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Impact of vernalization and heat on flowering induction, development and fertility in root chicory (*Cichorium intybus* L. var. *sativum*)

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ABSTRACT

Root chicory (*Cichorium intybus* var. *sativum*) is a biennial plant that requires vernalization for flowering initiation. However, we previously showed that heat can induce root chicory flowering independently of vernalization. To deepen our understanding of the temperature control of flowering in this species, we investigated the impact of heat, vernalization and their interaction on flowering induction and reproductive development. Heat increased the flowering percentage of non-vernalized plants by 25% but decreased that of vernalized plants by 65%. After bolting, heat negatively affected inflorescence development, decreasing the proportion of sessile capitula on the floral stem by 40% and the floral stem dry weight by 42% compared to control conditions, although it did not affect the number of flowers per capitulum. Heat also decreased flower fertility: pollen production, pollen viability and stigma receptivity were respectively 25%, 3% and 82% lower in heat-treated plants than in untreated control plants. To investigate the genetic control of flowering by temperature in root chicory, we studied the expression of the *FLC-LIKE1* (*CiFL1*) gene in response to heat; *CiFL1* was previously shown to be repressed by vernalization in chicory and to repress flowering when over-expressed in Arabidopsis. Heat treatment increased *CiFL1* expression, as well as the percentage of bolting and flowering shoot apices. Heat thus has a dual impact on flowering initiation in root chicory since it appears to both induce flowering and counteract vernalization. However, after floral transition, heat has a primarily negative impact on root chicory reproduction.

1. Introduction

During the last few decades, the average global temperature has increased, and this trend is expected to continue, with a mean rise of 2.5–4.5 °C expected by the end of the 21st century (IPCC, 2014). Moreover, heat waves will become more frequent and intense (IPCC, 2014). Temperature increase is detrimental to the growth and productivity of many plant species, especially in the summer months and in warm and temperate climatic regions (Asseng et al., 2015; Fahad et al., 2017; Huang and Xu, 2008; Zhao et al., 2017). Fruit and seed crops are expected to be particularly affected by heat as the reproductive phase is more vulnerable to abiotic stresses than the vegetative phase (Driedonks et al., 2016; Prasad et al., 2015). However, vegetative crops—those cultivated for root or leaf production, such as sugar beet, root chicory, carrot, cabbage and lettuce—are also sensitive to heat stress (Mathieu et al., 2014; Ober and Rajabi, 2010; Park et al., 2013; QingJun et al., 2011; Saha et al., 2016). Exposure of vegetative crops to above-optimal growth temperatures decreases the rates of photosynthesis, canopy expansion, root growth and sucrose accumulation (Mathieu et al., 2014; Ober and Rajabi, 2010; Park et al., 2013; Saha et al., 2016). Heat may also reduce vegetative crop production by hastening floral transition and decreasing the duration of vegetative growth (Dally et al., 2018;

Abbreviations: FLC, FLOWERING LOCUS C; FT, FLOWERING LOCUS T; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CO 1; PIF4, PHYTOCHROME INTERACTING FACTOR 4; FLM, FLOWERING LOCUS M; SVP, SHORT VEGETATIVE PHASE; MAF1−5, MADS AFFECTING FLOWERING 1−5; CiFL1, FLC-LIKE 1; PVPP, polyvinylpyrrolidone.

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Mathieu et al., 2014; QingJun et al., 2011). For field production, vegetative crops must not enter the reproductive phase before the vegetative parts of the plant are harvested (Dally et al., 2018; Dielen et al., 2005). However, flowering and seed set is necessary for breeding and seed production (Dally et al., 2018; Dielen et al., 2005). Identifying environmental factors that affect floral transition of vegetative crops is thus crucial for the future adaptation of these crop plants to climate change.

Root chicory (*Cichorium intybus* var. sativum) is a vegetative crop cultivated primarily for its production of inulin, a fiber source with dietary and medicinal uses, in the roots (Dielen et al., 2005; Vandoorne et al., 2012). Like most biennial plants, root chicory requires vernalization to flower (Dielen et al., 2005; Gianquinto, 1997; Pimpini and Gianquinto, 1988) i.e., it requires prior prolonged exposure to cold to undergo bolting and flowering (Périlleux et al., 2013). The effectiveness of cold treatment in inducing bolting depends on its duration, the variety, and the plant's age at the start of vernalization (Dielen et al., 2005). Vernalization treatment at 4 °C applied at the imbibed seed stage for 6 weeks is effective in inducing flowering in root chicory (Dielen et al., 2005; Périlleux et al., 2013). In nature, root chicory plants form a rosette of leaves and a taproot during their first year of growth; bolting and flowering occur during the second year after vernalization over the intervening winter. During the first year, the taproot accumulates inulin that is mobilized by the plant during the second year to sustain reproductive development. After bolting, the plant initiates a floral stem called a stalk (Périlleux et al., 2013). This is composed, from bottom to top, of cauline leaves, lateral flowering branches and sessile capitula in clusters of decreasing size. Capitulum inflorescences comprise 15–25 hermaphroditic ligulate flowers surrounded by involucral bracts. In general, about 30 days elapse between the start of bolting and the anthesis of the first capitulum, and 15 days from flower initiation to anthesis (Habarugira et al., 2015; Périlleux et al., 2013). All flowers of a capitulum open synchronously, and anthesis lasts for 1 day (Habarugira et al., 2015).

In the field, chicory is cultivated as an annual plant for inulin production, and bolting is avoided since it reduces the inulin yield of the roots. However, early bolting is sometimes observed in field conditions, mainly due to vernalization by low temperatures during early spring just after sowing (Dielen et al., 2005). Bolting induced by high temperatures during hot summers has also been reported (Dielen et al., 2005; Mathieu et al., 2014). High temperatures (> 35 °C) can induce bolting and flowering in root chicory independently of vernalization (Mathieu et al., 2018, 2014). High temperatures can also erase the effects of vernalization: seedling exposure to 25 °C for 2 weeks directly after vernalization has a de-vernalization effect (Périlleux et al., 2013). Similar observations have been reported for *Arabidopsis thaliana* (Arabidopsis; Bouché et al., 2015) and a number of cold-requiring plants (Bernier et al., 1981).

How temperature variations regulate flowering at the molecular level in root chicory remains largely unknown, and the molecular control of flowering is less understood in root chicory as compared to model plants such as Arabidopsis. In Arabidopsis, flowering control by vernalization involves the gene *FLOWERING LOCUS C* (*FLC*), a suppressor of flowering (Michaels and Amasino, 1999; Sheldon et al., 1999). *FLOWERING LOCUS C* encodes a MADS-box domain transcription factor that inhibits the expression of the flowering integrator genes *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) (Helliwell et al., 2006). Vernalization promotes flowering through epigenetic repression of *FLC* (Bastow et al., 2004; Sheldon et al., 2000). Flowering can also be accelerated in Arabidopsis by elevated growth temperature (Balasubramanian et al., 2006). The mechanisms underlying this effect have recently been identified and involve the transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4) or the FLOWERING LOCUS M (FLM)–SHORT VEGETATIVE PHASE (SVP) complex (Balasubramanian et al., 2006; Capovilla et al., 2015; Jagadish et al., 2016; Kumar et al., 2012; Lee et al., 2013; Posé et al., 2013; Theißen et al., 2018). Binding of PIF4 at the *FT* promoter is temperature dependent (Capovilla et al., 2015; Kumar et al., 2012; Kumar and Wigge, 2010; Thines et al., 2014). *FLOWERING LOCUS M* (*FLM*, also called *MADS AFFECTING FLOWERING 1*, *MAF1*) is a MADS-box gene of the *FLC* clade whose gene product undergoes a temperature-dependent alternative splicing (into *FLM-β* and *FLM-δ*) (Balasubramanian et al., 2006; Lee et al., 2013; Mateos et al., 2015; Scortecci et al., 2003, 2001; Theißen et al., 2018). At low temperature, the amount of FLM-β increases and the SVP/ FLM-β complex represses the floral integrator genes *SOC1* and *FT*, whereas higher temperatures destabilize the SVP protein and thus favor flowering (Lutz et al., 2015; Posé et al., 2013; Theiβen et al., 2018). Besides *FLM*, other members of the *FLC* clade, the *MAF2−5* gene cluster, also appear to be involved in ambient-temperature flowering response, but the molecular mechanism is not completely understood (Theiβen et al., 2018). Moreover, in this species, fluctuating warm temperature also accelerate bolting in genotypes with high *FLC* levels (Burghardt et al., 2015)

