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Phytohormone profile and *CiFL1* expression in young seedlings of *Cichorium intybus* L. var *sativum* exposed to high temperature in relation to vernalization and de-vernalization processes

A.S. Mathieu[a], P.I. Dobrev[b], D. Tarkowská[c], J. Pospíšil[c], V. Motyka[b], G. Jacquemin[a], M. Quinet[a], S. Lutts[a,*]

[a] Groupe de Recherche en Physiologie végétale, Earth and Life Institute – Agronomy (ELI-A), Université catholique de Louvain, 5 (Bte 7.07.13) Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium
[b] Institute of Experimental Botany of the Czeck Academy of Sciences, Rozojová 263, CZ-165 02 Prague-Lysolaje, Czechia
[c] Laboratory of Growth Regulators, Institute of Experimental Botany of the Czech Academy of Sciences & Palacky University, Šlechtitelu 19, CZ-783 71 Olomouc, Czechia

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ABSTRACT

Cichorium intybus is a biennial plant species forming a taproot and a leaf rosette during the first year and which requires low temperature during vernalization to flower during the second year of its cycle. Heat stress, however, is known to induce premature flowering during the first year. The present work aims to compare the effect of heat treatment (38 °C during 3 days) and vernalization (4 °C during 6 weeks) on the phytohormonal profile and the expression of two chicory genes (*CiFL1* and *CiFT*) homologous to the Arabidopsis flowering genes *FLOWERING LOCUS C* (*FLC*) and *FLOWERING LOCUS T (FT)*. In Arabidopsis, *FLC* and *FT* code, respectively, for a repressor and an activator of flowering. Although *CiFL1* was inhibited by vernalization, its expression was increased by heat treatment in both vernalized and non-vernalized seedlings while high temperature unexpectedly inhibited *FT* expression in vernalized seedlings. Vernalization induced a modification in the hormonal profile of *C. intybus* (cv. Melci) in a sense of a decrease in ethylene production, abscisic acid and total jasmonates content, while the level of salicylic and benzoic acids as well as polyamine spermidine increased. A limited effect on the gibberellins' profile was observed. The subsequent impact of heat stress on phytohormone content was different in non-vernalized and vernalized seedlings, with a higher abscisic acid and jasmonates and a lower 1-aminocyclopropane-1-carboxylic acid concentration in the former than in the latter. These data are discussed in relation to the putative involvement of phytohormones in stress-induced flowering. It appears that heat stress implies different pathways than vernalization to hasten flowering process.

1. Introduction

Root chicory (*Cichorium intybus* L. var *sativum*) is a biennal plant species which forms a leaf rosette and a taproot during the first growing season and then bolt and flower during the second growing season after vernalization occurred during winter period. The plant stores polysaccharide inulin as a very rich energy source in its taproot during the first growing season and is therefore cultivated as an annual to avoid bolting which reduces the inulin yield. However, early bolting could be observed in field condition during the first year mainly due to vernalization resulting from low temperatures occurring during early spring just after sowing (Dielen et al., 2005).

Although root chicory requires vernalization for flowering initiation (Pimpini and Gianquinto, 1988; Gianquinto, 1997), high temperature could also induce bolting and flowering in this species independently of vernalization (Mathieu et al., 2014, 2018). Paradoxically, high temperature may also have a de-vernalization effect by suppressing flowering in vernalized plants (Périlleux et al., 2013). Hence, high temperature has a dual impact, promoting stress-induced flowering and suppressing it through a de-vernalization process.

Abbreviations: ABA, abscisic acid; BA, benzoic acid; BRs, brassinosteroids; *cis*-OPDA, *cis*-(+)-12-oxo-phytodienic acid; CKs, cytokinins; DHZ, dihydrozeatin; DPA, dihydrophaseic acid; GAs, gibberellins; IAA, indole-3-acetic acid; IAA-Asp, IAA-aspartate; IAAox, indole-3-acetaldoxime; JA, jasmonic acid; JA-Ile, (+)-*7-iso*-jasmonoyl-L-isoleucine; SA, salicylic acid; DEPC, diethyl pyrocarbonate; EDTA, ethylenediamine tetraacetic acid; oxIAA, 2-oxindole-3-acetic acid; PA, phaseic acid; PAs, polyamines; PAR, Photosynthetic active radiation; PCA, principal component analysis; PVPP, polyvinylpyrrolidone; Put, putrescine; Spd, spermidine; Spm, spermine

* Corresponding author.
E-mail address: stanley.lutts@uclouvain.be (S. Lutts).

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The molecular control of flowering induction by vernalization and by high temperatures in root chicory remains poorly understood. In *Arabidopsis thaliana*, vernalization implies the down-regulation of the gene *FLOWERING LOCUS C* (*FLC*) which normally acts as a suppressor of flowering by inhibiting the expression of the flowering integrator genes *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) (Michaels and Amasino, 1999; Sheldon et al., 1999; Helliwel et al., 2006). The control of flowering by elevated growth temperature in Arabidopsis involves two mechanisms (Capovilla et al., 2015): one is based on the transcription factor *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*), and the other involves a complex between proteins encoded by *FLOWERING LOCUS M* (*FLM*) and *SHORT VEGETATIVE PHASE* (*SVP*) (Balasubramanian et al., 2006; Lee et al., 2013; Posé et al., 2013; Kumar et al., 2012; Capovilla et al., 2015). In root chicory, a MADS box sequence similar to *FLC* and *FLM* has been identified and named *FLC-LIKE 1* (*CiFL1*) (Périlleux et al., 2013). Vernalization represses *CiFL1* expression in root chicory and overexpression of *CiFL1* in Arabidopsis delays flowering (Périlleux et al., 2013).

High temperature was recently reported to negatively affect plant growth in chicory and to reduce sugar translocation from leaves to root leading to a decrease in inulin production (Mathieu et al., 2014, 2018). Several phytohormones are involved in the plant response to heat stress. Generally, ethylene, abscisic acid (ABA) and salicylic acid (SA) are rapidly synthesized following heat stress while cytokinins (CKs), auxin and gibberellins (GAs) contents decrease (Larkindale and Huang, 2005). Polyamines (PAs) titers could also increase in plant exposed to high temperature and these compounds are reported to assume key protective roles in response to heat stress (Sagor et al., 2013; Glaubitz et al., 2015).

Hormones, such as CKs and ethylene were suspected to be directly involved in flowering of root chicory (Joseph et al., 1985). In other biennal species, GAs can also substitute for vernalization (Lang, 1957; Mutasa-Göttgens and Hedden, 2009) and promote bolting (Lang, 1957). Depending on the species, ethylene could induce or repress flowering (Abeles, 1973,1992). In the rosette plant spinach, Crèvecoeur et al. (1986, 1988) have shown that there was a burst of ethylene release when plants were induced to flower by long days. In radish and pea seedlings, ethylene release increased gradually during vernalization (Suge, 1977). Jasmonates (JA) (Pedranzani et al., 2003) and SA (Takeno, 2016) are also involved in stress-induced flowering. In *Pharbitis nil*, auxins and PAs are involved in the induction of flowering by stress (Koshio et al., 2015).

It is still unclear if vernalization process induces a modification in the hormonal profile in *Cichorium intybus* and if heat-induced de-vernalization also involves a modification in the phytohormonal status. Similarly, the promoting impact of high temperature on flowering may rely on modifications in gene expression, phytohormonal content or both processes but data regarding *C. intybus* are crucially lacking and the impact of high temperatures on vernalized and non-vernalized plants were never compared. The present study was therefore undertaken in order to compare the impact of high temperature on the expression of *CiFL1* and phytohormonal content in vernalized and non-vernalized young seedling.

2. Materials and methods

2.1. Plant material and growing conditions

Experiments were conducted in growth chambers on chicory seedlings (*Cichorium intybus* L. var. *sativum*) cultivar Melci (kindly provided by Warcoing S.A.). Young seedlings were exposed to four treatments as shown in Fig. 1: (1) non-vernalized plants grown at 17 °C (control condition), (2) vernalized plants grown at 17 °C (control condition), (3) non-vernalized plants grown at 38 °C (heat treatment) and (4) vernalized plants grown at 38 °C (heat treatment). Seeds were sown on a substrate containing sand and loam (3:1, v/v) in small tube of PVC (8 cm high and 2.5 cm diameter) and incubated in a growth chamber at 4 °C during 6 weeks for the vernalization treatment (Dielen et al., 2005; Périlleux et al., 2013) or at 17 °C during 3 weeks for the non-vernalization treatment. All vernalized seedlings were then maintained in a growth chamber at 17 °C during 1 week before application of heat treatment. Vernalized seedlings slowly grew when exposed at 4 °C; after one week of exposure to 17 °C, they reach exactly the same stage as non-vernalized one (three unfolded leaves). Half of vernalized and non-vernalized plants were then maintained under a constant temperature of 17 °C for the control condition while the other half were exposed to 38 °C for the heat treatment. In all cases, plants were maintained at 16 h of photoperiod, 70 % relative humidity and exposed to 150 µmol m^{-2} s^{-1} PAR (provided by Sylvania VHO 250 W) at the top of the canopy. On the Fig. 1, arrows represent time when measurement and harvests were performed depending on the analyses. A sub-sample of 80 plants remained unharvested under these conditions for 12 weeks in order to estimate the bolting percentages which were 0% for non-vernalized plants in control condition, 100 % for vernalized plants in control condition, 25 % for non-vernalized plants at 38 °C and 30 % for vernalized plants at 38 °C (supplemental data Fig. S1).

