software.

2.4. Microscopy

Morphological changes of the main bud (meristem region) from the end of May until the beginning of September were monitored with an Olympus Stereomicroscope System SZ61 and photographs were taken with a Nikon DS-Fi2digital camera.

2.5. Hormone determination

The hormone contents were determined in the main buds of the corms growing under field conditions on 8 different dates throughout the life cycle of the saffron, including the vegetative and reproductive developmental stages. The identification of the different samples is related to those carried out for the transcriptomic analysis: S0) 24 April; S1) 29 May; S2) 21 June; S3) 19 July; S6) 17 Sept; S7) 3 October; S8) 6 November; S9) 30 January (Supplemental Fig. S1). Hormone levels were also measured in non- competent to flower axillary buds, but only during the period from leaf senescence until flower induction: S0) 24 April; S1) 29 May; S2) 21 June; S3)19 July. For each date, buds from 70 to 80 corms were dissected with a scalpel and pooled together to form one biological replicate. Three replicates per date and type of bud were used. IAA, ABA, GA_1 and GA_4 gibberellins levels were quantified by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) using a Q-Exactive spectrometer (Orbitrap detector; ThermoFisher Scientific) by the Plant Hormone Quantification Service, IBMCP, Valencia, Spain.

3. Results

3.1. Shoot growth and flower initiation

The transition to flowering in saffron starts after leaf withering and coincides with the rise of temperatures in summer. In this study, the drying of the leaf occurred at the beginning of the dry season, from the end of April to the beginning of May. The time-course of flower development both during summer and before flowering occurs (October-November) is presented in Fig. 1. The morphological changes in the main bud during the studied period (throughout 5 dates from the end of May until mid-September) are in accordance with what has previously been described (Molina et al., 2005a). No growth was detectable in the buds during the first 40 days after leaf withering, either in the underground corms under field conditions [F], or in the lifted corms incubated under controlled conditions (25 °C) [C]. Around 16–19 July, the meristem was covered by developing leaf primordia, and bracts were initiated at the edge of the meristem (Fig. 1). The formation of the stamens, preceding the initiation of the perianth and the formation of the gynoecium, took place between the end of July and the beginning of August, both in the corms coming from the field and those stored at a constant temperature. All the flower organs were already differentiated by early September (Fig. 1). However, at this point, the corms coming from the field showed a more advanced development of flower primordia, with the developing stamens, style, and stigma showing the typical reddish and yellow colors.

3.2. Transcriptome assembly and annotation

In order to determine the changes in gene expression related to the floral transition process, a transcriptomic analysis was performed in the buds of corms on different dates (stages S1 to S5), including flowering initiation and differentiation. Both the corms sampled in the field and those in the growth chamber were used in the study. 248 million clean reads from pair ends were assembled with Trinity and, after filtering and gene clustering, a total of 56,824 clusters and 93,448 transcripts were obtained. To simplify the gene expression analysis, the more expressed transcripts by cluster were selected, so the number of transcripts was reduced to 56,824. The average length of transcripts is 1042 pb and the size distribution is shown in the supplemental data (Supplementary Fig. S2). The transcriptome was annotated with Trinnotate and, finally, 26,987 transcripts were associated with a GO term. As expected, most of the annotated GO are at levels 5 and 6 (Supplementary Fig. S3). To evaluate the quality of the transcriptome, a BUSCO analysis was

performed at embryophyta taxon level dataset. From the 1440 genes included in the dataset, 1071 were detected as completed, 121 fragmented and 248 were not detected; therefore, 82 % of simple copy orthologous genes are included in the transcriptome.

3.3. Differential analysis expression

The reads from the 5 dates of the two independent experiments were mapped against the reference transcriptome and their expression/expressions quantified. In spite of the differences between chamber storage and field conditions (Supplementary Fig. S4), the PCA analysis of samples indicates that the highest percentage of variability is related to the time course. Taking into account both these results and the morphological differences during the later stages, both experiments, field and chamber, were analysed independently.

We have identified transcripts with differential expression between all the combinations of samples from the same experiment. To reassure DEGs linked to time course effects, we have selected the transcripts that have been identified as differentially expressed in both experiments independently. We have selected 6427 transcripts whose expression changes by a factor of at least two in both experiments (Supplementary Table S2). We have performed a cluster analysis of the expression of these transcripts by using the expression data of both experiments together (Fig. 2). Six different clusters have been detected: three clusters with a clear inhibition pattern (Clusters 1, 3, 5) and another three with an activation one (2, 4, 6). Clusters 3 and 5 show lightweight differences in the pattern of both experiments. It is necessary to emphasize the coherence of the results between the experiment carried out in the chamber and that performed in the field. Correlation analysis of those selected DEGs also shows a clear time course linkage between experiments (Supplementary Fig. S5)

A GO enrichment analysis was conducted on the complete list of differentiate transcripts and in the clusters. The different expression patterns detected in the cluster analyses are related with different GO terms (Supplementary Table S3).

In order to establish the floral induction period and to identify marker genes of that process, we focus specifically on clusters containing genes that could be involved in flower development and related processes. The gene annotation and the GO terms overrepresented in these selected clusters are shown in Table 1. There are interesting GO terms, such as GO related with cell division, flower morphogenesis, the identity of flower organs, sexual reproduction, hormone pathways, the regulation of meristem growth, or those related with the dormancy process, showing that this collection of differentially expressed genes is enriched on those implicated in the flower induction and development.

Cluster 1, with a clear downregulation pattern, has “DNA-binding transcription factor activity” as the enriched GO term, with 63 different transcripts. Of these, it is noteworthy to highlight transcripts with a marked similarity to floral repressors, like *TFL1*, related to dormancy (*MARD1*), but there are also others involved in ABA biosynthesis (*NCED*), ABA signalling pathway (*ABI5*) or gibberellin-regulated proteins (*GASA*). Cluster 2, with an activation pattern, contains terms such

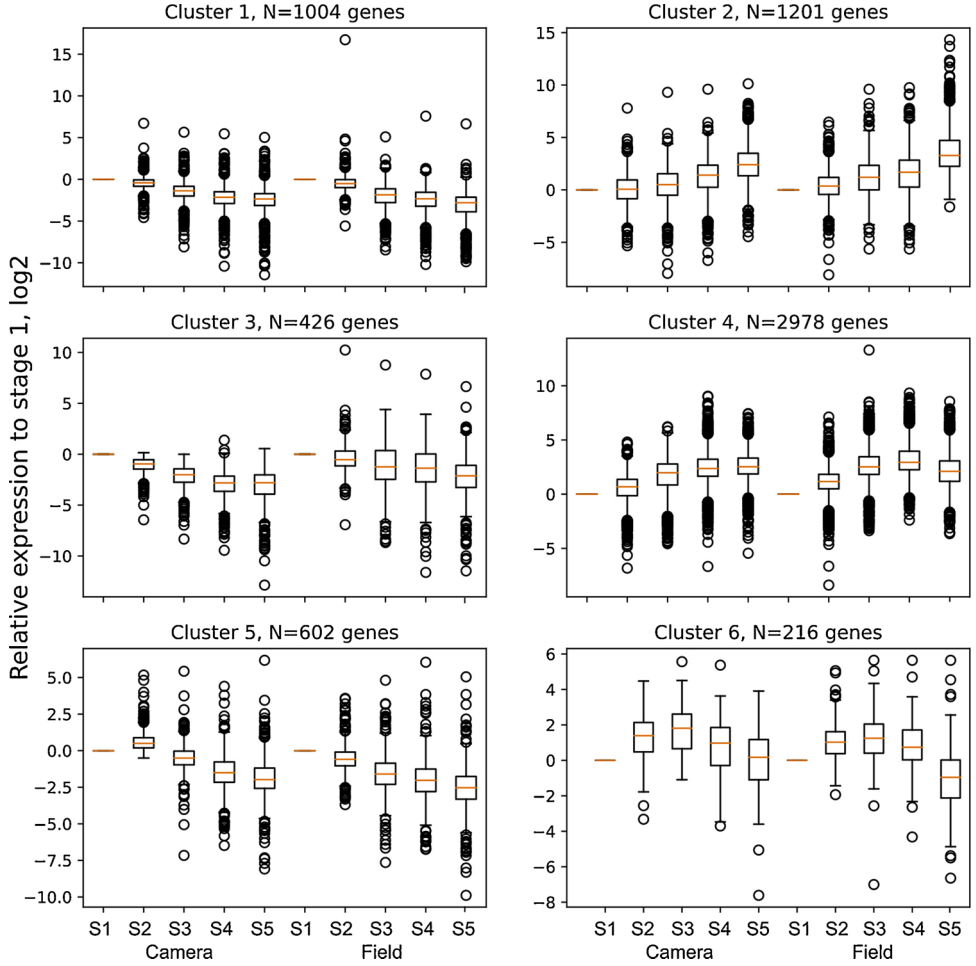

Fig. 2. Clustering of differentially expressed genes (DEGs) profiles. DEGs for each comparison have been grouped in one FPKM expression table and then separated into clusters by their expression profiles using Pearson correlation. Expression data were normalized for each DEG by dividing its value by that of stage 1at the same storage condition (chamber or field) and then transformed into log2.

Table 1
Gene annotation and GO terms overrepresented in clusters selected by including transcripts showing a high similarity with known transcription factors related to flowering process. The GO enrichment analysis was run with Blast2GO.