In root chicory, a MADS-box sequence similar to those of *FLC*, *FLM* and *MAF* has been identified and named *FLC-LIKE 1* (*CiFL1*) (Périlleux et al., 2013). Périlleux et al. (2013) showed that overexpression of *CiFL1* in Arabidopsis delays flowering and that vernalization represses *CiFL1* expression in root chicory, although the gene is reactivated after cold treatment. High temperature after vernalization leads to de-vernalization in both Arabidopsis and root chicory and to reactivation of *FLC* and *CiFL1*, respectively (Bouché et al., 2015; Périlleux et al., 2013). These results suggested that *CiFL1* is involved in the vernalization process in root chicory. However, the involvement of *CiFL1* in flowering induction by high temperature has not been investigated so far.

In addition to its effect on flowering induction, high temperature may also affect later reproductive development. In many species, heat negatively affects flower development and consequently reduces fruit and seed production (Bita and Gerats, 2013; Hedhly, 2011; Hedhly et al., 2005; Prasad et al., 2015; Sage et al., 2015; Young et al., 2004). Heat stress may reduce flower number, decrease flower size and cause abnormalities in flower development (Erickson and Markhart, 2002; Konsens et al., 1991; Teixido et al., 2016; Zinn et al., 2010). The maturation, viability and germination ability of pollen grains, as well as pollen tube growth, may also be reduced (Müller and Rieu, 2016; Zinn et al., 2010). In addition, heat stress can shorten the duration of stigma receptivity to pollen and thereby decrease the chances of successful fertilization (Dudley et al., 2018; Hedhly, 2011; Teixido and Valladares, 2015; Zinn et al., 2010). How high temperatures affect later flowering development in root chicory has not been studied so far. Controlling sexual reproduction in root chicory is required to allow seed production during the next year and for breeding programs aiming to produce hybrids (Dielen et al., 2005).

In the present work, we investigated the impact of high temperature, vernalization and their combination on flowering induction and reproductive development in root chicory. Our questions were (1) How do heat, vernalization and their combination affect bolting, inflorescence and flower development, and flower fertility? and (2) Is *CiFL1* involved in flowering induction by high temperature? We hypothesized that although high temperature may induce flowering, it would negatively affect inflorescence and flower development in root chicory, and that *CiFL1* may be involved in the flowering induction by high temperature in this plant.

2. Material and methods

2.1. Plant materials and growing conditions

Seeds of chicory (*Cichorium intybus* L. var. *sativum*) cultivar Melci were obtained from Chicoline (a division of Cosucra groupe Warcoing S. A.; Belgium). Eighty plants were grown in growth chambers and divided into four experimental treatments (20 plants per treatment): (1) non-vernalized plants grown at 17 °C, (2) vernalized plants grown at 17 °C, (3) non-vernalized plants grown at 35 °C and (4) vernalized plants

grown at 35 °C (Fig. 1A). Seeds were sown on a substrate containing sand and loam (3:1, v/v) in small PVC tubes (8 cm high and 2.5 cm diameter). Seeds were incubated in a growth chamber at 4 °C for 6 weeks for the vernalization treatment, as this was previously shown to be the most efficient vernalization method (Dielen et al., 2005; Périlleux et al., 2013). Non-vernalized seeds were sown 3 weeks later and placed in a growth chamber at 17 °C. After that, vernalized and non-vernalized seedlings were transplanted into pots containing 2 L of the same substrate and maintained in the growth chamber at 17 °C for 1 week before heat treatment. The sowing dates of non-vernalized and vernalized plants were adjusted so that all plants were at the four-leaf stage at the start of heat treatment. To apply heat treatment, half of vernalized and non-vernalized plants were exposed to 35 °C day/28 °C night, whereas control plants were maintained at a constant temperature of 17 °C. These temperatures were chosen based on previous experiments. The temperature of 17 °C was considered a control condition since no de-vernalization is observed at this temperature (Périlleux et al., 2013), and 35 °C was selected as high temperature because it induces bolting (Mathieu et al., 2014). In all cases, plants were maintained in a 16-h photoperiod at 70% ± 5% relative humidity and exposed to 300 µmol m^{-2} s^{-1} PAR (provided by Sylvania VHO 250 W) at the top of the canopy in both growth chambers. The temperature and relative humidity were measured continuously in the growth chambers. The total duration of the experiment was 12 weeks from the start of heat treatment.

2.2. Plant growth, inflorescence development and flower fertility

The number of rosette leaves was recorded every 2 weeks during vegetative growth from 5 plants per treatment. The area of the youngest fully expanded leaf (rosette leaf before bolting and cauline leaf after bolting) was also measured with a leaf area meter (ADC, Bioscientific Ltd.) every 2 weeks on 6 plants per treatment.

To determine floral transition, visually detectable bolting plants were recorded regularly for all 80 plants. Inflorescence status and flower fertility were measured. Eleven weeks after the start of the heat treatment, inflorescence development was assessed by counting on all flowering plants (1) the number of different nodes (nodes with cauline leaves, nodes with lateral flowering branches and nodes with sessile capitula) on the floral stem as described by Périlleux et al. (2013) and (2) the number of flowers per capitulum. At the end of the experiment, the dry and fresh weight of the floral stem was also determined.

Flower fertility was assessed by quantification of the stigma receptivity, the number of pollen grains per flower and the pollen viability of 10 flowers per condition (flowers were randomly collected from different sessile capitula of 5–10 plants per condition on the same day). Stigma receptivity was estimated on the basis of esterase and peroxidase activity using a colorimetric test as described by Dafni (1992) and Dafni and Maués (1998). Stigma were observed under a stereomicroscope and classified as receptive (brown coloration), partly receptive (part of the stigma was brown) or not receptive (no brown coloration). For pollen grain quantification and viability assessments, flower buds were collected and the anthers were separated out under a stereomicroscope and individually crushed in a microcentrifuge tube containing 50 µL of Alexander's stain (Kearns and Inouye, 1993). They were then mixed and sonicated to disperse the pollen grains in the solution. The number of pollen grains per anther was counted in 5 µL in triplicate under a light microscope. Viable and non-viable pollen grains were separated based on their color. Viable grains appeared red, whereas non-viable grains exhibited a green coloration. The percentage of pollen viability was calculated based on the number of viable and total grains.

