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Cold deacclimation mechanisms and reacclimation potential in flower buds of blackcurrant (*Ribes nigrum*)

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As a consequence of global climate change, cold acclimation and deacclimation cycles are becoming increasingly frequent during winter in temperate regions. However, little is known about plant deacclimation and in particular reacclimation mechanisms, although deacclimation resistance and the ability to reacclimate may have wide-ranging consequences regarding plant productivity in a changing climate. Here we report time-dependent responses of freezing tolerance, respiration rates, metabolite contents (HR-MAS NMR) and fatty acid levels (gas chromatography) in flower buds of two ecodormant *Ribes nigrum* cultivars exposed to three different deacclimation temperatures followed by a reacclimation treatment at 4°C. The data reveal that despite differences in the progression of deacclimation, the capacity of blackcurrant flower buds to reharden in late winter is virtually non-existing, implying that increasingly irregular temperature patterns is critical for blackcurrant fruit yield. The early phase of deacclimation is associated with a transient increase in respiration and decreasing contents of amino acids, tricarboxylic acid (TCA) cycle intermediates and sugars, indicating an increased need for carbon sources and respiratory energy production for the activation of growth. Decreasing sugar levels may additionally cause loss of freezing tolerance. Deacclimation also involves desaturation of membrane lipids, which likely also contributes to decreased freezing tolerance but may also reflect biosynthesis of signaling molecules stimulating growth and floral organ differentiation. These data provide new insights into the under-researched deacclimation mechanisms

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and the ability of blackcurrant to reacclimate following different advancements of deacclimation and contribute to our understanding of plant responses to increasingly irregular temperature patterns.

Introduction

Plants native to temperate and boreal climates show natural low temperature acclimation during fall in preparation for winter frost. This process is termed cold acclimation. Upon exposure to warmer temperatures in spring, plants lose the freezing tolerance acquired during acclimation by deacclimation, while they resume growth and development (Xin and Browse 2000, Vitasse et al. 2014). If this transition takes place too late, the plants lose valuable time during the growth season. A premature transition on the other hand involves the danger of freezing damage under a late-season cold spell, unless the plants have the ability to reacclimate if the temperature drops again (Pagter and Williams 2011, Kovi et al. 2016). This situation is strongly influenced by the effects of global climate change. Global climate models predict an increase in the mean surface air temperature and in the frequency and severity of erratic temperature events (IPCC 2013). Hence, winters in temperate regions are becoming progressively milder and temperature patterns are becoming increasingly irregular. This will increase the frequency of unseasonable warm spells leading to more frequent acclimation and deacclimation cycles (Rapacz et al. 2014). In woody perennials, which undergo seasonal transitions between active and dormant states, sudden temperature increases may be particularly decisive in late winter and early spring, when plants are particularly vulnerable to cold-injury due to emergence from endodormancy (Pagter and Arora 2013).

While considerable effort has been directed towards understanding how plants cold acclimate and adapt to low temperature, deacclimation and in particular reacclimation mechanisms have not attracted much attention, although deacclimation resistance and the ability to reacclimate may have wide-ranging consequences regarding global ecosystems and crop yield in a changing climate (Kreyling 2010, Smith and Katz 2013). Most extensively documented is the association between changing metabolite concentrations, especially compatible solutes, and changes in freezing tolerance (Zuther et al. 2015, Andersen et al. 2017, Pagter et al. 2017a). In particular, little is known about the role of membrane lipids in deacclimation (Yoshida 1986, Martz et al. 2006), although it has been suggested that freezing tolerance is a function of membrane fluidity (Steponkus 1984) and that changes in membrane fluidity itself trigger temperature responses in plants (Ruelland and Zachowski 2010). Also, an understanding of the importance of membrane lipids in a regain of freezing tolerance during reacclimation is completely lacking.