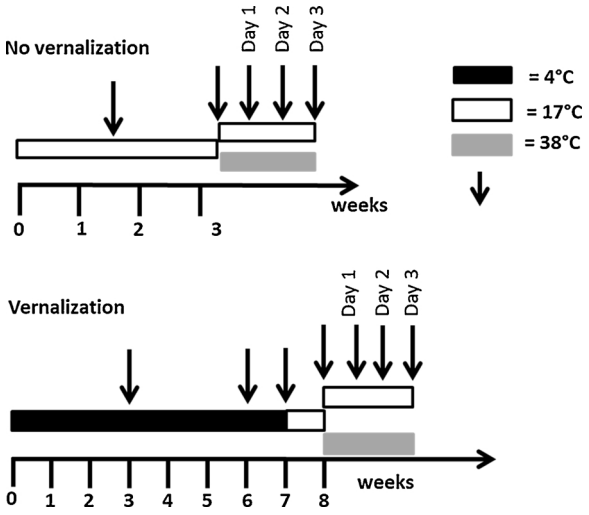

Fig. 1. Schematic description of the experiment conducted in controlled environmental conditions on root chicory (*Cichorium intybus* var *sativum*) seedling exposed to four conditions: no vernalization – control condition (grown at 17 °C), no vernalization – heat treatment (grown at 38 °C), vernalization – control (grown at 17 °C) and vernalization – heat treatment (grown at 38 °C). Seeds of chicory were sown at 4 °C during 6 weeks for the vernalization treatment or at 17 °C during 3 weeks for the non-vernalization treatment. Afterwards, vernalized seedlings were maintained in a growth chamber at 17 °C during 1 week before application of heat treatment at 38 °C. Arrow represent time when measures and harvests were performed depending on the analyses.

2.2. Gene expression

As indicated in Fig. 1, RNA extractions were performed on leaves harvested 10 days before the imposition of the heat treatment and 0, 1, 2 and 3 days after start of heat treatment for the non-vernalized seedlings or at 35, 14, 7 days before start of heat treatment and 0, 1, 2 and 3 days after start of heat treatment for the vernalized seedlings.

For each sample, 500 mg fresh material was ground in liquid nitrogen to extract total RNA. Seven mL of pre-heated (65 °C) extraction buffer (300 mM Tris HCl (pH 8.0), 25 mM EDTA (pH 8.0), NaCl 2 M, 2% (w/v) CTAB, 0.05 % (w/v) spermidine, 2% (w/v) polyvinylpyrrolidone (PVPP) and 2 % (w/v) β-mercaptoethanol added just before use) were added to the powder and samples were incubated for 10 min at 65 °C. Thereafter, the samples were centrifuged during 15 min at 4000 *g* and 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 vol of sodium acetate (3 M pH 5.2) and 0.6 vol of isopropanol were added to the supernatant to precipitate the RNA. After 30 min incubation at −80 °C and centrifugation (30 min, 8400 *g*, 4 °C), the pellet was dissolved in 1 mL of TE buffer. Then 0.3 vol of LiCl (10 M) was added and the samples were incubated at 4 °C during 12 h. After centrifugation at 4 °C 8400 *g* for 30 min, the pellet was washed with ethanol 70 %, dried and resuspended in 20 µL of DEPC-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 the manufacturer's instructions. Transcript levels were quantified in three independent qPCR (in triplicates for each PCR) using the GoTaq qPCR Master Mix (Promega) on a StepOnePlus Real-Time PCR system (Applied Biosystems) according to the procedure described by the supplier. PCR reactions were performed in triplicates using 0.2 µM of each primer, 5 µL of the 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 condition 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) and the primers *CiFTF* (5′-CGTTGGTT GTTGGACGTGTGATAGG-3′) and *CiFTR* (5′-CATACACAGTTTGTCGAC CCA–3′) were used to amplify a 381 bp PCR product for *CiFT* (sequence identified as described in supplement data including Fig.S2 and Fig.S3). Data were normalized according to *CiTUB* (AF101419) gene expression levels. The primers used were *CiTUBF* (5′- AGTTCTGGGAGGTTGTCTGC -3′) and *CiTUBR* (5′ − CCTTCGCCCAATTGTTACCG -3′) that 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). Expression of *CiFT* was also analyzed by RT-PCR on the cDNA synthetized for the main experiment as described in the material and methods and with the same primer than for the qPCR analysis. Amplifications were conducted using GoTaq DNA polymerase (Promega Benelux b.v., Leiden, The Netherlands) with 33 cycles and 57 °C of annealing temperature. The PCR products were resolved on agarose gels and expression differences were analyzed by gel densitometry using ImageJ software and expressed as relative values compared to *CiTUB* (same primer as for the qPCR analysis).

2.3. Total soluble sugar analysis

Leaf samples (*c.a.* 400 mg FW) were ground to a fine powder in liquid nitrogen and extracted three times with 4 mL of 70 % ethanol and centrifuged during 15 min at 6600 *g*. For total soluble sugar quantification, 200 µL of the pooled supernatant were added to 1 mL of anthrone solution (0.5 *g* anthrone, 250 mL H_2SO_4 95 % and 12.5 mL distilled water) during 10 min at 100 °C according to Yemm and Willis (1954). The absorbance was read at 625 nm (Spectophotometer UV,1800, Shimadzu, Kyoto, Japan) and a standard curve was established using glucose.

2.4. Polyamines quantification

Free polyamines (putrescine (Put), spermidine (Spd) and spermine (Spm)) were extracted and dansylated according to Quinet et al. (2014) from *c.a.* 500 mg FW of leaves. Samples were re-suspended in methanol, filtered (Chromafil PES-45/15, 0.45 µm; Macherey-Nagel, Duren, Germany) and injected onto a Nucleodur C18 Pyramid column (125 × 4.6 mm internal diameter, 5 µm particle size; Macherey-Nagel) maintained at 40 °C. Analyses were performed by a Shimadzu HPLC system coupled to a RF-20A fluorescence detector (Shimadzu,'s-Hertogenbosch, The Netherlands) with an excitation wavelength of 340 nm and an emission wavelength of 510 nm. The mobile phase consisted of a water/acetonitrile gradient from 40 % to 100 % acetonitrile and the flow rate was 1.0 mL min^{-1}.

2.5. Phytohormone analysis

For ethylene measurement, four seedlings were placed on two layers of filter paper Whatman n°1 moistened with Hoagland nutrient solution in a 1 L glass bottle tightly closed by a plexiglass plate with an inlet and an outlet for gas flow. The system was connected to an ethylene detector ETD-300 (Sensor Sense, Nijmegen, The Netherlands) to estimate the ethylene production through a photo-acoustic method (Cristescu et al., 2002). The measurements were conducted in continuous flow mode with each bottle being alternatively flushed with a flow of 4 L h^{-1} during 20 min over a total period of 24 h. A bottle without plant was used as reference and its ethylene level was subtracted from the emission rates obtained. Measurements were performed with 6 experimental repetitions per condition and analyzed with the Valve Controller software. Ethylene production was expressed as nanoliters per hour per g of plant FW.

Other phytohormones (except GAs) including ABA, auxins, SA, JA, BRs, benzoic acid (BzA), CKs and their metabolites were extracted and purified from fresh chicory leaves according to Dobrev and Kamínek (2002) and Dobrev and Vankova (2012). One hundred mg FW sample was homogenized with liquid nitrogen and extracted with 0.5 mL of extraction buffer (methanol/formic acid/water (15:1:4, by volume; cooled to −20 °C). The following internal standards (10 pmol per sample) were added to the extract: $^{13}C_6$-indole-3-acetic acid ($^{13}C_6$-IAA; Cambridge Isotope Laboratories), [$^2H_5$] indole-3-acetyl-L-[^{15}N] aspartic acid, [$^2H_5$]indole-3-acetaldoxine (DN-IAAOx) (Olchemim), $^2H_4$-SA (Sigma-Aldrich), $^2H_6$-ABA (NRC-PBI), $^2H_3$-phaseic acid ($^2H_3$-PA;NRC-PBI), $^2H_3$-DPA (NRC-PBI), $^2H_5$-JA (C-D-N Isotopes Inc.), $^2H_2$N-[(-)-jasmonyl-isoleucine, [$^2H_5$]*cis*-12-oxo-phytodienoic acid (Olchemim), $^2H_5$-*trans*Z, $^2H_5$-*trans*ZR, $^2H_5$-*trans*Z7G, $^2H_5$-*trans*Z9G, $^2H_5$-*trans*ZOG, $^2H_5$-*trans*ZROG, $^2H_5$-*trans*ZRM, $^2H_3$-DHZR, $^2H_3$-DHZ9G, $^2H_6$-iP, $^2H_6$-iPR, $^2H_6$-iP7G, $^2H_6$-iP9G, $^2H_6$-iPRMP (all CKs standards are from Olchemim: the system of CK abbreviations adopted was established by Kamínek et al. (2000)). After 1 h extraction at −20 °C solids were separated by centrifugation (15,000 *g*, 15 min) and re-extracted for 30 min at −20 °C with 0.5 mL of the same extraction solvent. Combined supernatants were purified using the dual-mode solid-phase method (Dobrev and Kamínek, 2002). Two phytohormone fractions were obtained: fraction A-containing acidic and neutral compounds (auxins, BRs, ABA, SA, JA), and fraction B-containing basic compounds (CKs and immediate precursor of ethylene 1-aminocyclopropane-1-carboxylic acid (ACC))). Hormonal quantification was performed by HPLC (Ultimate 3000, Dionex) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP; Applied Biosystems) as described previously (Djilianov et al., 2013) using isotope dilution method with multilevel calibration curves ($r^2 > 0.99$). Data processing was carried out with Analyst 1.5 software (Applied Biosystems).