2.3. Expression of CiFL1 in heat-treated plants

The expression of *CiFL1* was investigated in 40 plants grown in a

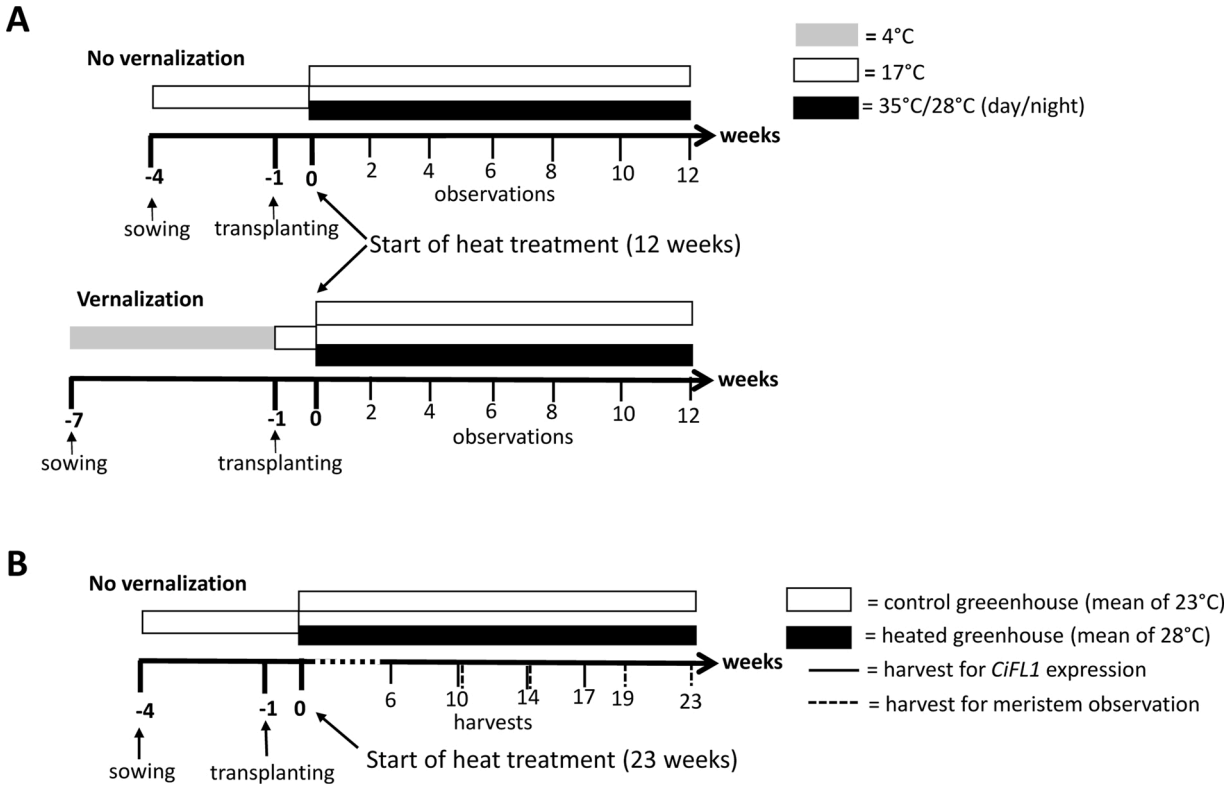

Fig. 1. Schematic description of the experiments conducted in (A) growth chambers where root chicory plants were exposed to four conditions: no vernalization – control condition (grown at 17 °C), no vernalization – heat treatment (grown at 35 °C), vernalization – control (grown at 17 °C) and vernalization – heat treatment (grown at 35 °C); and (B) greenhouses where non-vernalized plants where grown either in control greenhouse (mean of 23 °C) or heated greenhouse (mean of 28 °C). Start of the heat treatment is indicated as 0. Time points when measurements and harvests were performed are indicated.

temperate greenhouse under control conditions (mean temperature 23 °C) or in a heated greenhouse for heat treatment (mean temperature 28 °C) (Fig. 1B). Plants were not vernalized. Sowing and transplanting were performed as previously described. Heat treatment started 4 weeks after sowing. The temperature and relative humidity were measured continuously in the greenhouses. Leaves of 6 plants per treatment were harvested 6, 10, 14 and 17 weeks after start of heat treatment for gene expression analysis. For each date and treatment, leaves of plants of the same development stage were pooled. In addition, shoot apices of 3–6 plants per treatment were harvested 10, 14, 19 and 23 weeks after start of heat treatment and fixed in FAA (70% ethanol : 37% formaldehyde : 100% acetic acid ; 90:5:5, v/v/v) to identify the physiological state of the plants. We defined three different physiological stages of the shoot apices: the 'concave' apex corresponding to the vegetative state, the 'domed' apex corresponding to the start of bolting process and the 'flowering' apex, with cauline leaves and the start of floral stack development.

For gene expression analysis, total RNA was extracted from 500 mg fresh leaf material ground in liquid nitrogen. Seven milliliters of pre-heated (65 °C) extraction buffer (300 mM Tris HCl (pH 8.0), 25 mM EDTA (pH 8.0), 2 M NaCl, 2% (w/v) CTAB, 0.05% (w/v) spermidine, 2% (w/v) polyvinylpyrrolidone (PVPP), with 2% (w/v) β-mercaptoethanol added just before use) were added to the powder and the mixture incubated for 10 min at 65 °C. Thereafter, the samples were centrifuged for 15 min at 4000 *g*, 4 °C. The supernatant was collected and an equal volume of chloroform:isoamyl alcohol (24 : 1, v/v) was added. A second centrifugation and extraction with chloroform:isoamyl alcohol was performed. After that, 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volume of isopropanol were added to the supernatant to precipitate the RNA. After 30 min at −80 °C and a centrifugation (30 min, 8400 *g*, 4 °C), the pellet was dissolved in 1 mL of TE buffer. Then 0.3 volume of LiCl (10 M) was added and the samples were placed at 4 °C for at least 12 h. After centrifugation at 4 °C for 30 min, the pellet was washed with 70% ethanol, dried and resuspended in 20 µL of DEPC-treated water. DNase treatment was performed using RQ1 RNAse-free DNase (Promega, Leiden, The Netherlands) according to the manufacturer's instructions. Total RNA was quantified and its purity was assessed using a NanoDrop spectrophotometer (ND-1000; Isogen Life Science). The RevertAid H Minus First Strand Synthesis kit (Fermentas, St. Leon-Rot, Germany) was used to synthetize the cDNA with 1 µg of DNAse-treated RNA according to manufacturer's instructions. Transcript levels were quantified in three independent qPCRs (in triplicate for each PCR) using the GoTaq qPCR Master Mix (Promega) in a StepOnePlus Real-Time PCR systems (Applied Biosystems) according to the procedure described by the supplier. PCR reactions were performed using 0.2 µM of each primer, 5 µL of GoTaq qPCR Master Mix (Promega) and 600 ng of cDNA in a final volume of 10 µL. Negative controls were included in each run. The PCR cycling conditions were as follows: initial denaturation at 95 °C for 90 s followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. Amplification was followed by melting-curve analysis to check the specificity of each reaction. The primers *CiFL1F* (5′-CTCGAAACGGAGAACGGGAT-3′) and *CiFL1R* (5′-CGGAGTGTCAGATCTTGGGG-3′) were used to amplify a 550-bp PCR product for *CiFL1* (HF549158). Data were normalized according to *CiTUB* (AF101419) gene expression levels. The primers used were *CiTUBF* (5′-AGTTCTGGGAGGTTGTCTGC-3′) and *CiTUBR* (5′−CCTTCGCCCAATTGTTACCG-3′), which generated a 255-bp PCR product. Gene expression was analyzed using iCycle iQ Real-Time PCR Detection System software (Bio-Rad) with a method derived from the algorithms outlined by Vandesompele et al. (2002). Three independent qPCRs were each performed in triplicate and the results are presented as means ± SE of the different technical replications.