Blackcurrant (*Ribes nigrum* L.) is an important soft fruit crop of cold and temperate regions. Floral primordia in blackcurrant are initiated in late summer/early autumn and overwinter on dormant stems (Tinklin and Schwabe 1970). Flowering will, therefore, only occur the following year if flower primordia are present and undamaged. The deacclimation response and the ability to reacclimate is likely an important part of the reproductive success of blackcurrant as one of the main limitations to the commercial production has been spring frost damage to dehardened flower buds and newly developed leaves and flowers (Atkinson et al. 2013). We have previously observed genotypic differences in the timing and rate of deacclimation and bud break of blackcurrant (Winde et al. 2017),

which may also set the limit for the different genotypes to reacclimate or halt the deacclimation process when experiencing a drop in temperature following a warm period. The aim of this study was to assess the ability of ecodormant *R. nigrum* to reacclimate following exposure to a warm period and to investigate the deacclimation and reacclimation mechanisms. Specifically, we used two genotypes of *R. nigrum* of contrasting origin to investigate time-dependent responses of freezing tolerance, metabolite contents and free fatty acid levels in fully differentiated floral primordia to deacclimation at three different temperatures followed by a reacclimation treatment at 4°C. It was hypothesized that (1) the capacity of ecodormant plants to reacclimate depends on the progress of deacclimation. (2) The timing of metabolic regulation during deacclimation and reacclimation varies between genotypes depending on their deacclimation resistance and phenological status and (3) changes in freezing tolerance during deacclimation and reacclimation are reflected by changing levels of membrane lipids.

Materials and methods

Plant material and treatments

Evaluations were carried out using 2-year-old vegetatively propagated *Ribes nigrum* L. 'Zusha' and 'Ben Hope'. 'Zusha' is a Russian cultivar (Pedersen 2008), while 'Ben Hope' is a Scottish cultivar from complex crossings involving 'Westra' and a backcrossed blackcurrant × gooseberry hybrid (238/36 × EM21/15) (Cerekovic et al. 2014). Both 'Zusha' and 'Ben Hope' are regarded as relatively low chill-requiring cultivars (Atkinson et al. 2013, Jones et al. 2013, Andersen et al. 2017), however, we have previously observed that 'Zusha' is released from dormancy earlier than 'Ben Hope' (KH Kjær and M Pagter, unpubl. res.), demonstrating a lower chilling requirement of the former cultivar. Before start of the experiment, plants were grown outside. Hence, the plants underwent cold hardening under natural conditions. Before initiation of the experiment, it was verified that both cultivars had emerged from endodormancy. Dormant status (or lack thereof) was estimated by moving potted plants into a greenhouse and inducing bud break at 20°C day/night, 18-h photoperiod and 100-300 µmol m^{-2} s^{-1}. In both cultivars, the majority of buds started developing within two weeks, implying that endodormancy had been broken. At the time of the experiment, the floral primordia of both cultivars were fully differentiated with primordia of all flower parts being visible in the primary flower. This corresponds to stage 6 of the Sønsteby and Heide (2013) scheme.

In early February, 11 fully cold-hardened plants of each cultivar were randomly selected to estimate maximum freezing tolerance (6 plants) and measure bud respiration and harvest material for destructive analysis (5 plants) at start of the experiment (0 days of deacclimation). Another 126 plants of each cultivar were randomly divided into three groups and moved into three climate chambers for a deacclimation period of 12 days. The deacclimation treatments consisted of an 8.5-h photoperiod (corresponding to natural day length) at 150-200 µmol m^{-2} s^{-1} combined with three day/night-temperatures of 16/11, 13/8 or 10/5°C. Measurements of floral bud respiration and harvesting of

material for HR-MAS NMR spectroscopy and lipid analysis were carried out after 4, 8 and 12 days of deacclimation (DOD). At each time point, samplings and measurements were randomly performed on five plants per treatment. Cold hardiness estimations of flower primordia were carried out after 12 DOD on six plants per treatment.

To evaluate the reacclimation ability and mechanisms of flower buds, the temperature in the three climate chambers were lowered to a constant 4°C, which is usually conducive for cold acclimation (Renaut et al. 2006), starting the night between day 12 and day 13. Measurements of bud respiration and harvesting of material for destructive analyses were carried out after 4, 8 and 12 days of reacclimation (DOR) using five replicates per treatment. Determination of freezing tolerance of flower primordia was carried out after 12 DOR using six replicates per treatment.