For specific quantification of GAs the sample preparation and analysis was performed according to the method described in Urbanova et al. (2013) with some modifications. Briefly, tissue samples of about 20 mg FW were ground to a fine consistency using 3-mm zirconium oxide beads (Retsch GmbH & Co. KG, Haan, Germany) and a MM 301 vibration mill at a frequency of 30 Hz for 3 min (Retsch GmbH & Co. KG, Haan, Germany) with 1 mL of ice-cold 80 % acetonitrile containing 5 % formic acid as extraction solution. The samples were then extracted overnight at 4 °C using a benchtop laboratory rotator Stuart SB3 (Bibby Scientific Ltd., Staffordshire, UK) after adding 17 internal gibberellins

standards ([$^2H_2$]GA_1, [$^2H_2$]GA_3, [$^2H_2$]GA_4, [$^2H_2$]GA_5, [$^2H_2$]GA_6, [$^2H_2$]GA_7, [$^2H_2$]GA_8, [$^2H_2$]GA_9, [$^2H_2$]GA_{15}, [$^2H_2$]GA_{19}, [$^2H_2$]GA_{20}, [$^2H_2$]GA_{24}, [$^2H_2$]GA_{29}, [$^2H_2$]GA_{34}, [$^2H_2$]GA_{44}, [$^2H_2$]GA_{51} and [$^2H_2$]GA_{53}) purchased from OlChemIm, Czech Republic. The homogenates were centrifuged at 36,670 *g* and 4 °C for 10 min, corresponding supernatants further purified using reversed-phase and mixed mode SPE cartridges (Waters, Milford, MA, USA) and analyzed by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS; Micromass, Manchester, UK). GAs were detected using multiple-reaction monitoring mode of the transition of the ion $[M-H]^-$ to the appropriate product ion. Masslynx 4.1 software (Waters, Milford, MA, USA) was used to analyze the data and the standard isotope dilution method was used to quantify the GAs levels.

2.6. Statistical analysis

Normality tests were performed using a Shapiro-Wilk and Levene's test. No further transformation of the raw data was required. The ANOVA 2 model was defined to evaluate the effects of the vernalization and heat treatment. The ANOVA 1 model was defined to evaluate the effects of heat treatment inside vernalized or non-vernalized plants. Following ANOVA, post-hoc analysis was performed at a significance level of $P < 0.05$ according to Student-Newman Keuls test. Data were analyzed using SAS Enterprise Guide 6.1 (SAS 9.4 system for windows). Differences between plant groups (vernalized *versus* non-vernalized) and treatment (temperature) were also visualized using principal component analysis (PCA) with R 3.2.2 Statistics software ('FactoMineR' package) and Pearson correlations between analyzed parameters were performed using the 'corrplot' package. If not indicated otherwise data are presented as means ± SE.

3. Results

3.1. Gene expression

As shown in Fig. 2, *CiFL1* expression increased in seedlings exposed to heat treatment for both vernalized and non-vernalized plants. In non-vernalized plants, *CiFL1* expression was significantly higher in heat-treated plants than in control after two days of heat treatment. In vernalized plants, *CiFL1* expression decreased during the vernalization period until 14 days before the imposition of heat treatment. After the imposition of heat treatment, *CiFL1* expression decreased in control plants and increased in plants exposed to high temperature. *CiFL1* expression was significantly higher in heat treated plants than in control plants after three days of heat treatment.

Fig. 3 presents the level of *CiFT* expression in non-vernalized and vernalized plants obtained with semi quantitative RT-PCR analysis. In vernalized plants, the *CiFT* expression remained stable before the start of heat treatment and then increased for control (with a peak after 1 day) but decreased in heat-treated seedlings. For the heat-treated non-vernalized plants, *CiFT* expression decreased after 1 and 2 days but then strongly increased in seedlings exposed to heat treatment for 3 days while *CiFT* expression decreased in controls.

The expression of *CiFT* was also analyzed by qPCR but the low level of *CiFT* expression in the non-vernalized seedlings did not allow us to obtain reliable data with qPCR approach. As far as vernalized plants are concerned (Fig. S4), *CiFT* expression remained stable until the start of heat treatment. Exposure to high temperature induced a strong decrease in *CiFT* expression while it slightly increased in control seedlings.

3.2. Total soluble sugars

Heat increased total soluble sugars concentration in both non-vernalized and vernalized seedlings (Fig. 4). In non-vernalized plants, difference between control and heat-treated seedlings was already visible one day after heat imposition. Total soluble sugars strongly increased during the vernalization period and peaked 14 days before the start of heat treatment. It then decreased, but re-increased again especially in response to heat stress after 2 and 3 days.

3.3. Polyamines concentration

Putrescine and Spd concentrations were slightly higher in vernalized compared to non-vernalized seedlings with the start of the heat treatment (Fig. 5). Heat increased Put and Spm concentrations for both vernalized and non-vernalized seedlings. However, it decreased Spd concentrations at the exception of heat-treated non-vernalized seedlings 3 days after treatment. The Put concentration increased also after 3 days of treatment in control seedlings and was higher in vernalized than in non-vernalized plants. In contrast, after 3 days of treatment, Spm concentration was similar in vernalized and non-vernalized plants.

3.4. Phytohormone analysis

As shown on Fig. 6, heat increased ethylene production in both non-vernalized and vernalized seedlings. In non-vernalized plants, ethylene production increased during all the experimental period. In vernalized seedlings, ethylene production first decreased during the vernalization

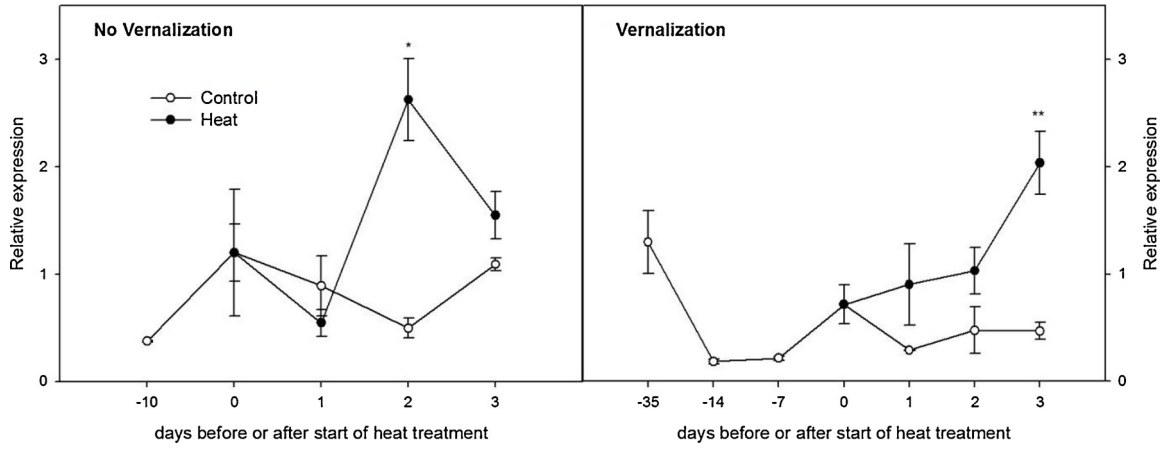

Fig. 2. Relative expression of *CiFL1* transcripts in leaves of non-vernalized and vernalized root chicory seedlings (*Cichorium intybus* var. *sativum*) grown at 17 °C for control condition or 38 °C for heat treatment. *CiFL1* transcript levels were estimated by quantitative RT-PCR. Data were normalized using *CiTUB* as internal control. Data are means ± SE of 3 replicates. The stars indicate the presence of a statistical difference between the treatments (*: $P < 0.05$; **: $P < 0.01$ and ***: $P < 0.001$).