2.4. Statistical analysis

Normality tests were performed using a Shapiro-Wilk test. No further transformation of the raw data was required. The ANOVA models were defined to evaluate the effects of the vernalization and heat treatments and their interaction on the number of rosette leaves, the leaf area, the number of nodes on the floral stem, the floral stem dry weight, the number of flowers per capitulum, the number of pollen grains per bud, the pollen viability and the *CiFL*1 expression level. *Post-hoc* analyses were performed using the Student-Newman Keuls test at a significance cutoff level of $P < 0.05$. Bolting rates, stigma receptivity and flowering stage of shoot apices were compared among treatments using chi-squared tests. Data were analyzed using SAS Enterprise Guide 6.1 (SAS 9.4 system for windows). If not indicated otherwise, data are presented as means ± SE. Relative differences between conditions expressed as percentages were calculated as (Vc – Vh)/ Vc *100 were Vc and Vh are respectively the values under control and heat conditions.

3. Results

3.1. Interacting effects of vernalization and high temperature on root chicory growth and flowering

3.1.1. Heat and vernalization affected leaf production and bolting rate

Vernalized and non-vernalized plants were grown under heat and control conditions for 12 weeks (Fig. 1A). The number of rosette leaves increased along the experiment in all of the experimental groups except for the vernalized plants exposed to the control temperature (Fig. 2A). For these plants, the number of rosette leaves increased during the first 28 days of growth after the end of vernalization and never exceeded 10 leaves, as the plants stopped producing rosette leaves and bolted. For the other treatment groups, the number of rosette leaves increased faster in

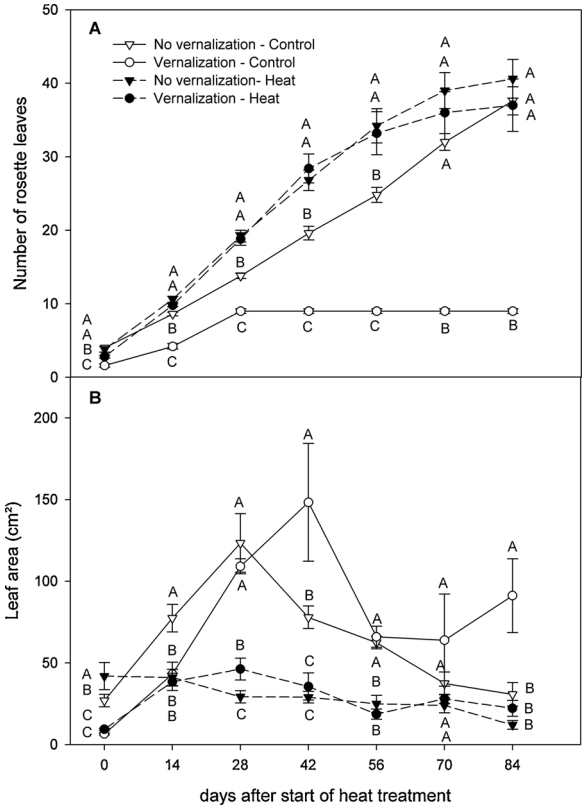

Fig. 2. Impact of vernalization and heat treatment on leaf production in root chicory. (A) The number of rosette leaves and (B) the area of the youngest expanded leaf (rosette leaf before bolting and cauline leaf after bolting) in vernalized and non-vernalized chicory plants exposed to 35 °C/28 °C day/night for heat treatment or to 17 °C for control conditions. Different letters indicate significant differences at $P < 0.05$ between treatments for a same date.

plants exposed to heat treatment than in plants exposed to control conditions. At the end of the experiment, no significant differences were observed in the number of rosette leaves between vernalized and non-vernalized plants exposed to heat treatment and non-vernalized plants exposed to control conditions. However, heat reduced the area of the youngest expanded leaf in both vernalized and non-vernalized plants (Fig. 2B). The leaves of the plants exposed to high temperature were more rigid and curly compared to control plants, and some also showed necrotic spots and a purple coloration (data not shown).

After 84 days of growth under control conditions (17 °C), no bolting was recorded for non-vernalized plants, whereas all vernalized plants bolted (Fig. 3). Under heat conditions (35 °C), the bolting percentage reached 25% for non-vernalized plants and 35% for vernalized plants after 84 days of treatment (Fig. 3). Thus, heat increased the flowering percentage of non-vernalized plants by 25% but decreased that of vernalized plants by 65%. Bolting started between 28 and 42 days after the start of treatment depending on the temperature conditions (Fig. 3). Plants bolted after production of 8.6 ± 0.51, 39.5 ± 3.09 and 35.33 ± 3.84 rosette leaves among, respectively, vernalized plants at 17 °C, non-vernalized plants at 35 °C and vernalized plants at 35 °C. All bolted plants produced a floral stem and inflorescences.

3.1.2. *Heat affected floral stem development*

After bolting, plants initiated a floral stem bearing cauline leaves, lateral flowering branches and sessile capitula (Fig. 4A-C). Flowering plants produced 35–38 nodes on the main floral stem whatever the treatment (Table 1). Heat treatment did not affect the total number of nodes with cauline leaves, but it increased the number of nodes with lateral branches by 30% and decreased the number of nodes with sessile capitula by 40% on average (Table 1, Fig. 4C). However, the number of flowers per capitulum did not differ among treatments, averaging 17.44 (Table 1). At the end of the experiment, the dry weight of the main floral stem was on average 42% lower in vernalized and non-vernalized plants exposed to heat treatment than in vernalized plants under control conditions (Table 1). Moreover, the main floral stem was taller in vernalized control plants than in plants exposed to heat treatment (Fig. 4A). In heat-treated plants, no significant difference was recorded between the treatment groups. The main floral stem of heat-treated plants ceased its development after a short period, when around four secondary floral stems had appeared (Fig. 4A and C), as a consequence of apical dominance release.

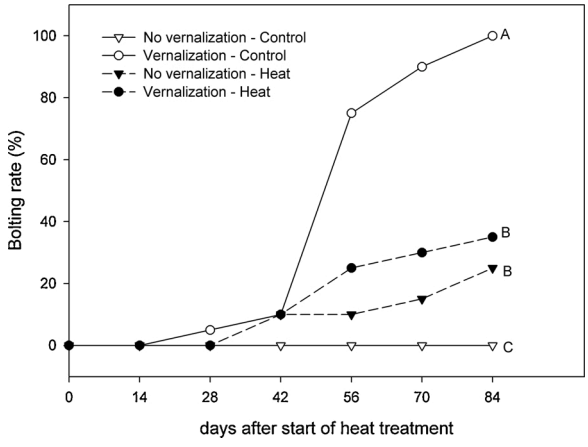

Fig. 3. Impact of vernalization and heat treatment on bolting rate in root chicory. Bolting rate in vernalized and non-vernalized chicory plants exposed to 35 °C/28 °C day/night for heat treatment or to 17 °C for control conditions. Different letters indicate significant differences at $P < 0.05$ between treatments at the end of the experiment.