Freezing tolerance of floral primordia

Freezing tolerance of floral primordia was determined at eight temperatures; one control (4°C) and seven subfreezing temperatures at -10, -15, -20, -25, -30, -40 and -50°C. For each temperature and replicate, two stems with one attached axillary bud each were pruned to equal lengths (1-2 cm), wrapped in moist paper towels to ensure ice nucleation and inserted into small sealed plastic bags. The samples were incubated in a pre-cooled temperature-controlled freezer on top of an aluminum grating and cooled at a rate of maximum 5°C per hour to 0°C and subsequently at 2°C per hour until the selected temperature was reached. The selected temperature was maintained for 2 h, thereafter the samples were withdrawn and thawed overnight on ice at 4°C. Thawed samples were left at 20°C for ca. 7 days. Subsequently buds were excised, cut longitudinally and incubated in 0.5% 2,3,5-triphenyl tetrazolium chloride (TTC) solution in 0.05 M phosphate buffer at 20°C for 24 h in darkness. Following incubation in TTC, the coloration of flower primordia was assessed with a dissecting microscope. Active dehydrogenases in mitochondria reduce colorless TTC to red triphenylformazan (Steponkus and Lanphear 1967), hence bright red and red floral primordia were assessed as vital, while weakly colored, brownish or colorless primordia were regarded as dead.

Respiration and development of floral buds

Respiration of floral buds was measured in the morning approximately 2 h into the light period. From each of five plants the first and second axillary bud from the top of a shoot was distributed to 2.37 ml glass vials with 25 mM imidazole buffer (pH 6.5). Extra buffer was added to allow closing the vials, with a screw cap containing a silicone inlet, without trapping air bubbles. Vials were immediately incubated in darkness in a thermomixer at 16, 13 or 10°C during the deacclimation treatment or 4°C before start of the deacclimation treatment and during the reacclimation treatment, corresponding to the day temperature in the three deacclimation treatments and the reacclimation treatment. Oxygen consumption was measured at 30, 75, 120, 170 and 210 min after incubation with a FireStingO2 oxygen meter and a needle-mounted optical oxygen micro sensor using the program Oxygen Logger

(Pyro Science, Aachen, Germany). Calibration was performed using ambient air (100% oxygen saturation) and a saturated 1% (w/v) sodium sulfite solution (0% oxygen saturation). For the measurements, the needle with the oxygen sensor was injected into the tube, the oxygen sensor was moved out of the needle and relative oxygen saturation was measured during 20 seconds, until a steady state value was reached. Plotting the readings against the exact times of the measurements approached linear oxygen consumption over time. Oxygen consumption rates with r^2 values lower than 0.7 and not covered by the confidence interval were omitted from further analysis and in the calculation of mean values. Fresh weights (FWs) of the buds were determined before the respiration measurements and dry weights (DWs) after drying at 70°C for 24 h. In order to compensate for bud swelling during the deacclimation and reacclimation treatments, the FW of the buds were set to equal the volume of water being suppressed in the flasks and subtracted from the full volume. The oxygen consumption was then calculated as nmol O_2 min^{-1} mg^{-1} DW.

Especially plants subjected to the warmest deacclimation treatment started to show signs of bud development at the end of the deacclimation treatment. Hence, bud development was recorded after 12 DOD and 7 and 9 DOR separately for the five top-most lateral buds of a single shoot. Bud burst was recorded using a rating of 0 to 4, where 0 = no bud development, 1 = green tip visible, 2 = visible leaves on the bud, 3 = grape stage and 4 = one or more flowers open on five plants per cultivar and treatment.