	GO ID	GO Name	P-value
CLUSTER 1	GO:0006351	Transcription, DNA-templated	9.84E-11
	GO:0003700	DNA-binding transcription factor activity	3.46E-12
	TFL1, WUS, Ghd7, MARD1, NCED, ABI5, KIN10, GAS14, PIF3, REM4		
CLUSTER 2		Sporopollenin biosynthetic process	
	GO:0080110	cell wall organization	1.26E-10
	GO:0071555	amino acid transmembrane transport	1.36E-08
	GO:0003333	specification of stamen identity	4.70E-07
	GO:0010097	flower morphogenesis	1.82E-06
	GO:0048439	hexose transmembrane transport	6.24E-05
	GO:0008645	lignin biosynthetic process	8.40E-05
	GO:0009809	cuticle pattern formation	1.24E-04
	GO:0035017	response to auxin	1.34E-04
	GO:0009733	flavonoid biosynthetic process	1.31E-04
	GO:0009813	fatty acid biosynthetic process	1.79E-04
	GO:0006633	cellular polysaccharide catabolic process	3.63E-04
	GO:0044247		4.43E-04
	GO:0009505	plant-type cell wall	2.40E-04
	GO:0000977	RNA polymerase II transcription regulatory region sequence-specific DNA binding	3.21E-07
	GO:0010328	auxin influx transmembrane transporter activity	1.87E-04
	AP3, AG1, SEP3, MADS2, MADS16, DL, PUB4, FTM2, GPAT6, JMJ706, AUX1, PIN1, SAURs		
CLUSTER 4		microtubule-based movement	
		DNA replication checkpoint	
		xyloglucan metabolic process	
	GO:0007018	DNA replication initiation	5.60E-16
	GO:0000076	mannan catabolic process	1.27E-10
	GO:0010411	cellulose catabolic process	1.37E-07
	GO:0006270	determination of dorsal/ventral asymmetry	1.94E-07
	GO:0046355		4.44E-06
	GO:0030245	regulation of mitotic spindle organization	6.18E-06
	GO:0048262		1.61E-05
	GO:0060236	regulation of meristem growth	1.87E-05
	GO:0010075	guard mother cell differentiation	6.04E-05
	GO:0010444	positive regulation of meiotic cell cycle	7.28E-05
	GO:0051446		7.28E-05
	GO:0010223	secondary shoot formation	1.43E-04
	GO:0000911	cytokinesis by cell plate formation	4.20E-04
	GO:0042547	cell wall modification involved in multidimensional cell growth	4.73E-04
	GO:0010068	protoderm histogenesis	5.23E-04
	GO:0009558	embryo sac cellularization	5.40E-04
	GO:0010497	plasmodesmata-mediated intercellular transport	5.40E-04
	GO:0009664	plant-type cell wall organization	5.82E-04
	GO:0048653	anther development	6.50E-04
	GO:0019953	sexual reproduction	6.78E-04
	GO:0003700	DNA-binding transcription factor activity	1.82E-08
	GO:0005199	structural constituent of cell wall	4.46E-07
	GO:0015250	water channel activity	8.39E-07
	GO:0016157	sucrose synthase activity	3.20E-04
	FT3, LFY, FTIP1, RBG1, TAR2, PIN1, VAN3, SAURs, PP2C		

as the specification of stamen identity, morphogenesis and response to auxins. Seventeen transcripts are related to floral organ development, including well known *MADS-BOX* genes controlling flower development, like *AP3*, *AG1* or *SEP3*. Furthermore, some mRNAs related to auxin responsive proteins (*SAURs*) and auxin transport (*AUX1*) have been identified. The expression of genes included in cluster 4 increased steadily from the 1st sampling (end of May) onward, until it reached a plateau of maximum expression around the 4th sampling (end of July). This cluster shows terms related with cell division, meristem growth and the initiation of flower development, such as DNA replication initiation, plasmodesmata-mediated intercellular transport, the regulation of meristem growth, the positive regulation of meiotic cell cycle, sexual reproduction, embryo sac cellularization, and anther development. This interesting cluster contains transcripts similar to *FT* (a floral integrator), *LFY* (an identity floral gene) and *FTIP1* (required for FT protein transport). All of them are involved in the vegetative to reproductive phase transition of the meristem (Andres and Coupland, 2012). Other interesting mRNAs are those encoding TAR2, involved in the pathway for IAA biosynthesis, VAN3, which regulates auxin transport-mediated plant morphogenesis (Naramoto and Kyozuka, 2018), or auxin responsive genes, such as *SAUR*s.

Clusters 3, 5 and 6 showed little relationship with the flowering process. Nevertheless, some overrepresented GO terms can be highlighted (Supplemental Table S3). Cluster 3, with differences in the patterns of both experiments, includes GO terms related with responses to environmental factors. Cluster 5, also showing a gene expression which decreases over time, has the abscisic acid biosynthetic process as the enriched GO term. Finally, cluster 6 has meiotic nuclear division as the significant term, and two genes annotated to this cluster are *XRI1*, essential for male and female meiosis (Dean et al., 2009), and *MCM8,* required for a pathway of meiotic double-strand break repair (Crismani et al., 2013). Overall, this analysis shows that floral induction and flower development take place in the time space of both analyses, the changes between 1–2 and 2–3 being the key steps of the flower induction process, as shown by the expression pattern (clusters 1 and 4) of the majority of genes related to this process.

3.4. Determination of the flower induction period and identification of putative genetic markers for the process

The analysis of the transcriptome revealed significant changes in the genes related to floral transition, starting at stage 2 (second half of June) but intensified at stage 3 (mid-July). In order to select genes to be used as genetic markers of the process, several genes potentially involved in the flower induction and development of saffron were highlighted. Genes were selected based on the homology to key regulators of the flowering time control in Arabidopsis, as well as in previously isolated saffron genes.

An increase in the level of a mRNA similar to a *CsatFT3* gene (CSATG01617), isolated by Tsaftaris et al. (2013) and proposed as a possible integrator of flowering time signals, was observed (Fig. 3A). Additionally, the transcript encoding a protein similar to FT-INTERACTING PROTEIN 1 of Arabidopsis (FTIP1) (CSATG26656), required for FT transport (Liu et al., 2012), showed an important increase in its mRNA level at stages 2–3 (Fig. 3B). Furthermore, mRNA levels of the *AtLFY*-like gene (CSATG38881) also exhibited a marked increase from stage 3 under field conditions (Fig. 3C). A progressive increase in CSATG38881 was observed under chamber conditions. It is interesting to note that although in mid-July (S3) an increase in the transcripts of the *CsatFT3* and *AtLFY-like* genes was identified both in corms under field conditions as well as in corms stored in growth chambers, the maximum expression is reached earlier in the buds of corms coming from the field. These data are in accordance with the fact that the corms kept in the field develop slightly earlier during the final stage.

In addition, a drop in the mRNA levels of two paralogous *TFL1 genes*, was also observed (Fig. 3D-E). One of them (CSAT51482) was isolated by Tsaftaris et al. (2012a) and named as *CsatCEN/TFL1-like* (hereinafter referred to as *CsatCEN/TFL1-like a*). The other (CSATG15553) has been identified in this work and we named it *CsatCEN/TL1-like b*. The transcripts number of the *CsatCEN/TFL1-like b* gene decreased markedly from stage 2. The expression pattern of *CsatCEN/TFL1- like a* showed a decrease of mRNA level later. It reached the highest expression when floral induction could be taking place and decreased from this time onwards. In other species, this gene represses the floral transition promoted by FT, but its function in saffron has not yet been confirmed

(Tsaftaris et al., 2012a). Also at this time, transcriptomic analysis showed a reduction in the mRNA levels of CSATG35780, a putative flowering inhibitor of the temperature-dependent pathway in saffron, which has been formerly isolated (Haghighi et al., 2020) and named as *CsSVP* (Fig. 3F). However, a significant decrease in the level of transcripts only took place in the buds of corms coming from the field.

To confirm these results, as well as the overall quality of our RNAseq assembly and DGE analysis, the mRNA levels of the flowering related genes (*CsatFT3, AtLFY and CsSVP*) were determined by qPCR on the buds coming from the field corms. The expression patterns obtained (Fig. 4A) were quite similar to those reported in the RNAseq analysis. Furthermore, expression analyses were performed in a second year in main buds from corms grown in the field. Samples were taken on dates corresponding to stages S1, S2, S3 and S5 of the first analysed year. Transcripts levels patterns of *CsatFT3*, *AtLFY*, *CsSVP*, *TFL1a* and *TFL1b* genes were related to the time course of flower induction and development (Fig. 4B). It has to be noted that flower development in the field was slightly delayed when compared to the first studied year, as by mid-September flower primordia pigmentation was not produced. Accordingly, the patterns of *CsatFT3, AtLFY, CsSVP, TFL1a, and TFL1b* expression levels in the second year resembled those of corms developed in the chambers at 25 °C in the first experiment (Fig. 4A), which showed a delayed flowering time when compared to corms developed in the field (Fig. 1).

Taken together, our results allowed the floral transition period to be determined quite precisely. In our experimental conditions, it took place between the end of June (S2) and mid-July (S3). In addition, we have identified *CsatFT3*, *AtFTIP1-like*, and *AtLFY*-like genes that could be considered as genetic markers of flower induction in saffron. *CsatCEN/TFL1-like b* gene seem to be playing a role as floral transition repressor.

3.5. Changes in hormonal levels related to floral transition

With the aim of finding out how hormonal changes are involved in the floral transition of saffron, the hormone content (GA_4, GA_1, IAA and ABA) in the main bud of the corm was measured (Fig. 5) during different developmental stages of the plant life cycle in the field (Supplemental Fig. S1). The quantification was also performed in axillary buds during the flower induction period for the purposes of knowing whether the changes in hormone levels were related exclusively to floral transition. These axillary meristems lack the ability to flower.