3.1.3. *Heat reduced flower fertility*

High temperatures reduced stigmatic receptivity. The peroxidase and esterase activities were on average 82% lower in stigmas exposed to high temperature compared to those of plants in control conditions in both vernalized and non-vernalized plants (Table 2). Under heat treatment, stigmas of vernalized plants exhibited lower peroxidase and esterase activity than stigmas of non-vernalized plants.

High temperatures also reduced pollen production and viability. The number of pollen grains per floral bud was 25% lower in plants exposed to high temperature than in plants exposed to control conditions (Table 2). A lower pollen grain viability was also observed in the plants exposed to high temperatures, although pollen viability remained above 90% (Table 2) in all conditions. Vernalization had no impact either on pollen production or on pollen viability.

Impact of high temperature on expression of CiFL1

Shoot apices were observed in non-vernalized plants grown in greenhouses under control (mean temperature 23 °C) or heat condition (mean temperature 28 °C) (Fig. 1B, Fig. 5). We defined three different physiological stages of the shoot apices: the 'concave' apex corresponding to the vegetative state (Fig. 6A), the 'domed' apex corresponding to the start of bolting (Fig. 6B) and the 'flowering' apex with cauline leaves and the start of floral stack development (Fig. 6C). All control plants were in the vegetative stage until 14 weeks after treatment, and 18% and 22% of the shoot apices had begun swelling by 19 and 23 weeks after treatment, respectively (Fig. 6D). However, neither macroscopic bolting nor flowering was observed in control plants during the course of the experiment. In heat-treated plants, half of the apices were domed at 10 weeks after the start of treatment, and flowering apices were observed starting from the 19th week after the start of treatment. More than 80% of shoot apices were at the domed or flowering stages 19 and 23 weeks after the start of treatment, respectively. Macroscopic bolting was observed in heat-treated plants starting from the 16th week after start of treatment, and the macroscopic bolting percentage was 42% at the end of the experiment. Most bolted plants produced inflorescences.

The expression of *CiFL1* was higher in heat-treated plants than in control plants except at 6 weeks after the start of treatment (Fig. 7). In control plants, *CiFL1* expression remained low and stable throughout the experiment. By contrast, *CiFL1* expression increased over time in heat-treated plants before macroscopic bolting was observed (up to 14 weeks after start of treatment). Seventeen weeks after start of treatment, *CiFL1* expression was higher in non-bolted plants than in bolted plants subjected to heat treatment; *CiFL1* expression in bolted heat-treated plants was about the same than in control plants.

4. Discussion

4.1. *Heat induces bolting of non-vernalized plants but de-vernalization of vernalized plants*

As for most vegetative crops, controlling bolting and flowering is important in root chicory. Indeed, under field conditions, flowering must be avoided, to maximize root and inulin production (Dielen et al., 2005). However, for seed production and breeding purposes, flowering must be easily activated (Dielen et al., 2005). Root chicory is a biennial plant that has a vernalization requirement that must be met before it is competent to respond to long days for flowering initiation (Dielen et al., 2005). Our results confirmed its requirement for vernalization, as all vernalized plants grown at 17 °C bolted. A vernalization requirement is a common trait for the flowering of biennial plants, and other vegetative crops, such as sugar beet, carrot, cabbage, etc., have an obligatory cold requirement to be competent to flower under normal conditions (Wiebe, 1990). However, flowering is also induced by heat in root chicory, independently of vernalization (Dielen et al., 2005; Mathieu et al., 2014). Indeed, we observed that 25% of non-vernalized plants bolted when cultivated at high temperature, whereas no bolting was observed

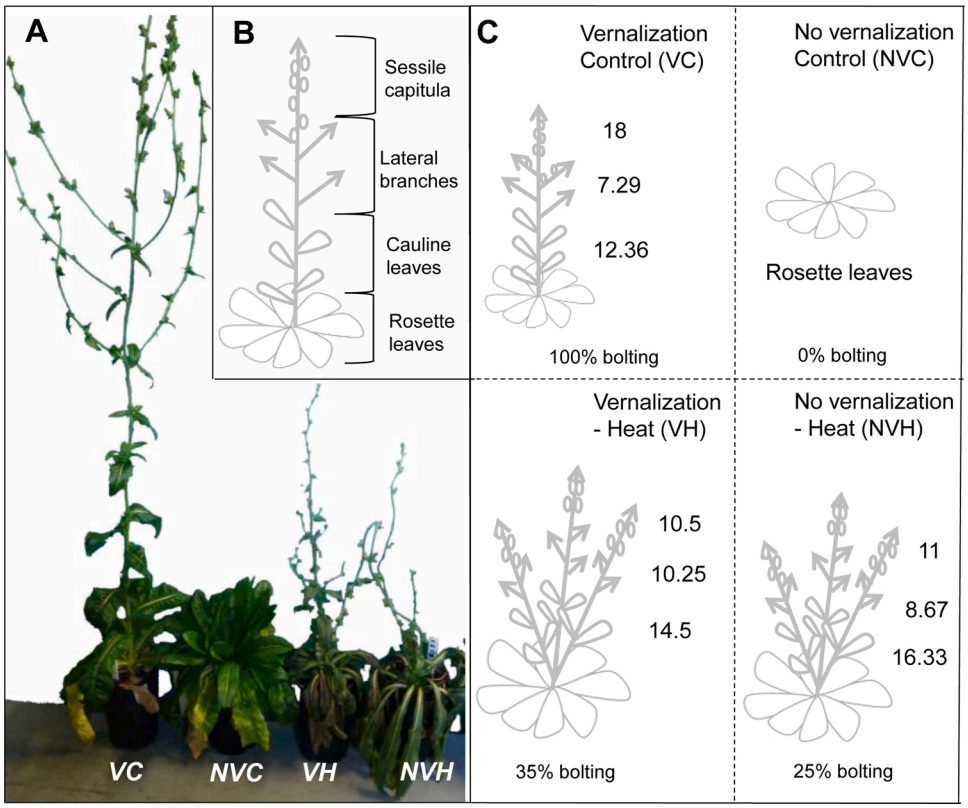

Fig. 4. Root chicory plants. (A) Vernalized and non-vernalized chicory plants exposed to 35 °C day/28 °C night for heat treatment or to 17 °C for control condition. VC: vernalized bolted plants in control condition; NVC: non-vernalized plants in control condition; VH: vernalized bolted plants in heat condition; NVH: non-vernalized bolted plants in heat condition. (B) Schematic representation of an adult bolted chicory plant. The adult bolted plant has rosette leaves and a floral stem bearing cauline leaves, lateral branches and nodes with sessile capitula. (C) Schematic representation of adult plant in each condition (VC, NVC, VH and NVH). The mean number of each type of node on the main floral stem and the percentage of plants that had bolted by the end of the experiment are indicated.

Table 1
Floral stem and capitulum structure in vernalized (6 weeks at 4 °C) and non-vernalized chicory plants exposed to 35 °C/28 °C (day / night) for heat treatment or to 17 °C for control condition.