HR-MAS NMR of flower primordia

Metabolic fingerprinting of flower primordia were analyzed using the high-resolution magic angle spinning (HR-MAS) technique, which has previously proved valuable in profiling metabolites of flower primordia (Pagter et al. 2017b). Just before measuring respiration, two of the uppermost positioned axillary buds were harvested from five plants per treatment, immediately frozen in liquid nitrogen and stored at -80°C. The buds were later placed on dry ice and dissected free of the bud base, adjacent bud scales and leaf primordia to uncover the flower primordia. The flower primordia were fitted into disposable 50 µl pre-weighed inserts (Bruker Biospin, Rheinstetten, Germany) followed by addition of 10 µl of 0.05% (w/v) D_2O containing 0.05% (w/v) trimethylasilylpropionic acid, sodium salt (TMSP-d_4). The inserts containing TMSP-d_4 and plant material were weighed again to obtain the exact weight of the primordia before frozen again at -80°C until HR-MAS NMR analysis. All HR-MAS NMR measurements were performed at 280 K on a Bruker AVANCE 600 NMR spectrometer operating at 14.1 Tesla, observing ^1H at 600.13 MHz and equipped with a four channel ($^1H/^2H/^{13}C/^{31}P$) probe (Bruker BioSpin, Rheinstetten, Germany). Upon measurement the insert (sample) was put into a 4 mm zirconium rotor and ^1H NMR spectra were acquired with a CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence (Bruker cpmgpr1d) including presaturation to suppress signals from water molecules and attenuation of broad signals from macromolecules. The acquisition parameters for the spectra were as follows: 5 kHz spin rate, 64 scans

collected into 32k data points, a spectral width of 12.15 ppm, an acquisition time of 2.25 s, a total spin–spin relaxation delay of 100 ms, a spin-echo delay of 1 ms and a recycle time of 3 s. The spectra were processed by the application of an exponential multiplication of the FIDs by a factor of 0.3 Hz prior to Fourier transformation. Each spectrum was automatically phased, baseline corrected and referenced to TMSP-d_4 signal at 0.00 ppm as internal reference.

To aid spectral assignment, a 2D ^{13}C–^{1}H heteronuclear single quantum coherence (HSQC) experiment on a selected sample was performed. The HSQC correlating spectrum were acquired with a spectral width of 12.15 ppm in the ^{1}H dimension and 180.00 ppm the ^{13}C dimension, a data matrix with a size of 2048 × 512 data points, 128 transients per increment and a recycle delay of 3 s.

Signal assignments was performed according to earlier literature and available databases; the Human Metabolome Database (Wishart et al. 2007) and the Biological Magnetic Resonance Databank, University of Wisconsin (http://www.bmrb.wisc.edu, Ulrich et al. 2008). The Chenomx NMR Suite 8.1 (Chenomx, Edmonton, Canada) software was applied to assign and quantify metabolites by determining the area of each metabolite and comparing the area with the integral of the TMSP-d_4 signal. Afterwards, the metabolites were normalized to the FW of the primordia in each sample. A first step multivariate data analysis was performed on the whole NMR spectra excluding the water signal. Misalignments of the spectra were corrected using the icoshift procedure (Savorani et al. 2010). The regions 0.2-4.80 ppm and 5.02-10.0 ppm of the ^{1}H NMR spectra were segmented into 0.0132 ppm bins and integrated. The binned NMR-data were normalized to total area. Principal Components Analysis (PCA) was applied to the binned NMR spectra, which indicated that seven samples were outliers. Hence, these replicates were excluded from further analysis. Following HR- MAS NMR the flower primordia were lyophilized and their DWs recorded in order to calculate the metabolite concentrations on a DW basis. Since dissection of flower primordia is very time consuming and since each sample has be loaded manually onto the NMR spectrometer and subsequently manually shimmed to ensure homogeneity of the magnetic field, metabolomics of flower primordia was only carried out on plants subjected to the warmest (16/11°C) and the coldest (10/5°C) deacclimation treatments.

Determination of free fatty acids in buds

Simultaneously with the respiration measurements, three to four of the uppermost positioned axillary buds from five plants per treatment and cultivar were harvested and their FW determined. Samples were frozen in liquid nitrogen and stored at -80°C. For analysis of fatty acids, approximately 15 mg of lyophilized sample was extracted by 1 ml heptane containing 0.4 mg ml^{-1} of C12:1 triglyceride (Nu-Chek prep, MN, USA) as internal standard and 200 µl of 25% sodium methoxide solution in methanol (Sigma Aldrich, Steinheim, Germany) at 50°C for 10 min. Transesterification of fatty acids to methyl esters was carried out adding 1.5 ml of 10% methanolic HCl, heating at 90°C for 30 min, and subsequently cooling in an ice bath. Still on ice, samples were mixed with 10 wt% K_2CO_3 in water,

and phase separation was carried out spinning the samples at 500 *g* for 5 min. Hereafter, the heptane layer containing the fatty acid methyl esters was transferred to an empty vial.