There are no significant changes in GA_1 content in the main buds during the period of floral transition and floral whorls development (Fig. 5A), and nor can any differences be observed between the main and axillary buds. Therefore, GA_1 might not be related to the floral induction process. However, there is a marked increase in GA_1 content when the flower emerges above the soil surface. These changes should be related to the growth of both the scape and the perianth tube. GA_4 content decreased progressively from the end of the vegetative period and during flower initiation and development (Fig. 5B). After flowering, there was an increase in the GA_4 level during the following vegetative life cycle, and it peaked when the daughter corms growth was maximum, in

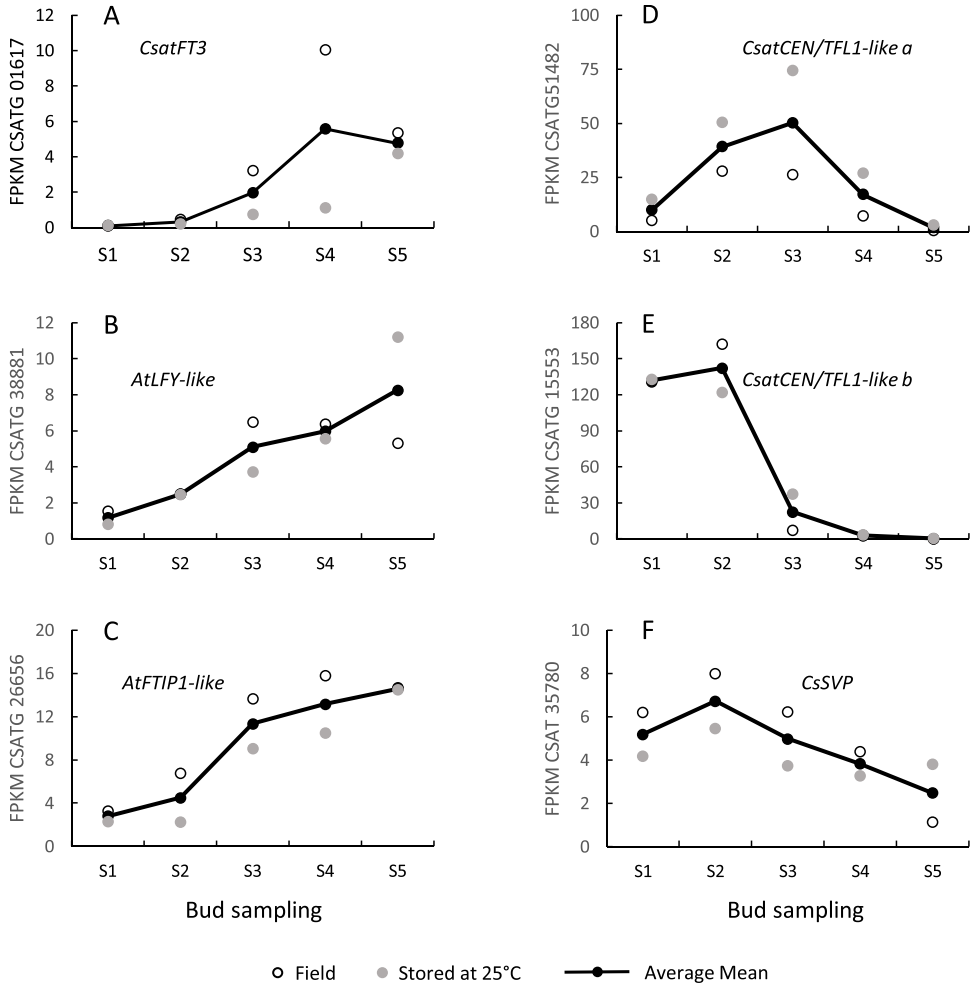

Fig. 3. Expression patterns of saffron flowering genes transcripts in the main bud of corms maintained in the field or stored at 25 °C, as well as the mean average. Transcript levels (FPKM) of (A) *CsFT3*, (B) *AtFTIP1*-like, (C) *AtLFY*-like, (D) *CsatCEN/TFL1*-like a, (E) *CsatCEN/TFL1*-like b and (F) *CsSVP* genes were shown at five different stages (S1 to S5) from the end of May until early September.

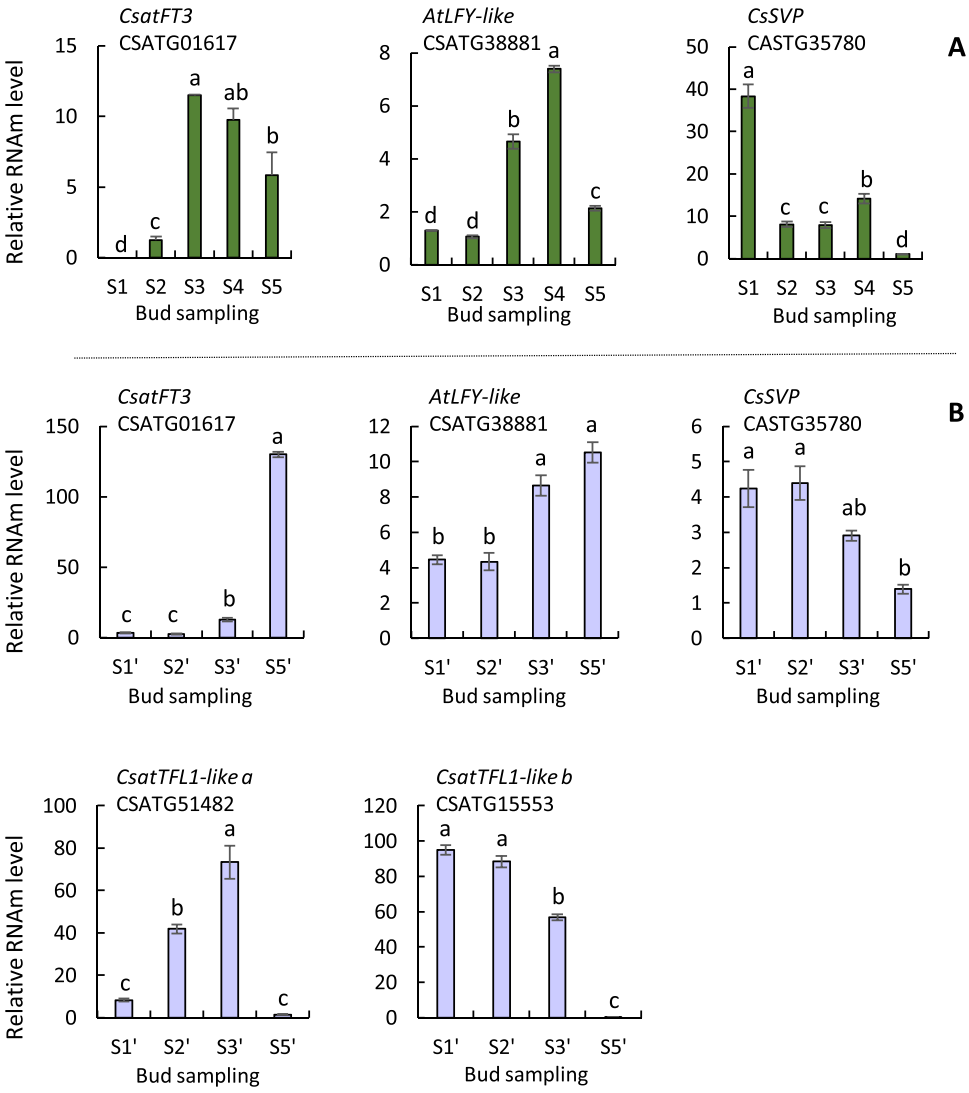

Fig. 4. Expression analysis by RT-qPCR of saffron flowering genes in the main bud of corms growing in the field in two years (2017, 2019). A) Expression levels of *CsFT3*, *AtLFY*-like and *CsSVP* genes in buds of corms grown in 2017. B) Expression levels of *CsFT3*, *AtLFY*-like, *CsSVP*, *CsatCEN/TFL1-like a* and *CsatCEN/TFL1-like b* genes in buds of corms grown in 2019. Buds were sampled from the end of May until early September (stages S1 to S5). Relative expression levels were normalized to the lowest value. Different letters indicate significant differences (LSD test; $P < 0.05$) among developmental stages for each gene. Each value is the mean of three biological replicates.

January. The axillary buds also exhibited a decrease in GA_4 level during the studied period. These results suggest that GA_4 could be related to the arrest of vegetative growth. Furthermore, an inhibitor role in the flowering process cannot be ruled out.

Interestingly, the IAA content showed a marked increase during the floral transition and the subsequent flower development (Fig. 5C). Afterwards, the IAA content declined and dropped significantly during the vegetative growth. By contrast, no changes in IAA content were observed in axillary buds during the floral transition period. These results point to the involvement of this hormone, promoting flower primordia initiation and development in saffron.

The ABA levels were highest at the end of the vegetative period of growth, after leaf senescence (Fig. 5D). From this point, the ABA content progressively decreased until September. A similar pattern of ABA content was observed in the axillary buds. These results suggest that ABA might be negatively related to corm dormancy release in both the main and axillary buds. Nevertheless, a possible inhibitory role in the floral induction process could also be considered.

3.6. Hormonal signalling pathways involved in flowering induction and initiation

The transcriptomic data were also analysed to support the previously proposed role of the different hormones in the process. We analysed the expression pattern of genes involved in hormone signalling pathways related to flowering control in other species (Yamaguchi et al., 2016; Wang et al., 2013; Arro et al., 2019).