Parameters	Control	Heat	
	Vernalization ($N = 16$)	Vernalization ($N = 6$)	No vernalization ($N = 5$)
Floral stem			
Nodes with cauline leaves	12.36 ± 0.74^{A}	14.5 ± 2.53^{A}	16.33 ± 6.67^{A}
Nodes with lateral flowering branches	7.29 ± 0.63^{B}	10.25 ± 1.89^{A}	8.67 ± 2.33^{AB}
Nodes with sessile capitula	18 ± 1.2^{A}	10.5 ± 2.53^{B}	11 ± 5.51^{B}
Total number of nodes	37.64 ± 1.37^{A}	35.25 ± 3.35^{A}	36 ± 8.39^{A}
Floral stem dry weight	26.37 ± 2.23^{A}	17.5 ± 1.38^{B}	13.2 ± 3.58^{B}
Capitulum			
Number of flowers per capitulum	18.27 ± 0.71^{A}	17.05 ± 0.53^{A}	17.00 ± 1.61^{A}

Different superscript letters indicate significant differences at $P < 0.05$. N indicates the number of bolting plants analyzed for each treatment.

Table 2
Flower fertility parameters of vernalized (6 weeks at 4 °C) and non-vernalized chicory plants exposed to 35 °C/28 °C (day/night) for heat treatment or to 17 °C for control condition.

Parameters	Control	Heat	
	Vernalization ($N = 10$)	Vernalization ($N = 10$)	No vernalization ($N = 10$)
Stigma receptivity			
Peroxidase activity	$100\%^{A}$	$0\%^{C}$	$16.7\%^{B}$
Esterase activity	$100\%^{A}$	$20\%^{C}$	$33.4\%^{B}$
Pollen			
Pollen grains per bud	4009.67 ± 481.11^{A}	3092.38 ± 473.22^{B}	2896.67 ± 175.47^{B}
Pollen viability	$97.27 \pm 0.48\ \%^{A}$	$93.31 \pm 1.39\ \%^{B}$	$94.39 \pm 0.5\ \%^{B}$

Different superscript letters indicate significant differences at $P < 0.05$. N indicates the number of flowers analyzed for each treatment.

for non-vernalized plants maintained at 17 °C. Our results nevertheless showed that the bolting percentage was lower after heat treatment than after vernalization and that heat-treated plants produced more rosette leaves before flowering than vernalized plants. These results suggest that both low and high temperatures can induce flowering in root chicory but that vernalization is a primary flowering signal, whereas heat is most likely an alternative flowering cue. Flowering induction by heat has not been deeply investigated in other biennial vegetative crops, to the best of our knowledge, but bolting induction under high temperature has been reported for sugar beet (Dally et al., 2018). Moreover, bolted sugar beet plants can be observed in the field during the heat of summer, as has previously been observed for root chicory (Dielen et al., 2005; Mathieu et al., 2014).

The observed flowering induction at 35 °C may be due to the stressful effect of high temperatures. Various stress factors, such as drought, poor nutrition, and high and low temperatures, induce flowering in different plants (Kazan and Lyons, 2016; Takeno, 2012; Wada and Takeno, 2010). Kazan and Lyons (2016) suggest that plants have the ability to sense the strength of stress factors and respond appropriately. If the intensity of stress is low, the plants delay flowering until the return of optimal conditions. If the intensity of stress is high, however, flowering is accelerated to ensure reproduction, even at the expense of yield (Kazan and Lyons, 2016). Thus, the induction of flowering can be the ultimate adaptation to stress, as flowering results in reproduction and seed production that ensures the survival of the species (Kazan and Lyons, 2016; Takeno, 2016). In plants, a stress can be defined as a situation in which growth and development are inhibited (Lichtenthaler, 1998). In a previous experiment, we observed that high temperature reduced growth and yield in root chicory (Mathieu et al., 2014), and we made similar observations in the experiments reported here. We could thus hypothesize that the observed flowering is due to a stress effect induced by high

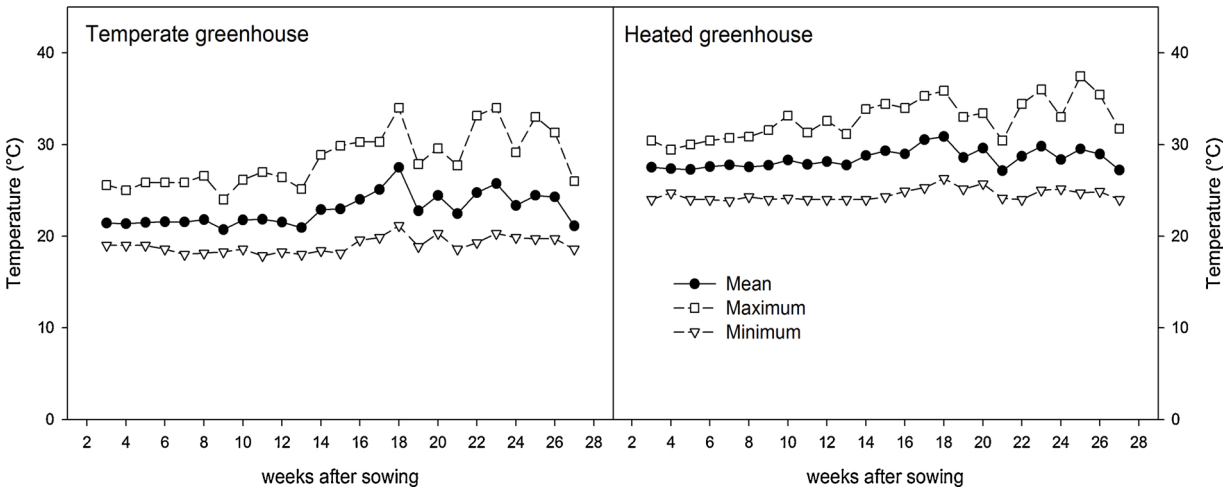

Fig. 5. Temperatures in the temperate greenhouse for control conditions and in the heated greenhouse for heat treatment. Weekly average of the mean, maximum and minimum daily temperatures.

temperatures. However, we cannot exclude the possibility that heat is an alternative treatment that can more directly supersede vernalization to induce flowering in root chicory, as was previously observed for cytokinin, ethylene and anoxia treatments (Joseph et al., 1985; Michniewicz and Kamieńska, 1964). For example, in Arabidopsis, it has been recently shown that fluctuating warm temperatures can overcome a vernalization requirement (Burghardt et al., 2015).

Although heat can induce flowering in root chicory, it has the opposite effect when combined with vernalization. Thus, we observed that the bolting rate of vernalized plants decreased by 65% when they were cultivated at 35 °C rather than 17 °C. High temperature thus induced de-vernalization, as previously observed by Périlleux et al. (2013). In root chicory, temperatures above 25 °C result in de-vernalization, whereas temperatures below 20 °C stabilize the vernalized state (Périlleux et al., 2013). A de-vernalization effect due to exposure to high temperature just after vernalization is known in many species, including Arabidopsis, sugar beet, radish, carrot, cabbage and onion (Bouché et al., 2015; Trap-Gentil et al., 2011; Wiebe, 1990; Yamasaki et al., 2000). The temperature at which de-vernalization occurs depends on the species, but de-vernalization is observed at as low as 20 °C in several species (Wiebe, 1990).

Our results thus show that high temperature can induce flowering of non-vernalized plants and inhibit flowering of vernalized plants in root chicory. Consequently, the recent increase in the frequency of summer heat waves due to climate change would be expected to negatively affect root chicory production. Indeed, in the field, bolting rates due to high temperature are expected to increase in the coming years, leading to a decrease of inulin production. Moreover, if vernalized plants are transferred to the field for seed production, heat will induce de-vernalization, leading to decreased seed production. It is likely that such assumptions will also hold true for other vegetative biennial crops. However, the mechanisms underlying this effect are not fully understood.