Separation and identification of methyl esters were performed using a HP 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA), equipped with a Restek fused silica column (model Rt-2560, 100 meter, 0.25 mmiD, 0.2 µm df; Restek Corp, PA) coupled with a flame ionization detector. Helium was used as a carrier gas at a constant flow of 1 ml min^{-1}, detector and injector temperatures were 250°C. Oven temperature profile was 140°C for 2 min, increased by 8°C min^{-1} to 170°C held for 9 min, increased by 6°C min^{-1} to 200°C held for 16 min, increased by 6°C min^{-1} to 240°C and held for 15 min. Peak identification was performed using an external standard mix of pure methyl esters (FAME mix C4-C24, Supelco, Bellefonte, PA). Quantification was performed by normalization of the data against the internal standard and comparing peak area of identified fatty acids methyl esters to standard curves of the external standard mix. Relative values for the identified fatty acids in % of the total content were used to analyze treatment effects on the fatty acid composition of the floral buds. The analysis was carried out on five biological replicates per treatment, with two analytical replicates for each biological replicate.

Data analysis

Freezing tolerance expressed as freezing temperature (°C) was estimated as LT_{50}-values, defined as the temperature where 50% of the buds contained dead flower primordia. For each sampling date and treatment, the LT_{50} values were determined by fitting a curve to the percentage of surviving flower primordia for all six replicates to temperature using a four-parameter sigmoid model in SAS (PROC NLIN, SAS Institute, Cary, NC). Differences between LT_{50} estimates were regarded as significant, if the 95% confidence intervals did not overlap.

The effects of cultivar, deacclimation temperature and treatment duration on bud respiration rates, metabolite concentrations and relative fatty acid values were analyzed using a three-way ANOVA in R (R Core Team 2015) with correction for multiple testing using the Benjamini-Hochberg method (Benjamini and Hochberg 1995). The statistical analysis of metabolite concentrations and relative fatty acid values only included data from 4 DOD onwards, as no treatment was employed at 0 DOD. However, data collected at 0 DOD are included in Fig. 4 and Figs 6-7 to further illustrate changes depending on treatment duration.

Principal component analysis (PCA) was performed using the FactoMineR package in R (Lê et al. 2008). Hierarchical clustering of metabolite concentrations normalized against the concentration determined in flower primordia of cold acclimated plants (0 DOD) was performed in MultiExperiment Viewer (MeV) v. 4.9.0 (Saeed et al. 2003) using a Pearson correlation and average linkage. Spearman rank order correlations were carried out in SAS (PROC CORR).

Results

Freezing tolerance of flower primordia, bud break and bud respiration

Flower primordia of 'Ben Hope' were considerably more cold hardy than primordia of 'Zusha', both at initiation of the experiment and after 12 DOD and 12 DOR (Table 1). 12 DOD caused a significant decrease in freezing tolerance of both cultivars. In 'Ben Hope', deacclimation progressed faster at temperatures of 16/11°C compared with 13/8 and 10 /5°C, whereas in 'Zusha', floral buds of plants exposed to 16/11 and 13/8°C deacclimated faster than buds of plants in the coldest deacclimation treatment. None of the plants were able to reacclimate in response to 4°C. Hence, freezing tolerance of flower primordia of 'Ben Hope' deacclimated at 16/11 and 10/5°C remained stable, whereas at 12 DOR flower primordia of plants deacclimated at 13/8°C had lost further freezing tolerance. Following the reacclimation treatment, flower primordia of 'Zusha' were heavily damaged by all freezing test temperatures, making it impossible to estimate LT_{50} for this cultivar and implying further significant deacclimation.