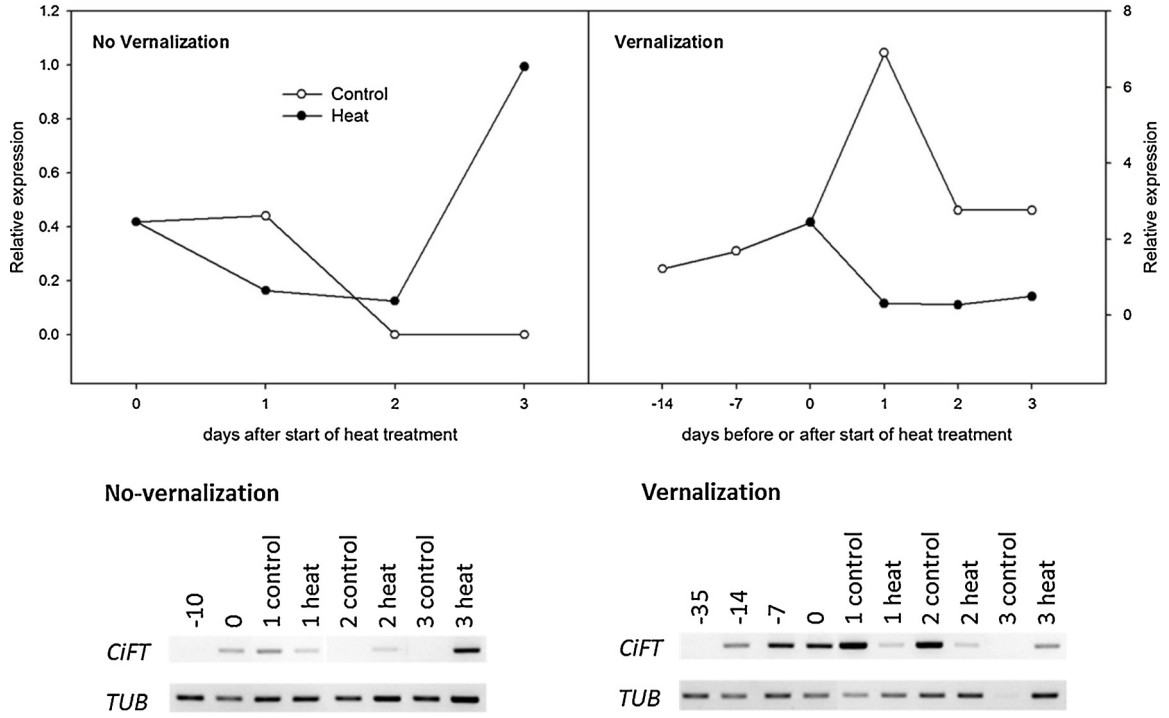

Fig. 3. Semi-quantitative RT-PCR of *CiFT* in leaves of vernalized and non-vernalized chicory seedling grown at 17 °C for control condition or 38 °C for heat treatment. Relative expression levels were analyzed by gel densitometry using *CiTUB* as a PCR control.

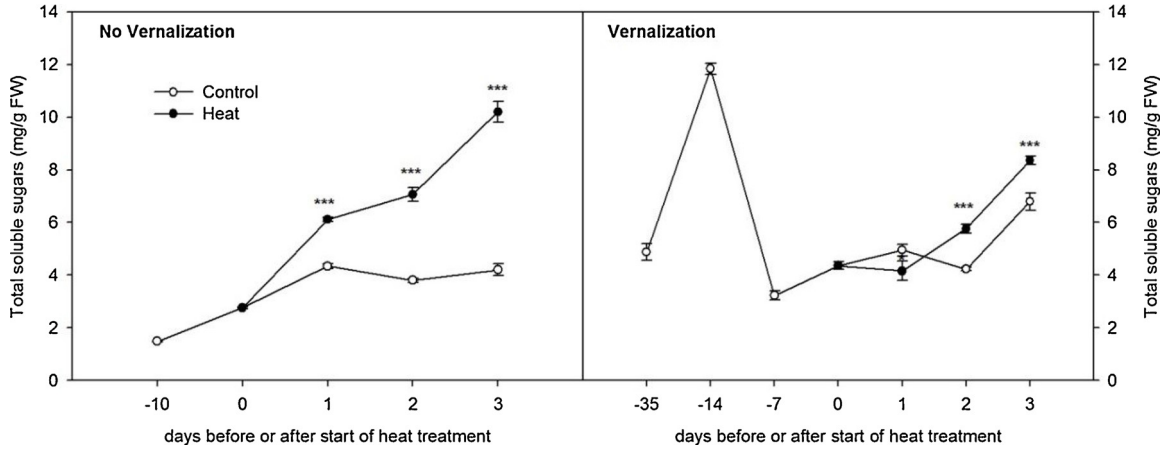

Fig. 4. Total soluble sugars (in mg. g^{-1} FW) in leaves of non-vernalized and vernalized root chicory (*Cichorium intybus* var. *sativum*) seedlings grown at 17 °C for control condition or 38 °C for heat treatment. Data are means ± SE of 6 replicates. The stars indicate the presence of a statistical difference between the treatments (*: $P < 0.05$; **: $P < 0.01$ and ***: $P < 0.001$).

period. It then remained stable after vernalization for control treatment but increased in heat treated plants.

As shown on Fig. 7, at the time of heat imposition, the concentration of ABA, jasmonates and auxins were higher in non-vernalized seedlings than in vernalized ones while an opposite trend was recorded for SA. No significant differences were observed between vernalized and non-vernalized seedlings at the start of heat treatment for ACC and CKs.

After 3 days, heat treatment increased total ABA concentration in the non-vernalized seedlings while it had no significant impact in the vernalized ones (Fig. 7). As detailed in Fig. 8A, treatments also modified the proportion of ABA-metabolites such as dihydrophaseic acid (DPA) and phaseic acid (PA). In the vernalized plants, the most important fraction was ABA itself, except two days after start of heat treatment where DPA was more important than the other forms. In non-vernalized plants, DPA was the most important form at the start of the heat treatment but the proportion of PA strongly decreased for both control and heat-treated seedlings. After 3 days of treatment, ABA constituted the majority of the recorded compounds in both control- and heat-treated plants.

The total jasmonates concentration was higher in non-vernalized compared to vernalized ones whatever the heat treatment. After 3 days of treatment, the concentration of total jasmonates was higher in heat treated plants than in control plants for both vernalized and non-vernalized plants (Fig. 7). Total jasmonates were composed of 3 different forms: bioactive compound jasmonic acid (JA), its main catabolite (+)-*7-iso*-jasmonoyl-L-isoleucineisoleucine conjugate (JA-Ile) and JA biosynthetic precursor *cis*-(+)-12-oxo-phytodienic acid (*cis*OPDA) (Fig. 8B). The proportion of isoleucine conjugate was very low in each sample compared to the others JA forms. The heat treatment increased JA proportion after 2 and 3 days in both vernalized and non-vernalized plants at the expense of the JA precursor, *cis*OPDA. No obvious difference was recorded between vernalized and non-vernalized plants

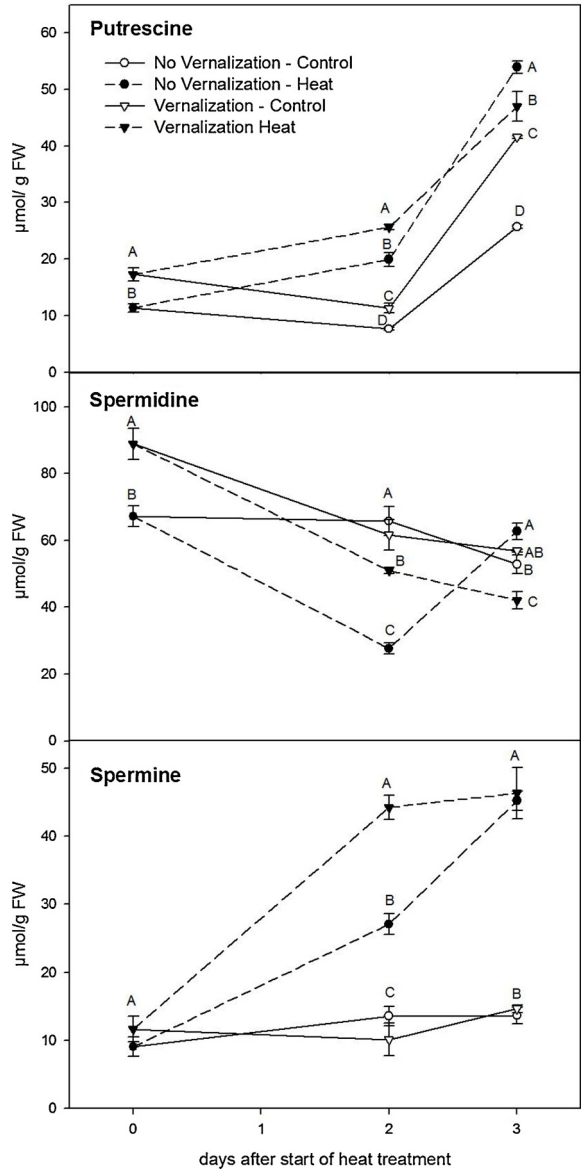

Fig. 5. Endogenous polyamines (putrescine, spermidine and spermine) concentrations in leaves of vernalized and non-vernalized root chicory (*Cichorium intybus* var. *sativum*) seedlings grown at 17 °C for control condition or 38 °C for heat treatment. Data are means ± SE of 3 replicates.

regarding the relative proportion of the different fractions.

At the beginning of the treatment, SA concentration was higher in vernalized than in non-vernalized seedlings. A similar picture was recorded for its immediate precursor BA which totalized 12.8 ± 1.7 nmol g^{-1} FW in the vernalized seedlings and only 5.9 ± 0.7 nmol g^{-1} FW in non-vernalized tissues. Heat significantly increased the concentration of SA in the non-vernalized seedlings but it had no significant impact on the vernalized seedlings. After 2 and 3 days of treatment, no significant differences were observed between treatments for SA content (Fig. 7). Heat increased the ACC concentrations especially in vernalized seedlings (Fig. 7). The highest ACC concentration was recorded for vernalized seedlings exposed to high temperature during 3 days.

High temperature increased total auxin concentration (Fig. 7) already after 2 days in vernalized plants and after 3 days in non-vernalized ones. Three major distinct forms were recorded for the auxin compounds (Fig. 8C): IAA, 2-oxindole-3-acetic acid (oxIAA) and IAA-aspartate (IAA-Asp). The conjugated IAA-Asp form was less important than the two others and only slightly increased in response to heat treatment. In contrast, heat decreased IAA proportion, except after 3 days of heat exposure in vernalized plants. The major primary auxin catabolite (oxIAA) represented the most important form, especially after 2 days of heat treatment.