4.1.1. Flowering induction by vernalization and by high temperatures may both involve CiFL1

Périlleux et al. (2013) isolated a root chicory MADS-box gene that belongs to the same clade as the Arabidopsis *FLC*, *FLM* and *MAF* genes and called it *FLC-LIKE 1* (*CiFL1*). They showed that *CiFL1* was repressed by vernalization, like *FLC* in Arabidopsis. By contrast, the abundance of *CiFL1* transcripts increased upon return to 'normal' or 'high' temperature: i.e., repression of *CiFL1* was not stably maintained after vernalization. In this work, we investigated the expression of *CiFL1* in response to heat treatment. We observed that heat increased *CiFL1* expression as compared to the control temperature. This increase was correlated with an increase in the percentage of domed and flowering shoot apices. An increase of *CiFL1* expression in response to high temperature was also observed by Mathieu et al. (2020). Although our results do not prove a link between *CiFL1* expression and bolting, we may not rule out a role for this gene in the floral transition in response to heat in root chicory. However, *CiFL1* expression seemed to decrease after floral transition and bolting since the amount of *CiFL1* transcripts was lower in bolted than in non-bolted plants 17 weeks after start of heat treatment. This may suggest that *CiFL1* could be required for floral transition but not for stalk development. Further analyses will be required to determine the exact role of *CiFL1* during floral transition in response to temperature in root chicory. In Arabidopsis, the *FLC* pathway has been suggested to modulate a multifaceted response to fluctuating temperature (Burghardt et al., 2015). It would thus not be surprising that *CiFL1* plays a role in flowering control by temperature in root chicory.

In Arabidopsis, the induction of flowering by elevated temperature is controlled especially by *FLM* (Posé et al., 2013). As *CiFL1* belongs to the same clade as *FLM*, we hypothesize that *CiFL1* may act like *FLM* in Arabidopsis. *FLM* undergoes alternative splicing depending on the temperature, and this alternative splicing is involved in the control of flowering (Lee et al., 2013; Posé et al., 2013; Verhage et al., 2017). It was initially suggested that the two main FLM protein splice variants, FLM-β and FLM-δ, compete for interaction with the floral repressor SVP (Posé et al., 2013). However, recent experimental evidence has cast doubt on the role of FLM-δ *in planta*, and it now appears that the control of flowering by ambient temperature depends mainly on the abundance of the SVP/FLM-β complex (Lutz et al., 2015; Theißen et al., 2018). It is thought that the SVP/FLM-β complex represses flowering at low temperature, and higher temperature destabilizes the SVP protein (Theißen et al., 2018). In addition to *FLM*, other *MAF* genes appear to be involved in the control of flowering by ambient temperature, and at least some of their gene products undergo temperature-dependent alternative splicing (Theißen et al., 2018; Verhage et al., 2017). FLC-like proteins may form part of higher-order complexes that integrate responses to endogenous and external cues to control flowering by targeting *FT* and *SOC1* in Arabidopsis (Theißen et al., 2018). *CiFL1* may play similar role in root chicory. Since our PCR experiment did not allow us to discriminate different transcripts, it would be interesting to explore the possible alternative splicing of *CiFL1* in response to temperature. Moreover, other genes such as *SVP* and *PIF4* are involved in the control of flowering by ambient temperature in Arabidopsis (Balasubramanian et al., 2006; Capovilla et al., 2015; Jagadish et al., 2016; Kumar et al., 2012; Lee et al., 2013; Posé et al., 2013; Theißen et al., 2018). Homologues of these genes have not yet been described in root chicory. The control of

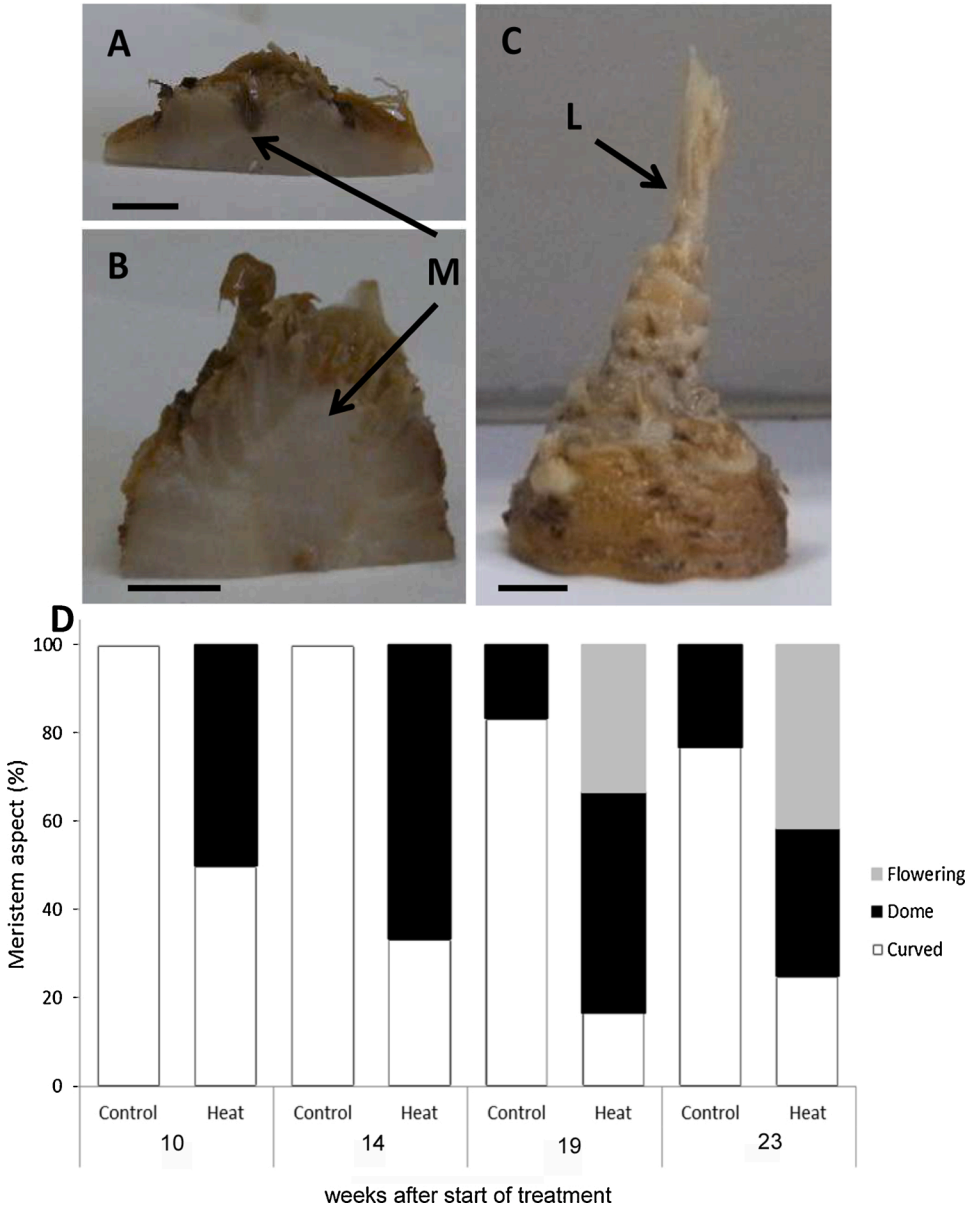

Fig. 6. Morphology of *Cichorium intybus* shoot apices. (A) A 'concave' apex that corresponds to the vegetative state, (B) a 'domed' apex that corresponds to the start of bolting process and (C) the 'flowering' apex with cauline leaves and the start of floral stack development. L: cauline leaves, M: meristem. Bars =1 cm. (D) Shoot apex state in chicory plants cultivated in control condition (mean temperature 23 °C) or in a heated greenhouse (mean temperature 28 °C). Heat treatment started 4 weeks after sowing, and shoot apices were harvested 10, 14, 19 and 23 weeks after the start of treatment.

flowering by temperature in root chicory is most probably a complex process that involved several genes in addition to *CiFL1* and yet to be discovered.