Flower buds of both cultivars started to break during deacclimation (Fig. 1). After 12 DOD 68, 48 and 8% of the five uppermost buds had reached the 'green tip' or 'visible leaves' stage in 'Zusha' at 16/11, 13/8 or 10/5°C, respectively. In 'Ben Hope' bud development was slower. Hence at 16/11°C approximately 32% of the buds showed visible signs of development after 12 DOD, whereas buds of plants at 13/8 and 10/5°C did not start to break. The following re-acclimation period at 4°C slowed down, but did not halt, bud break of both cultivars. Thus, after 9 DOR at least 80% of the buds of both cultivars deacclimated at 16/11°C and 'Zusha' deacclimated at 13/8°C showed signs of development. Flower buds of 'Zusha' deacclimated at 10/5°C also continued to develop during the reacclimation period, but did only reach a bud break percentage of 52% at the end of the reacclimation period. Buds of 'Ben Hope' deacclimated at 13/8 or 10/5°C started to burst during the reacclimation period, but slowest in plants deacclimated at 10/5°C.

The bud respiration rate was not affected by either cultivar or treatment, and therefore data for the two cultivars and treatments were pooled (Fig. 2). During 12 DOD the respiration rate decreased from approximately 70 nmol O_2 g^{-1} DW min^{-1} to less than 40 nmol 0_2 g^{-1} DW min^{-1}, where after a significant increase was seen during the reacclimation period.

HR-MAS NMR metabolite profiling of flower primordia

Principal component analysis (PCA) was used to identify the largest variance components in the metabolite data (Fig. 3). Principle component 1 (PC1) explained 54% of the variation and separated profiles of flower primordia of the two cultivars in different ways. Generally samples of 'Ben Hope' clustered rather closely together, however, for plants deacclimated at 10/5°C, PC1 separated samples deacclimated for 8 and 12 days from all other samples, while for plants deacclimated at warmer temperatures (16/11°C), it separated samples deacclimated for 4 or 8 days from all other samples. For 'Zusha', PC1 separated the profiles taken after cold acclimation and different durations of deacclimation and reacclimation. Especially for 'Zusha' deacclimated at 16/11°C did the distribution

of profiles follow an order from cold acclimated to 4 and 8 DOD, 12 DOD, 4 and 8 DOR and 12 DOR. In 'Zusha' deacclimated at 10/5°C, the samples followed an order from 4 DOD, 8 DOD, CA, 12 DOD, 12 DOR and 4 and 8 DOR, indicating that samples of plants reacclimated for 12 days at 10/5°C metabolically were approaching deacclimating samples. PC2 explained 11% of the variation and tended to separate profiles of flower primordia according to deacclimation temperature. According to the variables factor map (Fig. S1) most metabolites were highly correlated to the first dimension, while in the second dimension samples were mostly separated due to their content of fumarate and to a lesser extent choline.

Of the 22 metabolites identified from the HR-MAS NMR spectra, citrulline was the only metabolite whose concentration did not change significantly depending on cultivar, deacclimation temperature, treatment duration or an interaction as revealed by three-ANOVA (Table S1). Except for sucrose, the concentration of all significantly regulated metabolites changed according to treatment duration, while 13 metabolites were significantly affected by cultivar or an interaction between cultivar and deacclimation temperature. The concentrations of asparagine, choline, fumarate and succinic acid varied significantly with deacclimation temperature. Clustering of the metabolites normalized against the concentration determined in flower primordia of cold acclimated plants (0 DOD) revealed four major patterns of metabolic responses (Fig. 4): (a) Myo-inositol, TCA cycle intermediates (citric acid, succinic acid and malic acid) and most amino acids (glutamate, valine, glutamine, asparagine, lysine and isoleucine) remained stable during early deacclimation but increased during late deacclimation or reacclimation in 'Zusha'. For most of the metabolites, the concentration increase appeared earlier at 16/11°C than at 10/5°C. In 'Ben Hope', the tendency was the same, but in this cultivar the metabolites in cluster (a) initially decreased and concentration increases appeared later and/or were smaller than in 'Zusha', especially in primordia of plants deacclimated at 10/5°C. The concentrations of metabolites in cluster (b) also increased during late deacclimation and/or reacclimation, but in this cluster cultivar differences were smaller. Hence, only the concentration of one out of the four metabolites varied significantly between cultivars. This cluster contained quinic acid, proline, alanine and maltose. The concentrations of fumarate and choline in cluster (c) increased strongly during deacclimation and/or reacclimation. The increase appeared earlier in 'Zusha' than in 'Ben Hope' and earlier at warmer than at cooler deacclimation temperatures. Accordingly, the concentrations of these two metabolites were significantly affected by an interaction between cultivar, deacclimation temperature and treatment duration. Concentrations of glucose and fructose in cluster (d) initially decreased during deacclimation, whereafter they increased, with the increase being more pronounced in 'Zusha' than in 'Ben Hope'. In addition, three small clusters were identified, containing one or two metabolites, including O-phosphocholine, sucrose, citrulline and arginine. The concentration of these four metabolites varied only little during the experiment.