3.6.1. Signalling pathway for the inductive effect of IAA on corm dormancy release and floral initiation

The DGEs related to the auxin mediated pathway suggested a working model (Yamaguchi et al., 2016), pointing to an inductive effect of IAA on flower initiation and breaking dormancy (Fig. 6 and Supplemental Table 2). A transcript (CSATG33935) showing a high degree of similarity with the MP/ARF5 auxin responsive transcription factor in Arabidopsis was identified. An increased mRNA level of this gene was observed in the second half of June (S2), when the auxin level rises, just before the floral induction (Figs. 5C and 6). In addition, an enhanced expression of the *AINTEGUMENTA (ANT)-like* (CSATG01257, CSATG44960, CSATG44961) genes was observed from mid-June to mid-July. According to Yamaguchi et al. (2016), both ANT and MP/ARF5 act in parallel to promote *LFY* expression in Arabidopsis. *ANT-like* could also be a MP/ARF5-like direct target. As shown in Fig. 3C, an increase in *LFY-like* gene (CSATG38881) transcripts was also observed during this period in our study, suggesting the occurrence of this model in saffron. It has to be noted that, in parallel to *MP/ARF5* and *ANT* genes, high levels of transcripts from a *YUCCA-like* gene (CSATG33444) were observed during the flowering induction period

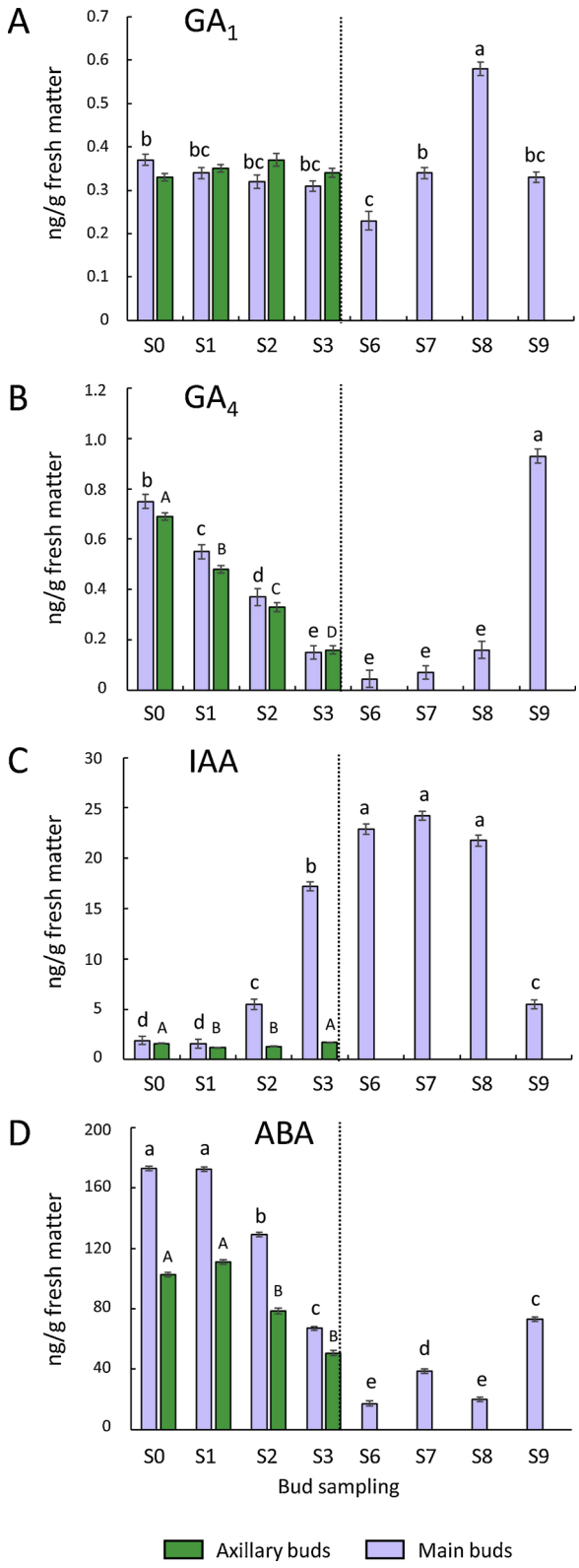

Fig. 5. Hormonal levels in the main and in axillary buds of saffron corms during their life cycle (S0-S9). The hormones contents in axillary buds were only determined during the period in which flower induction could take place. A) Gibberellin GA_1; B) Gibberellin GA_4; C) Indol acetic acid; D) Abscisic acid. Different lowercase letters indicate significant differences (LSD test; $P < 0.05$) among dates for the main bud. Different uppercase letters indicate significant differences (LSD test; $P < 0.05$) among dates for axillary buds. Each value is the mean of three replicates.

(Fig. 6). This mRNA codes for a flavin-containing monooxygenase, the rate-limiting enzyme of auxin biosynthesis. In addition, transcripts from a *TAR2*-like gene (CSATG19991), included in cluster 4 (Table 1), and also involved in the pathway for IAA biosynthesis, showed an increased level between the end of June and early July.

Additionally, we observed a progressive decrease in the mRNA levels of the *DRMH2-like* (CSATG45396) gene during the flowering induction period (Fig. 6). This bud dormancy-associated gene (*Dormancy-associated gene-1 /Auxin-repressed protein; DRM1/ARP)* is downregulated by high levels of auxins (Rae et al., 2014). This suggests a possible effect of auxins on breaking bud dormancy.

3.6.2. *Signalling pathway for the inhibitory effect of GA_4 in flower induction*

The DEGs related to GAs could be pointing towards a possible inhibitor role in saffron flower induction (Fig. 7). The significant decrease in GA_4 during the flower induction is related to the significant down-regulation of the flowering inhibitor *CsatCEN/TFL1-like b* (*CSATG15553*) (Fig. 3E). *TFL1* has been proposed as a direct target of GA-responsive transcription factors in perennial plants showing a repression of flowering by gibberellins GA_{4+7} (Elsysy and Hirst, 2019; Zhang et al., 2019). Increased mRNA levels of some genes encoding putative DELLA proteins, such as *GAI-like* (*CSATG32717; CSATG28592*) and *SLN1-like* (*CSATG28593; CSATG28594; CSATG28595; CSATG28597),* were observed from June, during the induction and initial development of the flowers. Fig. 7 shows the transcript level of *CSATG28594,* which displayed the highest mRNA level. Arro et al. (2019) revealed TFL1 as a new DELLA target in grapevines. Our results also showed high levels of *DELLA (SLN1-like)* transcripts while a reduction in the number of *CsatCEN/TFL1-like b* mRNAs is taking place (Figs. 3E and 7).

Our results also pointed towards an alternative pathway involving a PIF3 factor (Fig. 7). It has been shown that DELLA proteins inhibit phytochrome-interacting factors 3 and 4 (PIF3 and PIF4). Either the sequestration or degradation of PIF3 by DELLAs helps to weaken PIF3 binding to its target genes in Arabidopsis (Li et al., 2016). Our transcriptomic results revealed a significant drop in the transcription levels of *PIF3*-like gene (*CSATG37818*), especially pronounced from May (Fig. 7). This decrease is negatively correlated with the transcript levels of different *SLN1-like* (CSATG28593; CSATG28594; CSATG28595; CSATG28597) or *GAI-like* genes (CSATG28592). It could be hypothesized that DELLA proteins also influenced PIF3 expression. Remarkably, the antisense suppression of the PIF3 gene causes higher mRNA levels in floral activator genes, like *FT*, and leads to early flowering (Oda et al., 2004) in Arabidopsis. In saffron, the mRNA level of the floral integrator *CsatFT3* also increases when the level of *PIF3-like* transcripts decreases significantly (Figs. 3A and 7).

In addition, the transcriptomic analysis also explained the gibberellin content observed in the study. The decrease in GA_4 levels could be explained by the up-regulation of the *GA_2 oxidase-like* gene (*CSATG24054*) (Fig. 7). This enzyme catalyzes the 2-beta-hydroxylation of several biologically active gibberellins.

3.6.3. *DEGs for the components involved in ABA signalling during dormancy corm release and flower initiation*

The analysis of the DGEs pointed to an inhibitor role of ABA in corm dormancy release in saffron; however, a negative effect in flower initiation cannot be ruled out.

Transcripts showing a high degree of similarity with the three groups of components involved in the ABA signalling pathway: the receptor components, PYR/PYL/RCAR, the protein phosphatase 2C, PP2Cs, and the SNF1-related protein kinase2, SnRK2s (KIN10), were identified and showed differences in their mRNA levels during the studied period (Fig. 8 and Supplemental Table S2). The transcript levels of the *PYL-like* gene (CSATG21969) decreased from the end of June (S2), when the ABA levels also declined (Fig. 5D). In parallel, *PP2C-like* genes (CSATG07763,

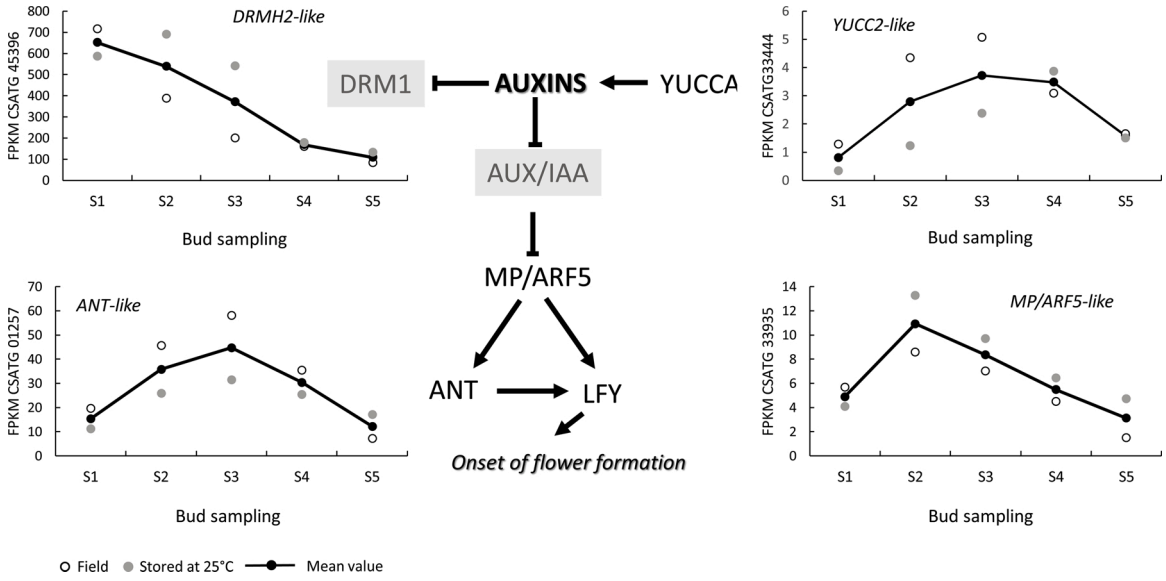

Fig. 6. Proposed model of the effect of auxins on saffron floral initiation and the expression patterns of the involved genes, from the end of May until early September (S1-S5). The auxin-activated MP/ARF5 transcription factor directly activates LFY. ANT is also a direct MP/ARF5 target and acts downstream of MP in initiation of flower primordia. Like MP/ARF5, ANT directly induce LFY upon auxin sensing (Yamaguchi et al., 2016). In addition, the decrease in the level of *DRM1-like* transcripts, downregulated by auxins, leads to corm dormancy release. The increased level of *YUCCA2-like* transcripts is related to a high level of auxins. The expressions of the involved genes have been analysed in corms coming from field or stored at 25 °C.