After two days of heat treatment, total CKs was higher in non-vernalized seedlings than in vernalized ones. No significant differences among treatments were observed after 3 days (Fig. 7). As shown in Fig. 9A, no clear trend was recorded for the proportion of *trans*-zeatin (*trans*Z), dihydrozeatin (DHZ), cis-zeatin (*cis*Z) and N^6-(Δ^2-isopentenyl) adenine (iP)-type CKs in vernalized and non-vernalized seedlings exposed to heat stress. Active CKs constitute a minor proportion of CK pool in *C. intybus* (Fig. 9B). This proportion increased after 2 days of heat treatment in non-vernalized plants while a similar increase was recorded after 3 days of culture in control conditions for the vernalized one. CK-phosphates are the primary products of CK biosynthesis but constitute a very small fraction in *C. intybus*. In contrast, CK *O*-glucosides was the most important fraction followed by CK *N*-glucosides.

Total GAs (Table 1) pool was hardly affected by the considered treatments, the maximal value being recorded in vernalized plants exposed to 38 °C during 3 days. Vernalization and high temperature had no impact on GA_7, GA_9, GA_{13}, GA_{34} and GA_{44}, whatever the considered period. High temperature increased GA_1 after 3 days, GA_{29} after 2 days, as well as GA_8 and GA_{20} after 2 and 3 days in both vernalized and non-vernalized plants. High temperature also increased GA_{53}, except in vernalized plants after 2 days. At day 0, vernalized seedlings presented higher concentration of GA_4 than non-vernalized ones. Vernalization decreased GA_{19} and increased GA_{51} in plants maintained at 17 °C but not in those exposed to 38 °C. Other gibberellins (GA_3, GA_5, GA_6, GA_{12}, GA_{15} and GA_{24}) remained below the detection limits and were thus not quantified in the present study.

3.5. Principal component and correlation analysis

In order to better understand the impact of heat and vernalization on the analyzed parameters and to identify correlations between them, we performed principal component (PCA; Fig. 10) and correlation analysis (Fig. 11). About 56.82 % of the total variance was explained by the principal component 1 (PC1) and the principal component 2 (PC2) while 55.38 % of the variance was explained by the PC1 and the principal component 3 (PC3) (Fig. 10A-D). Parameters that have the highest value factor coordinate for the PC1, with the highest variable contribution based on correlations, were total soluble sugar, Spm, Put, ABA, auxins and ethylene while it was only JA for PC2 and CKs and SA for PC3 (Fig. 10A-B). As shown on Fig. 10A-B, on PC1, Spd and CKs were negatively correlated to the others parameters. The Fig. 10C-D showed the position of the different treatments in the multivariate space of the PC1–2 and the PC1 − 3. PC1 showed a clear opposition between the control and heat treatment (data pooled for 2 and 3 days of heat treatment) while PC3 showed a clear opposition between vernalized and non-vernalized plants under heat treatment.

Analysis of correlations among parameters (Fig. 11) confirmed that CKs and Spd were negatively correlated to the others parameters except SA that was slightly positively correlated to CKs. Ethylene and *CiFL1* relative expression presented a very high value of negative correlation with Spm. Total soluble sugars were highly correlated with some hormones like Spm, Put, ABA, ethylene and SA. *CiFL1* relative expression was positively correlated to ethylene, SA and JA.

4. Discussion

4.1. Vernalization and high temperatures have contrasting effect on CiFL1 expression in Cichorium intybus

Heat has a dual impact on flowering in *C. intybus* since it could induce flowering on the one hand but counteract vernalization on the

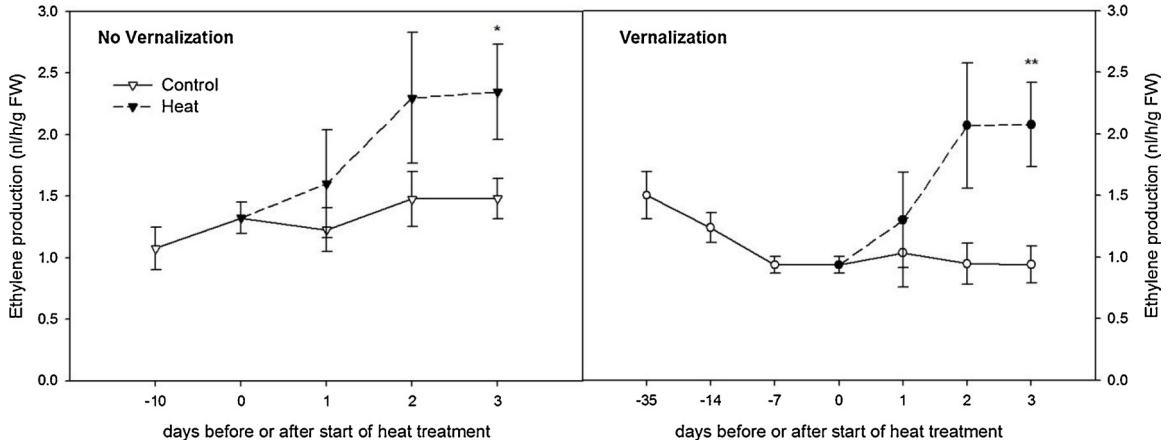

Fig. 6. Leaf ethylene production (expressed in nl/h/g FW) in vernalized and non-vernalized root chicory (*Cichorium intybus* var. *sativum*) seedlings grown at 17 °C for control condition or 38 °C for heat treatment. Data are means ± SE of 6 replicates. The stars indicate the presence of a statistical difference between the treatments (*: $P < 0.05$; **: $P < 0.01$ and ***: $P < 0.001$).

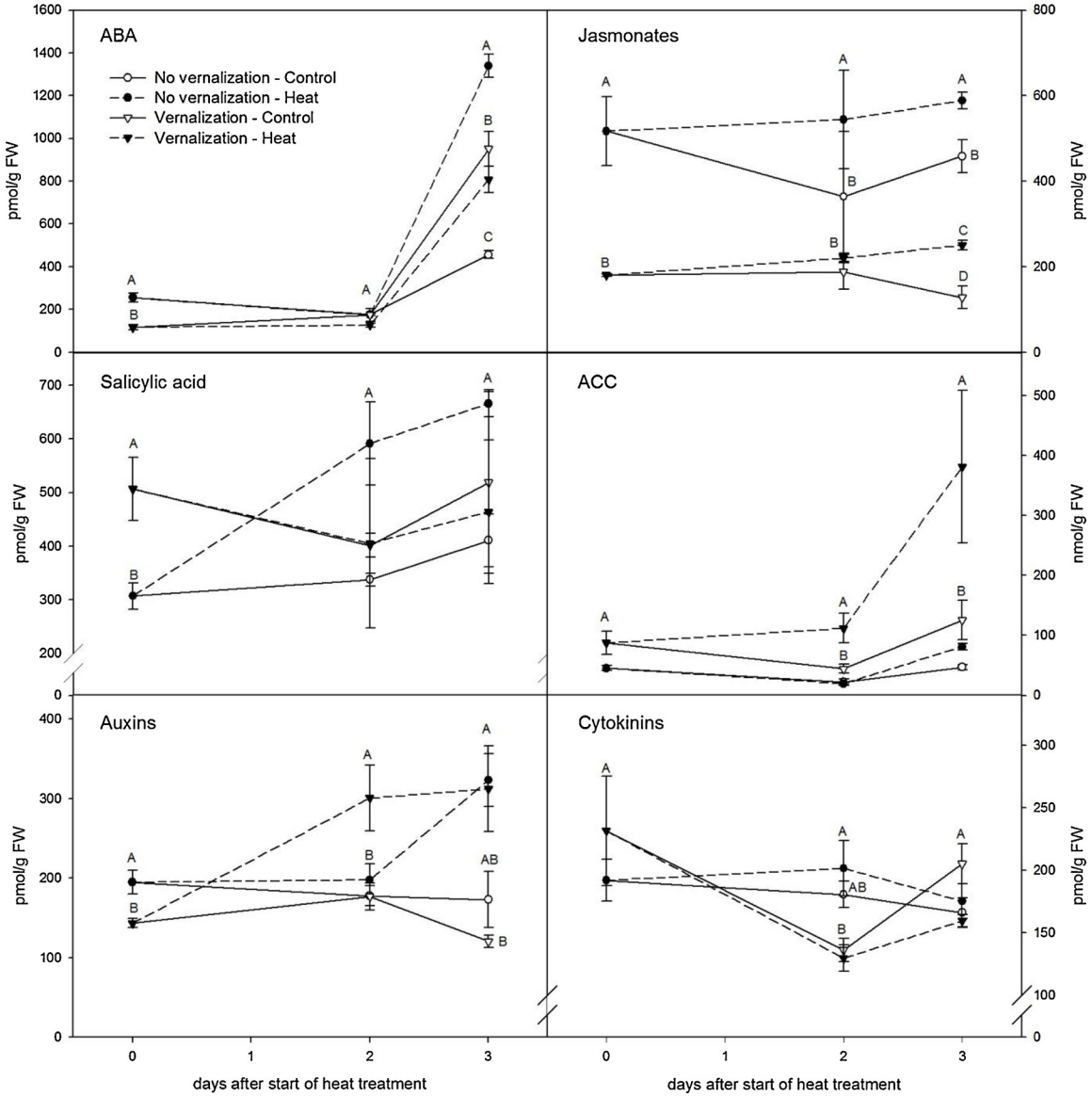

Fig. 7. Total abscisic acid (ABA), total jasmonates, salicylic acid, 1-aminocyclopropane-1-carboxylic acid (ACC), total auxins and total cytokinins concentrations in vernalized and non-vernalized root chicory (*Cichorium intybus* var. *sativum*) seedlings grown at 17 °C for control conditions or 38 °C for heat treatment. Data are means ± SE of 3 replicates.