Fatty acids in buds

Of the 37 fatty acid methyl esters (FAMEs) in the standard mix, eight FAMEs were identified in the blackcurrant buds. These were assigned to c16 (palmitic acid), c18:1 n9 (oleic acid), c18:2 n6 (linoleic acid), c20 (arachidic acid), c18:3 n6 (ɣ-linolenic acid), c18:3 n3 (α-linolenic acid), c20:2 (eicosadionic acid) and c22 (behenic acid). According to the PCA on the relative fatty acid values (Fig. 5) it was possible to distinguish the effects of treatment duration and deacclimation temperature for both cultivars as seen also for the HR-MAS profiles. PC1 explained 52% of the variance and separated profiles of cold acclimated and deacclimating buds from reacclimating buds. This was most clear for samples of 'Zusha', which were separated further apart depending on treatment duration than samples of 'Ben Hope'. PC2 explained 19% of the variation and tended to separate samples of 'Zusha' deacclimated for 12 d at the two warmest deacclimation temperatures (16/11 and 13/9°C) and samples of 'Ben Hope' deacclimated for 12 days at the warmest deacclimation temperature (16/11°C) from all other samples. According to the variables factor map (Fig. S2) cold acclimated and deacclimating buds were separated from reacclimating buds due to higher relative values of linoleic, arachidic, ɣ-linolenic and eicosadionic acids in cold acclimated and deacclimating buds and higher relative values of oleic and α-linolenic acids in reacclimating buds. In the second dimension, samples were mostly separated according to their relative values of palmitic acid.

The ratio of unsaturated to saturated fatty acids did not differ significantly with treatment duration, cultivar or deacclimation temperature, but the relative values of the individual fatty acids varied significantly depending on cultivar, deacclimation temperature, treatment duration or an interaction between two or all three factors (Table S2). All eight fatty acids varied between cultivars and with deacclimation temperature or an interaction between cultivar and deacclimation temperature. Except for palmitic acid, the relative values of all fatty acids also changed depending on treatment duration (Figs. 6-7). The relative content of oleic acid increased dramatically during reacclimation especially in 'Zusha' deacclimated at the warmest temperatures. Accordingly oleic acid was significantly affected by an interaction between all three factors. The relative contents of eicosadionic acid, ɣ-linolenic acid, behenic acid and arachidic acid decreased during late deacclimation (8 DOD-12 DOD) or during reacclimation. This accumulation pattern was most clear for 'Zusha' deacclimated at 16/11°C, whereas for other treatments and especially in 'Ben Hope' the pattern was less clear. The relative contents of α-linolenic acid and linoleic acid varied little in 'Ben Hope' during the experiment, while in 'Zusha' α-linolenic acid increased and linoleic acid decreased slightly during reacclimation.

A global correlation analysis among the eight fatty acids in flower buds indicated that levels of most fatty acids were regulated in a similar fashion in 'Zusha' during deacclimation and the reacclimation treatment. For an easier overview, Fig. 8 only shows the color-coded *P*-value ranges of these correlations. The numerical *P*-values can be found in Table S3. There were some exceptions though. Hence, the relative values of palmitic acid and behenic acid did not correlate with any other fatty acids. In 'Ben Hope', most correlations between fatty acids levels were reduced or absent. However, the persisting correlations were generally in the same direction as observed in 'Zusha'.