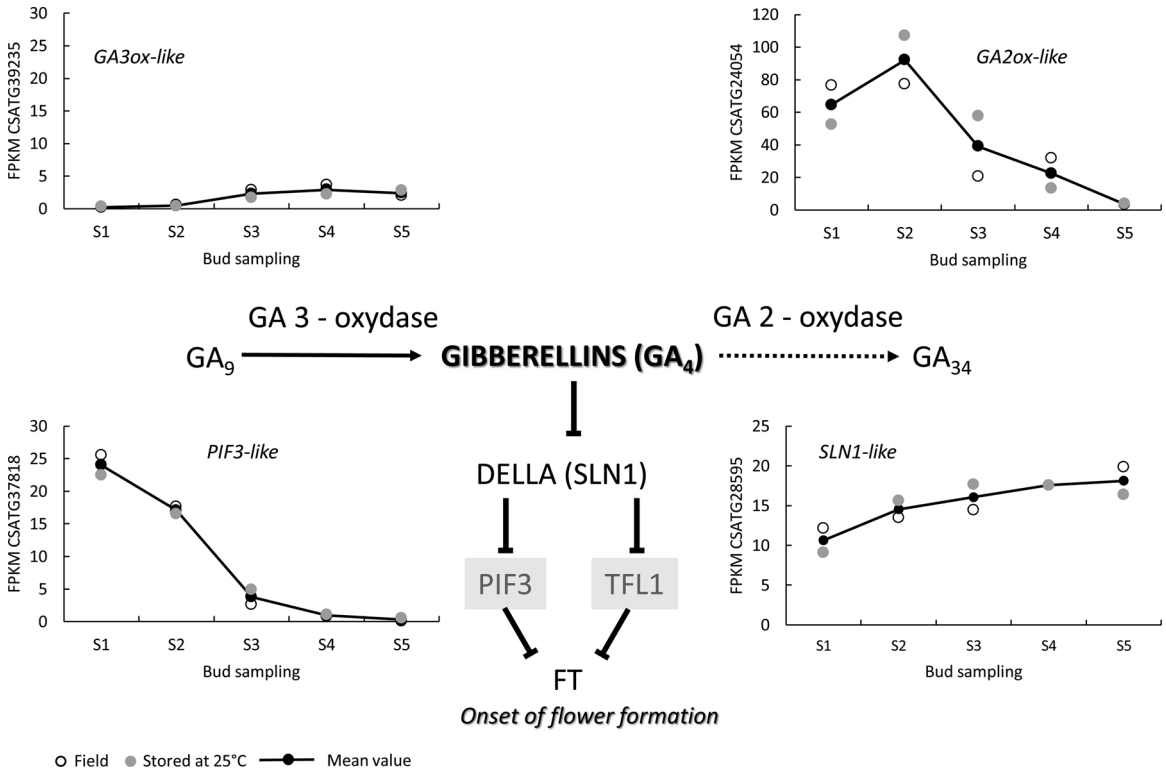

Fig. 7. Inhibition of the onset of flower formation in saffron by GA_4, following the models formulated by Oda et al. (2004) and Arro et al. (2019). An inhibitory effect of DELLA (SLN1-like) over *TFL1* in the shoot is proposed. An alternative pathway involving a drop in the expression of *PIF3* factor by DELLA, which prevents the binding of PIF3 to its target genes, including *FT*, is also hypothesized. The expression patterns of *GA3ox-like* and *GA2ox-like* are in accordance with a low GA level. The expressions of the involved genes have been analysed in corms coming from the field or stored at 25 °C from the end of May until early September (S1-S5).

CSATG07764, CSATG08180, CSATG17636, CSATG27029, CSATG32498, CSATG33114, CSATG35575, CSATG46791, CSATG46792), negative regulators of ABA, showed higher mRNA contents. Additionally, transcript levels of *KIN10*-like genes (CSATG12438, CSATG28435), which act as positive ABA regulators, decreased through the studied period. An inhibitory effect of ABA in corm dormancy release is mediated by ABSCISIC ACID INSENSITIVE 5 transcription factor (ABI5) in some ornamental geophytes (Wu et al., 2015). ABI5 is negatively regulated by PP2C. Interestingly, a significant reduction in *ABI5-like* transcripts (CSATG16773, CSATG49449) has been observed,

related to the drop of ABA levels in the saffron buds (Figs. 8. 5 D). These results point to a negative regulation of saffron corm dormancy release in saffron. In support of this assumption, we have also found transcripts of the *Mediator of ABA-Regulated Dormancy 1-like* gene (*MARD1*) (CSATG27859), which is upregulated by ABA, decreasing as time passes (Fig. 8 and Table 1).

According to the reduction in ABA content from late July onwards, we observed a decrease in the *NCED-like* transcripts, the key enzyme (*9-cis-epoxycarotenoid dioxygenase*) in ABA synthesis (CSATG06222, CSATG00963, CSATG06225, CSATG06224, CSATG06223, CSATG06226, CSATG01666, and CSATG01667). Fig. 8 displays the pattern of the CSATG06225 transcript, showing a high degree of similarity with the *NCED* from *Oncidium*.

A negative control of ABA over the floral induction in saffron cannot be dismissed. However, we did not find any key regulators of the known signalling pathways in other species. In Arabidopsis, the inhibitory effect of ABA in flowering is mediated by ABI5 through the activation of the FLC floral repressor (Wang et al., 2013). No homologous transcripts of *FLC* have been found in saffron.

3.7. DEGs related to the major B, C and E-classes of MADS-box genes

Most of the major players in the ABCDE model belonging to the MADS box gene family have been isolated in *Crocus sativus,* including two B-class genes (*CsatAP3/DEF*; *CsatPI/GLO*), one C-class gene (*CsatAG1*) and two E-class genes (*CsatSEP3*; *CsatAGL6*). In this study, an analysis of the transcripts showing a high degree of similarity with those major B, C, and E-classes of MADS-box genes has been carried out.

Interestingly, in any transcript that we have found, the amount of mRNA increased either from mid-July (Field) or at the end of July (Chamber), reaching its peak in September (S5) when there was a rapid increase in growth of the flower primordium (Fig. 9). In addition to the MADS-box transcription factors already isolated from *C. sativus*, we have identified 3 transcripts encoding genes belonging to the B-class, showing an analogous expression pattern. Two of them, CSATG03695 and CSATG39084, exhibited a high degree of similarity with the *MADS-box 2* from rice. A rice *MADS-box 16–like* transcript (CSATG18569) was also found.

Previous research, whose aim was to analyse the expression of the isolated B, C, and E-class genes in the different whorls (see Tsaftaris et al., 2010 for a review), reveals that the presence of C-class gene transcripts is restricted to the reproductive parts of the flower, stamen and carpels, and the E-class genes are expressed in all the whorls, according to the ABCDE model. The B-class genes extend their expression to whorl l, leading to the formation of tepals in this whorl instead of sepals, and supporting the modified ABC model for tulips (Kanno et al., 2003). However, these B-class genes are also expressed in whorl 4. So, some other factors are required for carpel specification.

Within this framework, it is important to highlight the identification of a mRNA encoding a *DROOPING LEAF (DL)-like* protein, a factor required for carpel specification in grasses (Nagasawa et al., 2003; Yamaguchi et al., 2004). Furthermore, the expression pattern of *DL*-like transcripts is similar to those corresponding to the other floral homeotic genes (Fig. 9).

4. Discussion

In ornamental geophytes, such as the *Crocus* genus, the physiological aspects of flowering related to the effects of environmental factors, particularly the temperature, have been well studied for major crops in order to provide diverse techniques for commercial forcing (Rees, 1992; De Hertogh and Le Nard, 1993; Kamenetsky et al., 2013). However,