4.2. Heat affects inflorescence production and decreases flower fertility

Heat induces bolting in root chicory, but it also affects floral stem development. Indeed, the dry weight and size of the floral stem were both reduced by heat in our experiment. Moreover, although the total number of nodes on the main floral stem was unaffected by heat, the proportion of each type of node was modified: the number of nodes with sessile capitula was reduced and the number of nodes with lateral branches was increased. As a result, the total number of capitula was reduced in plants exposed to heat compared to vernalized control plants. Nevertheless, the number of flowers per inflorescence was not affected by heat. Modifications of plant architecture and inflorescence production in response to high temperature have also been observed in other plants. For example, high temperatures reduce the number of flowering branches and thus the number of flowers or flower buds per plant in *Brachypodium distachyon* (Harsant et al., 2013) and in *Borago officinalis* (Descamps et al., 2018). In *Phaseolus vulgaris*, however, heat increases the number of flowers, although the number of fruit is not increased (Konsens et al., 1991). Heat also induced a release of apical dominance in our study, so that plants exposed to heat developed several floral stems, whereas vernalized control plants produced only one floral stem. In the same way, we also observed development of axillaries in non-bolted heat-treated plants (data not shown). Such axillary development at high temperature was previously observed in root chicory (Mathieu et al., 2014) and in other species such as *B. officinalis* (Descamps et al., 2018). Thus, in root chicory, heat induces release of apical dominance and modifies floral stem architecture, thereby affecting inflorescence production, so that heat induction of flowering is negatively correlated with inflorescence abundance.

In regard to flower fertility, heat decreased pollen grain number, pollen viability and stigmatic receptivity in our experiment. It is well known that the male gametophyte (pollen) is sensitive to high temperature (Bita and Gerats, 2013; Borghi et al., 2019; Djanaguiraman et al., 2018; Erickson and Markhart, 2002; He et al., 2017; Sage et al., 2015). Reduction of pollen grain number and pollen viability by heat is observed in many species. For example, several studies of crops such as

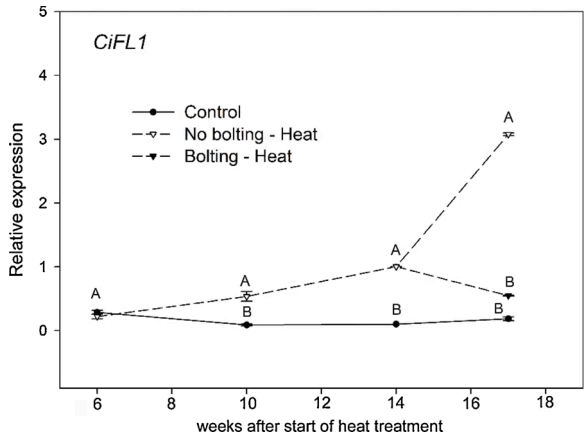

Fig. 7. Impact of heat treatment on *CiFL1* expression. Transcript levels were analyzed by real-time PCR in leaves of control and heat-treated plants 6, 10, 14 and 17 weeks after start of treatment. Heat treatment was applied 4 weeks after sowing. Bolting was only observed macroscopically from 16 weeks after start of treatment in heat-treated plants only, so at 17 weeks after start of treatment, expression of *CiFL1* was analyzed in bolted and non-bolted plants from the heat-treated group. Data were normalized using *CiTUB* as internal control. Different letters indicate significant differences at $P < 0.05$.

rice, sorghum and soybean have shown that the yield loss induced by high temperature is correlated with a decline in pollen production or viability (Djanaguiraman et al., 2013; Maruyama et al., 2013; Prasad et al., 2006). Heat can also negatively affect pollen maturation, pollen germination and pollen tube growth (Hedhly, 2011; Hedhly et al., 2009; Müller and Rieu, 2016). More analyses are required to assess whether these parameters are altered by heat in root chicory. Indeed, the sensitivity of flowers to high temperature depends on their developmental stage (Erickson and Markhart, 2002). Heat can also negatively impact the pistil in different ways, such as by altering stigma receptivity (Djanaguiraman et al., 2018; He et al., 2017). For example, Hedhly et al. (2003) observed a reduction of stigma receptivity by elevated temperature in sweet cherry (*Prunus avium* L.). However, pistil development is usually less sensitive to high temperature than stamen development (Erickson and Markhart, 2002; Monterroso and Wien, 1990; Warrag and Hall, 1984). These combined effects of heat on stigma receptivity and pollen viability are critical for the pollen-pistil interaction and thus can lead to pollination failure (Borghi et al., 2019; He et al., 2017). Heat may also affect post-pollination processes and inhibit fertilization and early seed development (Erickson and Markhart, 2002; Konsens et al., 1991). As root chicory is self-incompatible (Gonthier et al., 2013; Kiers-van der Steen, 2000), cross-pollination would be required to investigate whether fertilization, seed development and seed set occur under high temperature. Although root chicory is cultivated mainly for inulin production, these parameters are important for seed production and cultivar improvement. In nature, root chicory is mainly pollinated by insects (Funk et al., 2009). So, flower attractiveness to pollinators is also important to investigate. Heat also affects floral traits and floral rewards such as flower size, flower longevity, flower color, floral scents and nectar and pollen production (Borghi et al., 2019; Descamps et al., 2018; Dudley et al., 2018). In *B. officinalis*, for instance, heat-stressed plants are less visited by insects because of a reduction of pollen and nectar production (Descamps et al., 2018). Low seed set could thus be due to both low flower fertility and low insect visitations. These parameters remain to be investigated in root chicory.

5. Conclusions

In conclusion, heat had contrasting effects on flowering induction in root chicory: it induced bolting and flowering in non-vernalized plants, whereas it induced de-vernalization and suppressed flowering of vernalized plants. However, the genetic control of such flowering induction and repression is far from being understood. It was previously shown that *CiFL1* is repressed by vernalization in root chicory, and our results suggest that it could also be involved in heat-induced flowering. Later on, heat had a mainly negative impact on flower development by altering floral stalk development, decreasing the number of inflorescences and decreasing flower fertility. Together, our results suggest that future hot summers expected in the context of climate change may negatively affect root chicory by on the one hand increasing bolting in the field and subsequently reducing root growth and inulin production, and on the other hand decreasing flower production and floral fertility for seed production. This situation is most probably not limited to root chicory and could also be true for other biennial vegetative crops.

Author contributions

SL, MQ, AM and CP conceived and designed the research. AM, MR, GJ conducted experiments. AM and MQ analyzed data. AM, MQ, SL, CP wrote the manuscript. All authors read and approved the manuscript

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Declaration of Competing Interest

The authors report no declarations of interest.

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