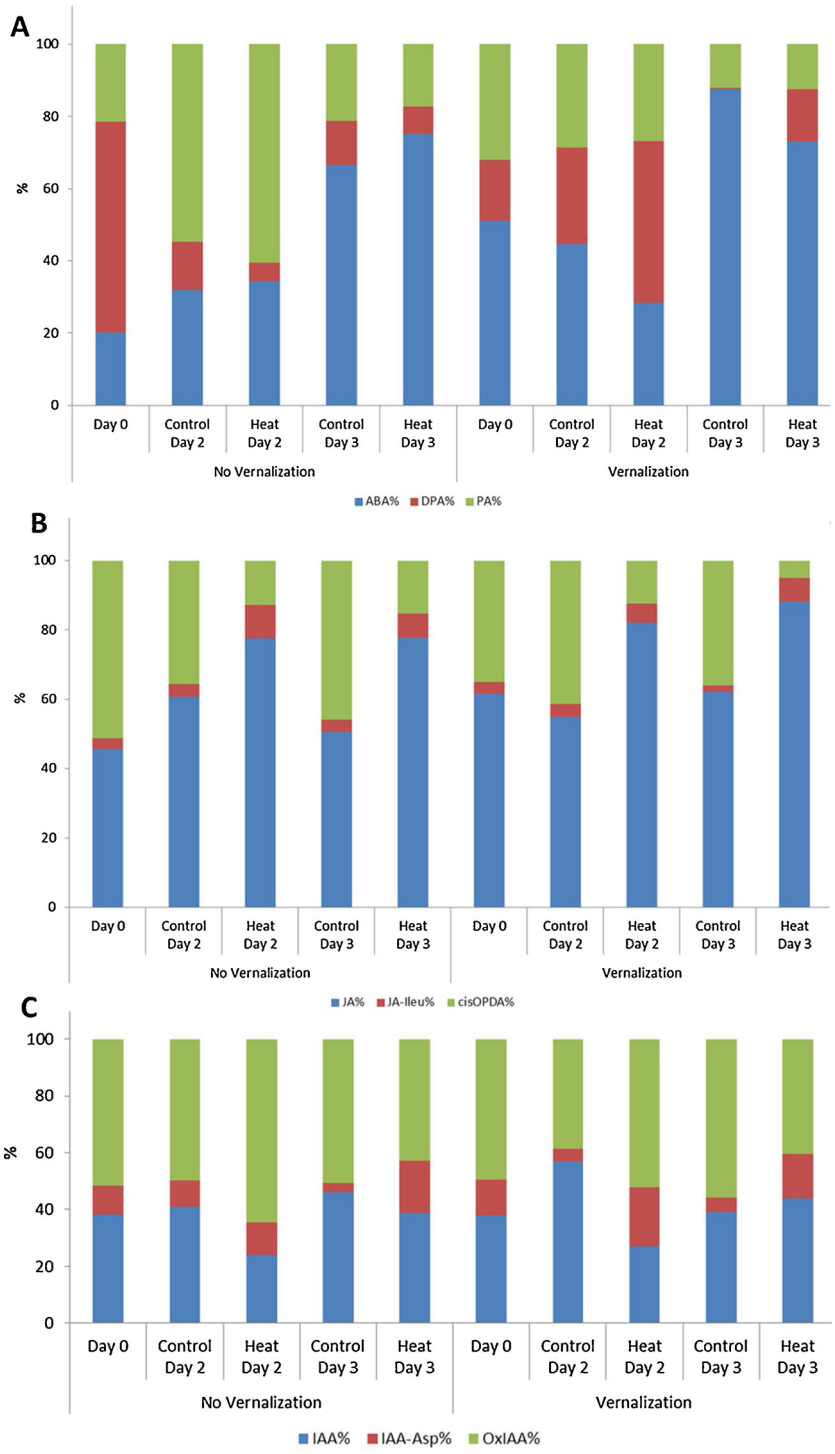

Fig. 8. Abscisic acid (A), jasmonates (B) and auxin (C) metabolites in leaves of vernalized and non-vernalized root chicory (*Cichorium intybus* var. *sativum*) seedlings grown at 17 °C for control condition or 38 °C for heat treatment. Abscisic acid metabolites were abscisic acid (ABA) itself, dihydrophaseic acid (DPA) and phaseic acid (PA). Jasmonates were represented by jasmonic acid (JA), its isoleucine conjugate (JA-Ile) and the JA precursor *cis*-(+)-12-oxo-phytodienic acid (*cis*OPDA). Among auxin metabolites, indole-3-acetic acid (IAA), its derivative 2-oxindole-3-acetic acid (oxIAA) and IAA amino acid conjugate IAA-aspartate (IAA-Asp) were included. Each value is the mean of 3 replicates.

other hand. Expression of *CiFL1* was inhibited during the vernalization phase as previously demonstrated (Périlleux et al., 2013) but heat treatment after vernalization induced an increase in *CiFL1* transcripts suggesting that the repressed state induced by vernalization was not maintained on a long-term basis.

In the present study, increase of *CiFL1* expression by high temperature was recorded not only for vernalized seedlings but also for non-vernalized plants confirming that heat alone induced *CiFL1* expression. A very high temperature was tested (38 °C) and should be regarded as a heat shock and not only as a de-vernalizing temperature. We suggest that *CiFL1* may have different functions in flowering induction in response to vernalization on the one hand or heat stress on the other hand. Theiße et al. (2018) recently reported that *FLM* in Arabidopsis forms protein complex in combination with other flowering regulators to induce or repress flowering and that different spliced proteins may associate with different partners to regulate flowering. A similar alternative splicing still needs to be tested for *CiFL1*.

The 20-kDa protein derived from the floral pathway integrator gene *FLOWERING LOCUS T (FT)* has been reported to induce flowering after vernalization and subsequent long days conditions in Arabidopsis (Corbesier and Coupland, 2005). The *FT* gene is involved in flowering induction by both vernalization and stress in Arabidopsis and homologs of *FT* are widely conserved among species (Takeno, 2016). Our results suggest a similar situation for *CiFT* in *C. intybus*. In the present study,

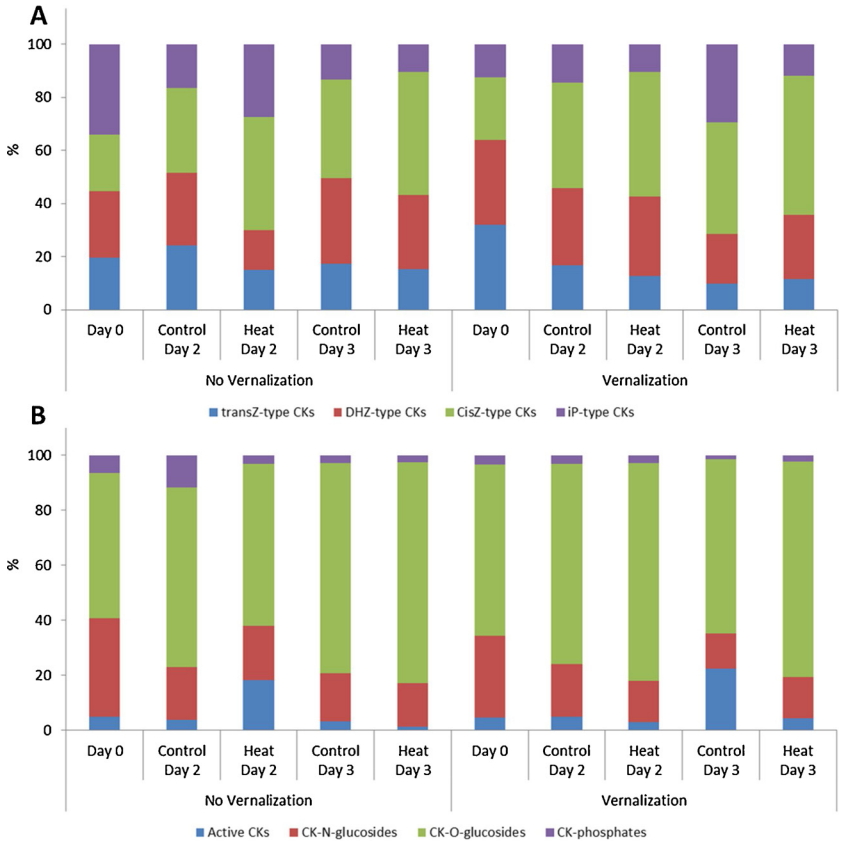

Fig. 9. Total cytokinins in leaves of vernalized and non-vernalized root chicory (*Cichorium intybus* var. *sativum*) seedlings grown at 17 °C for control condition or 38 °C for heat treatment. A: proportions of *transZ*-type CKS, DHZ-type CKS, *CisZ*-type CKs and iP-type CKs; B: proportions of active CKs, CK *N*-glucosides, CK *O*-glucosides and CK-phosphates. Each value is the mean of 3 replicates.

Table 1
Gibberellin concentration (in ng. g^{-1} FW) in leaves of non-vernalized (0 vern) and vernalized (+ vern) root chicory (*Cichorium intybus* var. *sativum*) seedlings grown at 17 °C for control condition or 38 °C for heat treatment. Data represent means of 3 biological replicates (each sample analyzed in technical triplicates). Standard errors are not provided for the sake of clarity. For a given compound, mean followed by the same letter are not significantly different at $P < 0.05$ according to Student-Newman Keuls test. Specific gibberellins whose content was modified by heat stress are indicated in bold italics while those which were affected by vernalization are underlined.