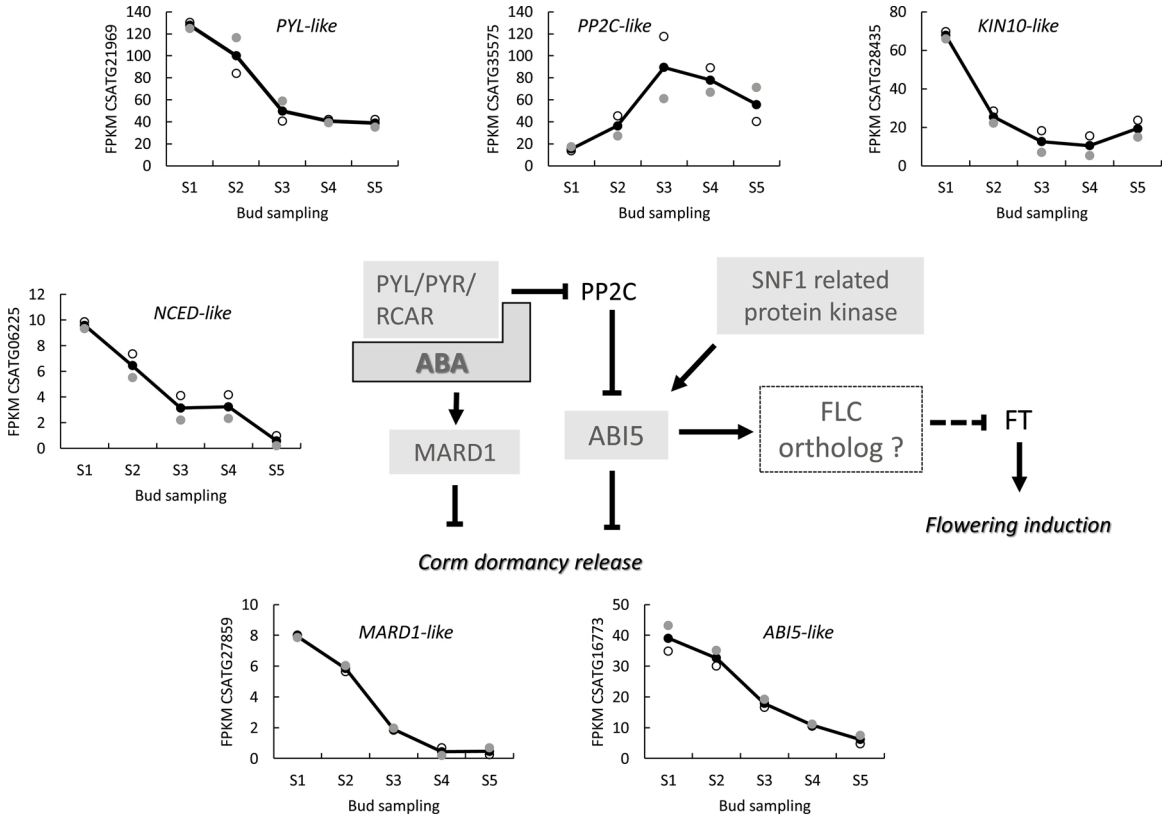

Fig. 8. DEGs of the components involved in ABA signalling pathway during the release of corm dormancy and in flower initiation. The inhibitory effect of ABA on corm dormancy is mediated by ABI5 and MADR1 transcription factors. Activation of ABI5 requires SF1 related protein kinase. ABI5 could also negatively regulate floral transition through an FLC ortholog, an inhibitor of FT. The expressions of the involved genes have been analysed in corms coming from the field or stored at 25 °C, from the end of May until early September (S1-S5).

there is still only limited information on the genetic control of meristem transition and flower development.

Although homologous of flowering genes from model plants have been isolated from geophytes, such as *Narcissus*, *Tulipa*, *Lilium* and *Crocus* (Tsaftaris et al., 2007, 2012a, 2012b, 2013; Noy-Porat et al., 2010; Li et al., 2013a, 2015; Leeggangers et al., 2018; Haghighi et al., 2020;), the molecular regulation of flower development in these plants differs from that of model plants (Kamenetsky et al., 2013). Furthermore, the physiological mechanisms and the different signalling pathways involved in the flowering process have been only partially elucidated in some species (Noy-Porat et al., 2013; Li et al., 2013a; Leeggangers et al., 2017). In saffron, molecular mechanisms regulating flower transition and development remain to be unveiled.

In this study, the analysis of transcriptional changes occurring prior to and during floral transition, has allowed for the accurate ascertainment of when flower induction takes place in saffron, as well as the identification of homologous of key conserved genes in floral initiation that can be used as molecular markers. In addition, the analysis of hormone levels in the main and axillary buds, and the relationship with the vegetative to reproductive phase change, has pointed out the possible involvement of IAA, GA_4 and ABA in saffron flower induction and development. Finally, the transcriptomic analysis was also used to support the proposed roles of the studied hormones and to provide new working models about hormone signalling pathways mediating flower induction in saffron.

Understanding flower induction and development in saffron could reveal ways to extend the harvesting period, and it would make the development of an industrial production easier. The limited information on the genetic control of floral transition available in geophytes is partially due to the large size of the main genera's genome and the lack of efficient transformation systems for these materials. Saffron genome size is greater than 10 Gb and it has polyploidy characteristics (2n = 3x = 24) (Brandizzi and Caiola, 1998). In this context, it is of great interest to gain knowledge into floral induction regulatory networks operating in saffron. In this study, we have obtained interesting information in relation to the questions initially raised to have a better understanding of saffron flowering.

4.1. Is it possible to determine, in an accurate way, the flower induction period by identifying molecular markers related to this process?

The fact that saffron flower induction took place between the end of

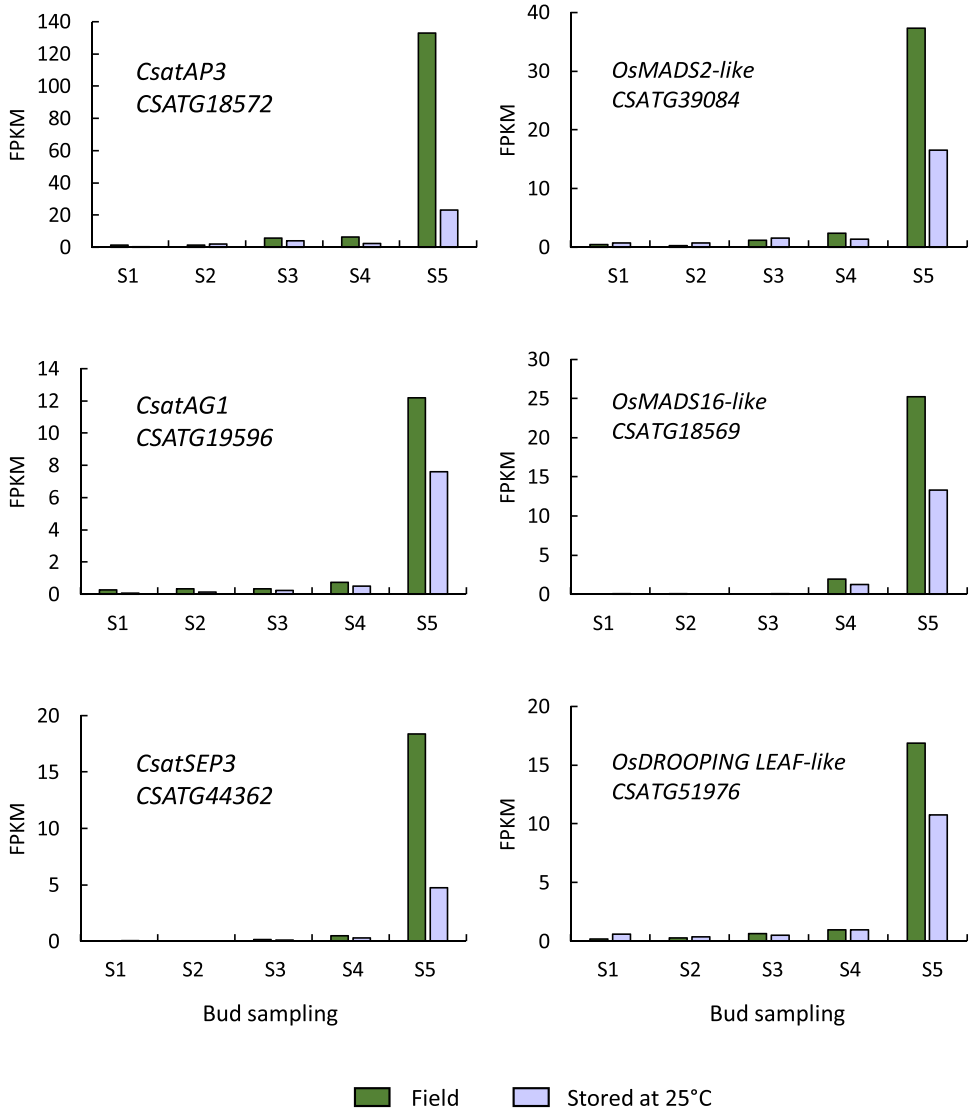

Fig. 9. Expression patterns of putative floral homeotic genes in the main bud of saffron corms. The transcripts levels of *CsatAP3*, *CsatAG1*, *CsatSEP3*, *OsMADS2*-like, *OsMADS16*-like and *OsDROOPONG LEAF*-like genes have been analysed in corms coming from the field or stored at 25 °C, from the end of May until early September (S1-S5).

June and mid-July is supported by different results: the clustering of DEG expression profiles, GO enrichment analysis, the identification and expression analysis of flowering and dormancy-related genes, as well as by meristem microscopy observations.

Around mid-July, it seems that dormancy release takes place (as indicated by an ABA drop and a decreased expression of *DRM1* and *MARD1* dormancy genes) and flower induction is initiated, as supported by differences in the gene expression of some homologs of key conserved genes in floral initiation like *FT*, *FTIP1*, *TFL1* and *LEAFY* (Andres and Coupland, 2012; Liu et al., 2012).

We have identified *CsCENT/TFL1 like b* as a potential repressor of flowering in saffron. In addition, *CsFT3* and a putative *LFY*-like gene could act as flowering inductors. FTIP1 has been described as a requirement for FT protein transport in Arabidopsis (Liu et al., 2012).

It is well known that *FT* and *TFL1* encode a pair of flowering regulators with homology to phosphatidylethanolamine-binding proteins (PEBPs), which share 60 % of the amino acid sequence identity but function in the opposite way (Hanzawa et al., 2005; Ahn et al., 2006). FT promotes the transition to reproductive development and flowering, whereas TFL1 represses this transition (Shannon and Meekswagner, 1991; Bradley et al., 1997; Kardailsky et al., 1999; Kobayashi et al., 1999). Species possessing flowering inductive *FT* genes include woody perennials, grasses, legumes, and ornamentals, among others. Likewise, flowering repressive *TFL1* homologs exist in woody perennials, grasses and ornamentals, among others (see Wickland and Hanzawa, 2015 for a review).

The cloning and characterization of a *TFL1-like* gene from *C. sativus* (*CsatCENT/TFL1-like a*) was carried out by Tsaftaris et al. (2012a). Its overexpression in Arabidopsis *tfl1* plants reversed the phenotype of early flowering, suggesting a repressor role in this species. However, an expression analysis was not carried out in saffron during the flower induction period and its involvement as flowering inhibitor has not yet been proven. In this work we have identified *CsatCEN/TFL1-like b,* a new *TFL1*-like gene. Taking into account its expression profiles and the significant decrease in transcripts from the end of June to mid-July (162 vs 7 on corms coming from the field), a potential use is proposed as a marker of flowering inhibition in saffron.

Leeggangers et al. (2017) have proposed that the *TFL1* gene function as a potential flowering repressor in Tulip, a bulbous species with summer dormancy, like saffron. The authors suggest that TFL1 could function in the ambient temperature pathway, being inhibited by high temperatures. In addition, they proposed the investigation of the function of TFL1 as an integrator of the photoperiod and ambient temperature pathways in monocots, as also occurs in dicots (Strasser et al., 2009; Rantanen et al., 2015). Although a bulb is an underground organ, specific genes could be induced when the plants still have green leaves that may translate a photoperiodic signal to the SAM in daughter bulbs. However, in saffron, *CsatCEN/TFL1* transcripts have not been detected in leaves during the vegetative cycle of this plant (Tsaftaris et al., 2012a). Therefore, we rule out the involvement of this gene in a photoperiodic pathway in saffron. However, its role is proposed in the GA_4 signalling pathway mediating flowering inhibition, and their involvement in ambient temperature pathway has to be investigated.

The expression profiles of *CsatFT3*, showing an increased number of transcripts from mid-July when the drop of *CsatCEN/TFL1* transcripts had taken place, suggest its involvement as a flowering inductor. The cloning and characterization of three *FT-like* genes from *C. sativus* was carried out by Tsaftaris et al. (2013). *CsatFT1* and *CsatFT2* were expressed from October to March in leaves when no expression was observed for *CsatFT3*. The *CsatFT3* expression was expressed just before the start of the flowering season (middle of September) in underground flowers and not on leaves and corms, suggesting a different functional role. However, its expression during the flower induction period was not measured.

FT protein is produced in the leaf, after a photoperiodic stimulus, and moves through the phloem to the apical meristems (Corbesier et al., 2007). In *C. sativus* and probably in other geophytes, however, this model is not applicable. Saffron flowers are formed underground, and flower induction and the increased expression of *CsatFT3* take place when the leaves have already withered. In addition, there is no expression of this flowering gene in saffron leaves. However, environmental factors, other than photoperiod, also modulate *FT* expression. Temperature-responsive *FT* regulators, which target the *FT* promoter or non-coding regions, include SHORT VEGETATIVE PHASE (SVP) (Hartmann et al., 2000; Lee et al., 2007), PHYTOCHROME INTERACTING FACTOR 4 (PIF4) (Kumar et al., 2012), or FLOWERING LOCUS C (FLC) in Arabidopsis (Michaels and Amasino, 1999; Searle et al., 2006). The role of SVP as a flowering inhibitor of the temperature-dependent pathway in saffron has already been shown (Haghighi et al., 2020) and our results support this hypothesis when corms are maintained in the field. In addition, we also proposed a modulation of *CsatFT3* by the transcription factors involved in hormone signalling pathways, like TFL1, or by PIF3 as shown by Oda et al. (2004). The up-regulation of *FT* homologs caused by high temperatures has also been observed in other geophytes in which flower initiation occurs in summer, such as *Narcissus tazetta* (Noy-Porat et al., 2013; Li et al., 2013a) and *Tulipa gesneriana* (Leeggangers et al., 2018)

Another possible key player in the floral transition, a putative *LFY-like* gene, has been identified in the transcriptomic analysis. Its expression pattern suggests involvement as a positive promoter of floral identity. *LFY* encodes a plant-specific transcription factor that regulates the change from the vegetative to the flowering stage (Blazquez et al., 1997; Hempel et al., 1997), and directs the development of initiated floral meristems through transcriptional activation of all the floral organ identity genes (see Siriwardana and Lamb, 2012 for a review). Floral transition is governed by the gradual increase in *LFY* expression, as observed in our results. The *LFY* genes are present in all terrestrial plants and their sequence is highly conserved throughout the plant kingdom (Maizel et al., 2005).

In a geophyte, resembling saffron in their life cycles and physiological requirements, *Narcisus tazetta*, a *LFY* homolog (*NFL*) has been isolated and characterized (Noy-Porat et al., 2010, 2013). *NLF* correlates with intrabulb florogenesis (Noy-Porat et al., 2010). A dramatic increase in *NLF* expression was observed during floral initiation as well as during the differentiation of flower primordia. However, unlike what occurs with *NtFT*, the expression of *NLF* is not regulated by photoperiod or temperature but might be affected by an endogenous signal (Noy-Porat et al., 2013). From our results, we also proposed that *LFY* could be a target of transcription factors involved in the auxin signalling pathway mediating flower induction.

The role of the FTIP1 has been less widely-studied, but its role as a requirement for FT transport through the phloem has been confirmed in monocots, like rice (Song et al., 2017). So, a rise in its expression prior to that taking place for FT could be an interesting marker.

4.2. What working models dealing with the effect of hormones on saffron floral transition could be suggested?

4.2.1. Auxin promotes reproductive meristems

The plant hormone, auxin, in particular indole-3-acetic acid (IAA), is a key regulator of virtually every aspect of plant growth and development (Mockaitis and Estelle, 2008). Its requirement for axillary meristem initiation or the promotion of reproductive meristems from the SAM has been studied in Arabidopsis, a plant with racemose inflorescences (see Wang and Jiao, 2018; Lee et al., 2019 for a review). Although the floral meristem has been considered as a specialized axillary meristem and the leaf as a bract or cryptic bract (Long and Barton, 2000), the effects of auxins differ.

Recent studies have shown that a low auxin environment is critical for axillary meristem initiation (Wang et al., 2014a, b, 2018). However, the initiation of a flower primordium is preceded by the establishment of a local maximum of the plant hormone, auxin (Benkova et al., 2003;

Heisler et al., 2005; Reinhardt et al., 2000). This maximum is generated by local auxin biosynthesis and polar auxin transport (Yamaguchi et al., 2014).

The stem anatomy, as well as the inflorescence in saffron, is quite different to that of Arabidopsis. The saffron corm is a modified stem. If the corm is large enough, a cymose inflorescence is generated from the apical bud. It becomes completely transformed into a single terminal flower or, in some cases, the inflorescence meristem yields two or three floral meristems. Axillary buds sprout and form new replacement corms (Molina et al., 2005a; Dadpour et al., 2012). Despite these differences, the level of auxins in saffron buds giving rise to flower primordia was also much higher than in axillary buds. In addition, two months after flowering, the main buds during vegetative development also show a lower level of auxins. It is interesting to point out that the difference between the auxins level in the axillary and main buds grows during flower induction and remains stable during flower development. Overall, our results suggest that auxin promotes reproductive meristems in saffron as well.

Translating local auxin concentration into specific gene expression outputs and flower initiation has been studied in Arabidopsis, and a key role of the auxin response factor, MP/ARF5 has been observed (Hardtke and Berleth, 1998; Przemeck et al., 1996; Zhao et al., 2010; Yamaguchi et al., 2013). Other genes also involved in the model formulated by Yamaguchi et al. (2016) for the onset of flower formation activated by auxins in Arabidopsis include *LFY* and *ANT,* as previously exposed. The identification and expression analysis of saffron genes similar to those of Arabidopsis involved in that auxin signalling pathway (*MP-like; LFY-like and ANT-like*) suggest that this could be a suitable working model for saffron. However, a great deal of additional functional work is needed to elucidate this mechanism.

The auxin level may be generated by auxin biosynthesis and polar auxin transport. The *YUCC2*-like gene, an auxin biosynthetic gene that could be involved in the auxin synthesis in saffron, has been identified and shows an increased number of transcripts from the end of June until mid-July. After that, it decreased in the main bud of the corms coming from the field and, after a while, in the stored corms. These results could be partially explained by taking into account that LFY inhibits auxin biosynthesis by suppressing *YUC* expression (Li et al., 2013b). In accordance with the expression pattern of *YUCC2-like* gene, a *TAR2*-like gene, involved in a previous step of the major pathway to IAA biosynthesis (Shao et al., 2017) is contained in cluster 4, exhibiting a similar expression pattern to *YUC*.

It must be noted that there is a great deal of interest in studying the dynamics of PIN1-mediated polar auxin transport (Okada et al., 1991; Leyser, 2010; Adamowski and Friml, 2015) as well as in the auxin biosynthesis in the different inner regions of the meristem, giving rise to floral primordium. It will be required for both a better understanding of the roles of auxins in specifying the meristem fate in the main bud, and also in order to learn about auxin signalling crosstalk with other phytohormones and signalling pathways. In this context, it is interesting to highlight that several transcripts encoding auxin efflux carriers (PIN1) have been identified in clusters 2 and 4. In addition, a gene encoding an auxin-influx carrier (AUX1) showing an expression pattern corresponding to cluster 2 has been found. These results, together with those related to the expression patterns of SAUR genes (clusters 2 and 4), which is a class of early responsive genes playing a role in auxin-mediated response, evidence the relevance of auxins in saffron flower induction and development.

Another interesting result from the transcriptomic analysis is the significant drop in the transcript coding for the DRM1 homolog 2 protein, downregulated by high levels of auxins (Rae et al., 2014). This drop matches the increase in the auxin level. DRM1/ARP is often used as a genetic marker for dormant meristematic tissues (reviewed by Rae et al., 2013). DRM1/ARP was first associated with dormancy through decapitation studies carried out in peas (Stafstrom et al., 1998). *PsDRM1* transcript levels are high in dormant buds located below the shoot apical meristem but are no longer detectable in these buds some hours after decapitation. Similar transcriptional profiles linking *DRM1/ARP* expression with dormant tissue, were observed in Arabidopsis (Tatematsu et al., 2005) and in kiwifruit (Wood et al., 2013). Our results suggest that DRM1/ARP is also involved in the bud dormancy release in saffron. The close correlation between auxin concentration and the transcript level of *DRM1/ARP* also points to the possible downregulation of this saffron protein produced by auxins.