Treatment	GA_1	GA_4	GA_7	GA_8	GA_9	GA_{13}	GA_{19}	GA_{20}	GA_{29}	GA_{34}	GA_{44}	GA_{51}	GA_{53}	**TOTAL**
Day 0														
0 vern	0.18 a	0.67 b	0.18 a	0.64 a	1.84 a	0.04 a	1.29 c	0.85 ab	0.22 a	0.75 a	0.51 a	0.79 c	1.93 b	**9.89**
+ vern	0.13 a	0.91 c	0.19 a	0.70 ab	1.72 a	0.01 a	0.68 a	0.65 a	0.15 a	0.85 a	0.48 a	1.13 d	2.05 b	**9.65**
Day 2														
0 vern-17	0.21 ab	0.68 b	0.19 a	0.68 a	1.62 a	0.02 a	1.04 b	0.57 a	0.24 a	0.76 a	0.49 a	0.30 a	1.38 a	**8.18**
0 vern 38	0.17 a	0.49 ab	0.16 a	***0.92 b***	1.47 a	0.05 a	***1.20 c***	***1.27 b***	***0.43 b***	0.84 a	0.40 a	0.35 a	***3.00 d***	**9.67**
+ vern-17	0.10 a	0.54 b	0.22 a	0.67 a	1.95 a	0.01 a	0.74 a	0.64 a	0.21 a	0.88 a	0.42 a	1.51 e	2.10 b	**9.99**
+ vern-38	0.19 ab	0.44 ab	0.21 a	***0.95 b***	1.66 a	0.02 a	***1.54 d***	***1.12 b***	***0.37 b***	0.87 a	0.57 a	0.23 a	***1.40 a***	**9.57**
Day 3														
0 vern-17	0.11 a	0.37 a	0.20 a	0.73 a	1.55 a	0.05 a	0.98 b	0.66 a	0.21 a	0.78 a	0.38 a	0.54 b	1.52 a	**8.08**
0 vern 38	***0.24 b***	0.51 b	0.16 a	***1.09 b***	1.87 a	0.06 a	***1.25 c***	***1.16 b***	0.32 ab	0.74 a	0.50 a	0.44 ab	***2.53 c***	**10.87**
+ vern-17	0.08 a	0.50 b	0.20 a	0.57 a	1.38 a	0.05 a	0.61 a	0.60 a	0.45 b	0.85 a	0.51 a	1.77 e	1.74 ab	**9.31**
+ vern 38	***0.27 b***	0.43 ab	0.21 a	***0.90 b***	1.32 a	0.05 a	***1.29 c***	***0.95 b***	0.44 b	0.86 a	0.57 a	0.44 ab	***2.47 c***	**10.20**

CiFT was activated in response to vernalization but it was repressed when vernalization was followed by heat. Interestingly, it was activated in response to heat in non-vernalized plants. This data contrast with the results obtained in *Brassica rapa* where high temperature was recently reported to induce the incorporation of the histone H2A.Z at *FT* chromatin leading to an inhibition of the gene expression (del Olmo et al., 2019d). It has however to be mentioned that several genes coding for FT-like proteins may exist in some plant species and might be differently regulated in response to environmental cues (Wada et al., 2010; Yamada and Takeno, 2014). The presence of *FT* homologs could indeed not be ruled out in *C. intybus*.

4.2. Vernalization modifies the hormonal profile in Cichorium intybus

Vernalization represents an adaptation of plant development to prevent transition from a cold-resistant vegetative phase to a cold-sensitive reproductive one. The present work shows a progressive decrease in ethylene synthesis occurring during this phase. Since ethylene shares a common precursor (S-adenosylmethionine) with polyamine, this could also explain the higher Spd concentration recorded in vernalized seedling comparatively to non-vernalized one at the time of heat imposition. A higher SA content in vernalized plants could also be directly related to a protective role of phenolic acids in cold acclimation during vernalization as reported by Vanková et al. (2014). Unexpectedly, total jasmonates levels were clearly lower in vernalized plants than in non-vernalized ones in *C. intybus*. JA-Ile did not increase

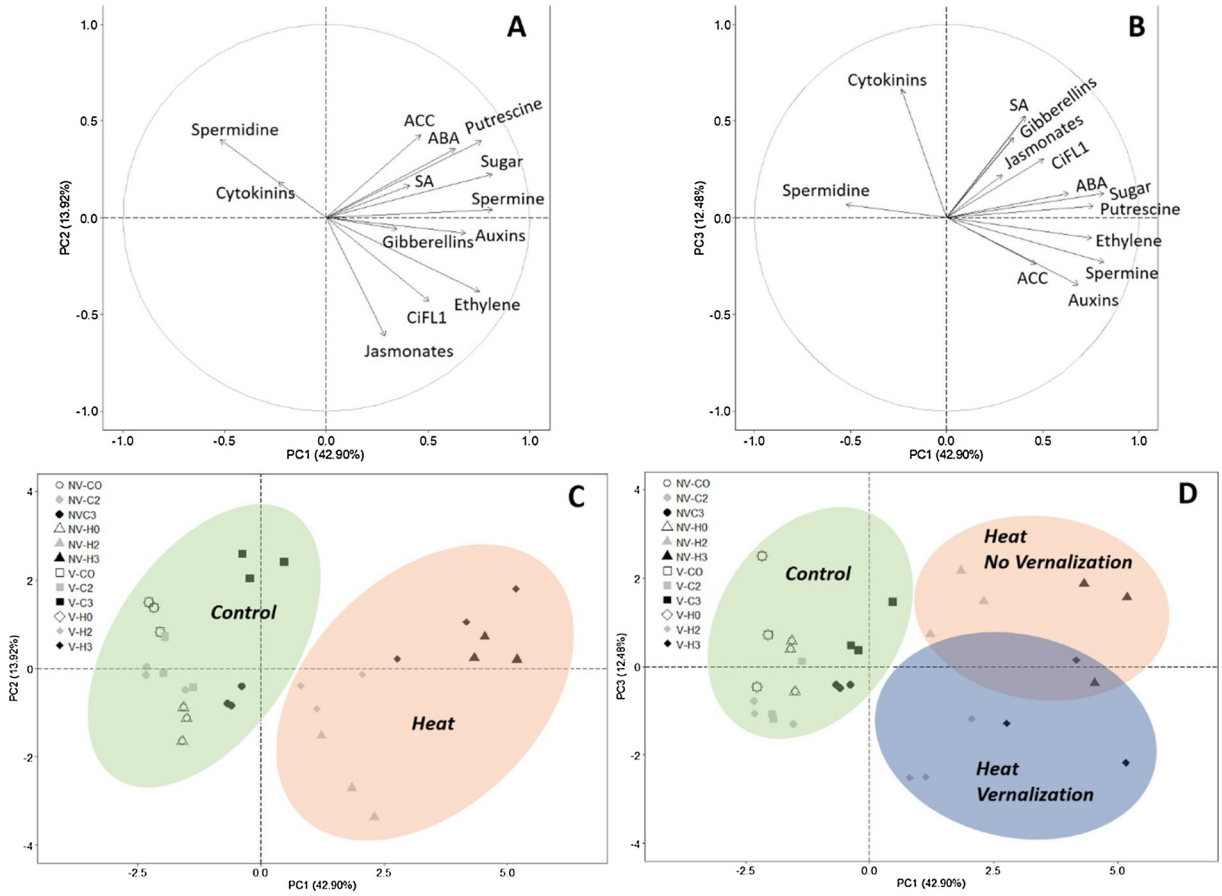

Fig. 10. Principal Component Analysis (PCA) of dim 1-2 (A-C) and dim 1-3(B-D) (A-B: variable graph and C-D: individual graph) of *CiFL1* relative expression, total soluble sugars and hormonal parameters in vernalized and non-vernalized root chicory (*Cichorium intybus* var. *sativum*) seedlings grown at 17 °C for control condition or 38 °C for heat treatment.

in vernalized plants of root chicory, although it represents a major bioactive form of JA and plays important roles in flowering, especially in anther maturation (Widemann et al., 2016). Similarly, data provided by Diallo et al. (2014) demonstrated that methyl jasmonate (MeJA) accumulated in vernalized plants in wheat and may have a role in floral transition.

Abscisic acid was also reported to be altered by vernalization in the biennial plant species *Beta vulgaris* (Liang et al., 2018). In *C. intybus*, the proportion of free ABA increased in vernalized seedlings (Fig. 8) while its catabolite DPA decreased. This implies that vernalization may cause slower degradation of ABA which is a key hormone involved in response to abiotic stress, including low temperature as those occurring during vernalization (Vanková et al., 2014).

Gibberellins are known to control flowering in plant species but data regarding their interaction with vernalization remains contradictory. In some biennal species, GAs can substitute for vernalization (Lang, 1957; Mutasa-Göttgens and Hedden, 2009) and promote bolting (Lang, 1957). Oka et al. (2001) considered that vernalization is a critical environmental cue initiating GA biosynthesis in *Eustoma grandiflorum* and *Arabidopsis thaliana* while MacMillan et al. (2005) reported that GA signaling is independent of vernalization in *Lolium perenne*. In a recent study, Tilmes et al. (2019) reported that GAs promote floral induction during vernalization in *Arabis alpina*. According to these authors, the transcription factor PEP1 (PERPETUAL FLOWERING) which represses flowering until its mRNA levels are reduced by vernalization also negatively regulates GA signaling and reduces GA levels before vernalization. Numerous studies, however, used very high concentration of exogenous GA from one single type (commonly GA_3) and did not discriminate between various endogenous GAs (Tarkowská and Strnad, 2018). In the present work, we showed that vernalization increased 13-nonhydroxylated bioactive GA_4 and its biologically inactive precursor GA_{51} (a degradation product of GA_9) but the higher concentration of the bioactive GA_4 remained transient and was recorded at day 0 only.