4.2.2. *GAs could inhibit flower induction but promote enlargement of the scape*

Gibberellins promote flowering in many species (Mutasa-Gottgens and Hedden, 2009). In Arabidopsis, GA is required for flower initiation under the non-inductive conditions (Wilson et al., 1992). The expression of the flower meristem identity gene, *LFY,* and the flowering time gene, *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1),* are both induced by GA (Blazquez et al., 1998; Moon et al., 2003). GA_4 is the GA that most actively promotes *LFY* expression upon flower induction (Eriksson et al., 2006), increasing sharply in the shoot apex immediately prior to flower initiation (see Sun, 2008; Mutasa-Gottgens and Hedden, 2009; Conti, 2017 for a review)

In the case of perennials, the role that GAs play in flowering has mainly been studied in fruit trees, in which GAs are generally inhibitory to flowering (reviewed by Wilkie et al., 2008; Mutasa-Gottgens and Hedden, 2009). Over the last 10 years, much of the research done to explain the molecular mechanisms of flower formation in these species has been carried out in apples and has focused on two main genes, *MdTFL1* and *MdFT1 (*Mimida et al., 2013; Xing et al., 2015*)*. It has become clear that gibberellin application (GA4 + 7 or GA3) inhibits flower formation by upregulating the inhibitory *MdTFL1* (Haberman et al., 2016; Elsysy and Hirst, 2019; Zhang et al., 2019)

The effect of GAs on the flowering of geophytes, also perennial species, has mostly been studied via their exogenous application. Treatments with growth regulators are routinely used to control growth and flowering in bulbous species (Kamenetsky et al., 2013). However, research providing an accurate link between the endogenous level of GAs and floral transition is very scarce (Naor et al., 2008) and, as far as we know, there are no hypotheses about the GA signalling pathways mediating flower induction.

In some species included in the florogenetic group to which saffron belongs, such as *Tulipa* or *Peonia*, the effect of GAs varies according to the temperature-dependent phase of the flowering process. Warm temperatures are required for flower differentiation in these species, but a prolonged cold period at 4−9 °C is required both for dormancy release and to prepare the shoot for the third phase, in which rapid floral stalk elongation and flower development occur, again at around 17 °C. (Hartsema, 1961; Rees, 1992; Kamenetsky et al., 2003). GA application can partly substitute the required cold treatment (Hanks, 1982; Evans et al., 1990; Halevy et al., 2002). However, GA treatment does not promote flower initiation and differentiation and it is not effective if applied before flower formation (Halevy et al., 1995). In saffron, warm temperatures are also required for flower initiation. However, although a prolonged cold period is not needed for dormancy release and flowering, the drop of temperature in autumn is the factor inducing the elongation of the scape and perianth tube (Molina et al., 2005a).

Our results suggest that an increase in GA_1 level could also be related to a very fast growth of the scape and perianth tube just before flowering in November. Nevertheless, as in other geophytes with summer dormancy, GAs does not seem to promote flower initiation. On the contrary, a significant drop in GA_4, the GA that most actively promotes *LFY* expression upon flower induction in model species, appears to be needed for floral initiation to take place. However, it seems that this drop may be a necessary, but insufficient, condition for flower induction. The axillary buds also show a significant decrease in GA_4 level. Auxins and sugars, together with the temperature, would also be involved in the signalling network mediating floral transition. Together,

our results might indicate that these factors would be increasing the expression of integrators of flowering time signals, like *FT*, as well as of some meristem identity genes, such as *LFY*.

The transcriptomic analysis supports the proposed role of the gibberellins by applying the models formulated by Oda et al. (2004) and Arro et al. (2019) to the onset of flower formation in saffron. Our results support a possible DELLA inhibitory effect on TFL1 in the shoot. An alternative pathway also involving DELLA, which prevents the binding of PIF3 to its target genes, including *FT* (inhibited by PIF3), is also hypothesized. In addition, DELLA could inhibit *PIF3* expression, given the negative correlation between their transcript level. Overall, these results bring forward a model of GA_4 signalling pathways mediating flower induction in saffron that requires further studies to confirm the role of GAs, as well as the possible roles of the proposed regulatory genes.

4.2.3. ABA could inhibit corm dormancy release through ABI5 and MARD1 transcription factors but its involvement in flower induction cannot be ruled out

Changes in the level of transcripts related to the three groups of components involved in the ABA signalling pathway suggest a negative relationship between the ABA signal and the development of a floral meristem in saffron. However, the drop in ABA could only be related to the dormancy release process. In Arabidopsis, the inhibitory effect of ABA in both dormancy and flowering is mediated by the transcription factor ABI5, showing a significant reduction in its transcript level during flower induction and the development period. Taking into account that ABI5 is an important transcription factor in ABA signalling that can enhance *Gladiolus* corm dormancy (Wu et al., 2015), in addition to its well-studied function in the dormancy of Arabidopsis seeds (Lopez-Molina et al., 2001), the involvement of a saffron *ABI5-like* gene in the inhibition of corm dormancy release could be hypothesized. Interestingly, a possible new player in corm dormancy release, *MARD1*, has been identified. Its expression is upregulated by ABA in imbibed Arabidopsis seeds. Seeds from a mard1 T-DNA insertional mutant germinate faster and are less responsive to ABA than wild type seeds (He and Gan, 2004). Taking into account that not much is known about molecular mechanisms related to the hormonal control of corm dormancy release (Wu et al., 2015), these results, together with those described for auxins and GAs, are relevant and the use of *ABI5 and MARD1* as molecular markers of bud dormancy in saffron could be of interest.

The ABI5 transcription factors regulate floral transition in Arabidopsis through the central mediator FLC, an inhibitor of FT (Wang et al., 2013). However, the FLC monocot clade consists solely of genes from *Poaceae*, except from MpFLC from *Musa paradisiaca*. FLC orthologs have not been found in other monocot species and neither have they been found in this transcriptomic analysis. So, the presence of another factor that mediates ABI5-dependent FT induction needs further research.

4.3. What expression patterns of floral homeotic genes are observed? Are there new players?

The saffron flower is bisexual, although sterile, due to its triploidy condition. The perianth consists of three tepals in the first whorl (outer tepals) and three tepals in the second whorl. The androecium consists of three distinct stamens and the gynoecium consists of a single compound pistil with: three carpels, a single three branched style, and an inferior ovary.

The homologous of the floral homeotic genes corresponding to the major B, C, and E-classes have been isolated in saffron, and the expression pattern of C and E- class genes fits the ABCDE model well (see Tsaftaris et al., 2010 for a review). The expressions of the B-class genes in whorls 1, 2, and 3, support the hypothesis that a modified ABC model could be responsible for the transformation of the sepals and petals into tepaloid organs. However, the B-class genes are also expressed in whorl 4, which does not fit the modified ABC model. Within this framework, new factors are required for carpel specification.

In all these studies, the expression analysis has been carried out in fully developed flowers. This may not be the most appropriate time. We have found a high expression of most of these genes at an earlier time, during early September, when the different whorls are developing but full flower growth has not yet been achieved. The definition of a period in which a progressive increase in the expression of these genes is taking place, offers the opportunity to carry out an accurate study of their expression in the different whorls, as well as their relationships, before flower development has been completed. Furthermore, we have found new players that could be involved in flower development. The identification of the transcripts encoded by a *DROOPING LEAF-like (DL)*gene is of particular relevance because this gene could be the new factor for carpel specification in saffron. DL has been shown to be a gene that controls carpel identity in rice (Nagasawa et al., 2003). We would like to emphasize that the saffron spice consists of the stigmas, by themselves or together with the styles, of the flowers and it is of particular interest to find out the genetic mechanism determining the floral organ identity. This information could allow a greater number of flower stigmas to be obtained.

Although *AP1-like*(*CsAP1a/b/c*) and *AP2-like* genes (*CsatAP2a/b/c*) have been isolated in saffron (Tsaftaris et al., 2004, 2012b), no function related to the control of floral organ identity has been described so far. The transcriptomic analysis carried out in this study has allowed us to identify mRNA encoded by *AP1-like* and *AP2-like* genes. However, their expression is not in line with those of the other floral homeotic genes, supporting a yet unidentified role of their corresponding proteins in saffron, as proposed by Tsaftaris et al. (2004; 2012b).

Taken together, we have shown that 20–30 days before morphological changes related to flower development are visible in the main saffron buds, the expression of floral repressor and dormancy genes decreases and a significant increase in potential flowering activators takes place, leading to the flower primordia initiation, which is clearly visible at the end of July-early August. However, floral homeotic genes do not reach their highest expression until early September when flower development is fast. We have identified many potential flowering regulators, involved in both floral induction and floral organ identity. The identification of a *DL-like* gene in saffron, which could determine carpel identity, is of great interest. However, a more detailed analysis will be needed to confirm the possible roles of these genes in the flowering process

Our results also suggested that IAA could be a positive regulator of floral transition and flower development in saffron. ABA might be negatively regulating the corm dormancy release, but its involvement in flower induction cannot be ruled out. GA_4 may be a repressor of floral induction, but GA_1 could be related to a very fast growth of the scape and perianth tube just before flowering. In addition, our transcriptomic results provide working models on hormone signalling pathways mediating flower induction and corm dormancy release. It is necessary to point out that a key strength of this study is the high similarity between the results obtained in the two environments where the floral transition and development have been tested.

Although further studies are required to confirm these models, this study paves the way to an understanding of the regulatory networks controlling flowering in saffron.

Author contributions statement

RM, BR and SN conceived the experiments; BR, SN, EG, MD and RM conducted the experiments; JC and VG carried out the transcriptome assembly and annotation, JC, VG, EG and RM analysed the results; RM, SN and BR wrote the paper, and all authors reviewed the manuscript.

CRediT authorship contribution statement

Begoña Renau-Morata: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Sergio G.**

Nebauer: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Víctor García-Carpintero:** Methodology, Software, Formal analysis. **Joaquín Cañizares:** Methodology, Software, Formal analysis, Data curation, Writing - review & editing. **Eugenio Gómez Minguet:** Methodology, Software, Formal analysis, Investigation, Writing - review & editing. **Marcelino de los Mozos:** Investigation, Writing - review & editing, Supervision, Funding acquisition. **Rosa V. Molina:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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