4.3. High temperatures may have different effects on phytohormones in vernalized and non-vernalized seedlings

Our PCA analysis demonstrates that a clear discrimination could be established between controls and heat-treated plants (Fig. 10). Perception of heat induces a signaling cascade in the developmental process which could involve phytohormones. Since vernalization occurring before heat stress modifies the hormonal status of seedlings, it was justified to compare heat effect on vernalized and non-vernalized seedlings. Once again, the PCA analysis allowed us to discriminate non-vernalized and vernalized seedlings within the group of heat-treated plants (Fig. 10D).

In heat-treated plants, the levels of SA and ABA were clearly higher in non-vernalized seedlings than in vernalized ones. Synthesis of SA from BA was already reported to play a key role in stress-induced flowering (Hatayama and Takeno, 2003). In *Pharbitis nil*, SA increased flowering under poor nutrition stress but did not induce flowering in unstressed plants suggesting that SA may be necessary but not sufficient to induce flowering. According to Wada et al. (2010), *PnFT2* (but not *PnFT1*) of *P. nil* was clearly expressed in flowering stressed plants. Yamada and Takeno (2014) demonstrated that exogenous SA stimulated *PnFT2* expression under both stress and unstressed conditions. In *C. intybus*, we observed that heat increased SA concentration and *CiFT* expression after 3 days of stress in non-vernalized plants suggesting that

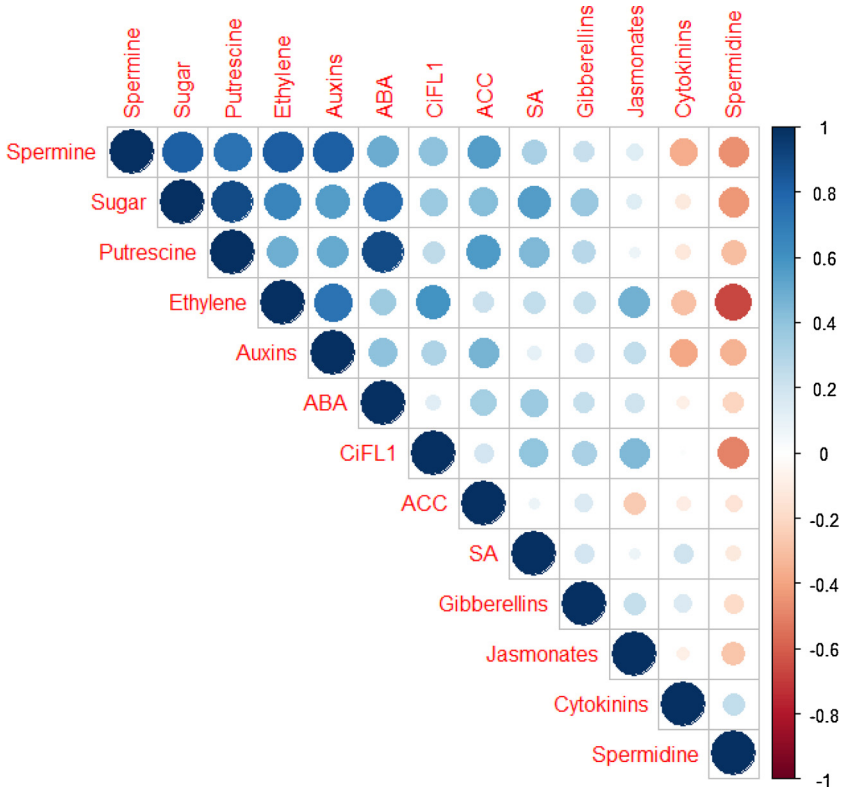

Fig. 11. Correlation graph of *CiFL1* relative expression, total soluble sugars and hormonal parameters in vernalized and non-vernalized root chicory (*Cichorium intybus* var. *sativum*) seedlings grown at 17 °C for control condition or 38 °C for heat treatment.

SA may be involved in flowering induction by heat stress.

Abscisic acid content reached the highest level after three days of heat stress in non-vernalized seedlings while the minimal value was recorded for non-vernalized plants maintained in control conditions and which will not flower in the time course of the experiment. ABA is acting as a stress hormone mediating gene expression in order to improve plant tolerance to heat (Abdelmrahman et al., 2017) but in our work, heat-induced ABA accumulation was two times higher in non-vernalized than in vernalized heat-treated seedlings confirming that the phytohormonal response of stressed plant to heat may be influenced by the preliminary vernalization step. In *Arabidopsis thaliana*, ABA was shown to promote flowering in stressed plants via up-regulation of *FT* (Riboni et al., 2016). We also observed a concomitant increase of ABA and *CiFT* in non-vernalized heat-treated plants 3 days after treatment. Nevertheless, Riboni et al. (2016) also reported that ABA has a dual impact on flowering and inhibits it by repressing *SOC1* expression, so that variation in ABA signaling provide different developmental information allowing the plant to co-ordinate the onset of the reproductive phase according to the environment. In our study we noticed that ABA accumulation occurred concomitantly with an increase in *CiFL1* expression and additional works are therefore required to precise if a causal link exist between ABA content and *CiFL1* expression.

It is also noteworthy that the level of some GAs formed via 13-hydroxylation biosynthetic pathway (such as GA_{20} – biosynthetic precursor of bioactive GA_1 and GA_1-inactive catabolite GA_8) were strongly elevated in response to high temperature but remained similar in the vernalized and non-vernalized material. Fukuda et al. (2009) reported that high temperature upregulates GA_1 content in lettuce, which belongs to the Asteracea family as *C. intybus*, but this effect was not observed in our work. In the same species, Liu et al. (2018) considered that both GA_3 and GA_4 contents increased as a consequence of heat stress. According to Han et al. (2016), although GAs regulate bolting in lettuce, high temperature promote flowering mainly by acting on MADS-box genes. Nevertheless, one must keep in mind that the present GA profile was established on the whole shoot in our study, while the GA status restricted to the shoot tip is also directly conditioning flowering process.

Our data also show an increase in Put and Spm concentration in heat-treated plants together with a transient decrease in Spd (Fig. 6). According to our correlation analyses (Fig. 11), Spd was negatively correlated with all other recorded parameters and this view is confirmed by the PCA approach (Fig. 10A and B) and the correlation analysis (Fig. 11). Spermidine is the precursor of Spm. Sagor et al. (2013) demonstrated that Spm synthase gene was induced by heat shock in *Arabidospsis thaliana* and that Spm may trigger synthesis of heat shock proteins protecting the plant tissues from the deleterious impact of high temperatures. Beside this protective role in vegetative tissues, Imamura et al. (2015) provided clear evidences that the gene coding for Spm synthase is transiently activated during vegetative to reproductive growth in *Gentiana triflora*. According to these authors, the expression of *FT* was significantly up-regulated with a concomitant down-regulation of *FLC* in transgenic lines of Arabidopsis over-expressing *GtSPMS*. Once again, *C. intybus* behaves in a contrasting way since accumulation of Spm was associated to an increase rather than a decrease in *CiFL1* expression. According to Zielińska et al. (2006), Put levels were lower in induced *Pharbitis nil* seedlings than in non-induced ones and a recorded decrease in Put in heat-treated plants might also be involved in heat-induced flowering.

Ethylene was increased by high temperature to a similar extent in vernalized and non-vernalized seedlings but ACC concentrations clearly exhibited a different trend and was 4 times higher in vernalized than in non-vernalized plants after 3 days of treatment. This leads us to hypothesize that synthesis of ACC precursor S-adenosylmethionine was increased in vernalized seedlings while ACC oxidase activity catalyzing the conversion of ACC to ethylene was similar in both types of plants. Such different behavior may explain the rather poor correlation found between ACC and ethylene synthesis. S-adenosylmethionine may also act as a methyl residue donor in DNA methylations such as those

occurring in the vernalization process. Heat stress may also induce DNA methylation responsible of epigenetic changes (Suter and Widmer, 2013).

Besides phytohormones, soluble sugars may also regulate bolting and flowering via increased expression of *FT* (Cho et al., 2018). We found that high tempearures increased total soluble sugars concentration to similar extent in non-vernalized and vernalized plants (Fig. 4). In *Lactuca sativa* (another species from *Asteracea*), Han et al. (2016) reported that bolting sensitive lines contained higher soluble sugars than bolting-resistant ones. According to Cho et al. (2018), sucrose is mainly involved in increasing FT expression. We did not discriminate individual sugars in the present work but we previously reported that high temperature decrease in leaves of *C. intybus* (Mathieu et al., 2018) which might explain the heat-induced decrease in *FT* expression of vernalized plants but not the induced increase recorded in non-vernalized ones.

Author statement

S.L. and M.Q. conceives the experiments. A.S.M. performed analyses in relation to plant culture and gene expression and made statistical analysis. G.J. isolated and sequenced FT gene. D.T. and J.P. quantified the gibberellin profile. P.I.B. and V.M. performed analysis of all other hormones. All authors contributed to the final redaction of the article and approved the submitted version.

Declaration of Competing Interest

The authors declare non conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2020.104127.

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