Discussion

Both cultivars displayed a significant drop in freezing tolerance of flower primordia after 12 DOD, but the deacclimation response varied with cultivar and deacclimation temperature. When exposed to the coldest deacclimation treatment (10/5°C), 'Ben Hope' had lost 36% of its freezing tolerance after 12 DOD (as percentage of LT_{50} of CA plants), whereas 'Zusha' had lost only 18% of its freezing tolerance. A more rapid loss of freezing tolerance in more tolerant genotypes has been reported before for other plants species (Pagter et al. 2011a,b, Zuther et al. 2015). In both genotypes, deacclimation progressed faster with increasing deacclimation temperature. However, after 12 DOD at 16/11°C and 13/8°C the more sensitive genotype 'Zusha' had lost 61 and 52% of its freezing tolerance, whereas the corresponding percentages for 'Ben Hope' were 48 and 26%. Differential loses of cold hardiness in response to different deacclimation temperatures indicates that the effect of temperature on the deacclimation response differs with genotype. Hence, at lower temperatures 'Zusha' may be more deacclimation resistant than 'Ben Hope' and vice versa at higher temperatures.

A strong potential for deacclimation was associated with bud break in both cultivars as evidenced by bud break percentages between 32-68% after 12 DOD at 16/11°C in both cultivars and at 13/8°C in 'Zusha'. In contrast, no or only few buds started to break in the other treatments, which were less effective in terms of decreasing freezing tolerance. This highlights the dual effect of temperature on deacclimation and ontogenetic development towards bud burst in plants released from endodormancy. In accordance with observations across four temperate deciduous tree species (Vitra et al. 2017), bud break was earliest in 'Zusha', which generally showed the highest dehardening potential. 'Zusha' is also known to be an early flushing genotype under natural conditions (Andersen et al. 2017, Winde et al. 2017).

The rehardening potential of both cultivars after 12 DOD at various temperatures was nil, as all plants, except for 'Ben Hope' exposed to the lowest deacclimation temperatures (10/5°C), lost further freezing tolerance during the reacclimation treatment. Hence, the reacclimation treatment did not reverse but only delayed deacclimation at best. It is very likely that the ability of blackcurrant to reharden decreases with the progression of winter as observed in other plants species (Pagter and Williams 2011, Vitra et al. 2017), and that the reacclimation capacity would have been greater during the endodormancy phase. However, the fact that none of the plants were able to increase their freezing tolerance following 12 DOD emphasizes the vulnerability of temperate woody perennials to irregular temperature patterns and late spring frost during ecodormancy and supports the proposition that spring temperatures are decisive in shaping cold range limits of temperate woody perennials (Vitra et al. 2017). It is additionally in line with the suggestion, that dehardening becomes irreversible and tissues lose their capacity to reharden after development and elongation growth has been initiated in the spring (Rapacz 2002, Jouve et al. 2007, Vitasse et al. 2014). Interestingly, bud development continued during the reacclimation treatment. Although an increasing length of natural chilling has been shown

to increase the ability for budburst at low temperatures (Junttila and Hänninen 2012), it is surprising that low, non-freezing temperature that are usually conducive for cold acclimation (i.e. 4°C) did not halt bud flushing. This may suggest that at non-freezing temperatures not only dehardening but also bud flushing is irreversible, when tissues have started to become active.

Since the reacclimation treatment did not reverse but only delayed deacclimation, the observed metabolite and fatty acid patterns and changes likely reflects metabolic regulation during deacclimation and bud opening rather than metabolic regulation during deacclimation and reacclimation. In accordance with our second hypothesis, both genotypic and treatment differences in loss of freezing tolerance and bud burst were visible at the metabolic level. Thus, dynamic changes in primary metabolism were in most cases greater and occurred earlier in 'Zusha' than in 'Ben Hope', consistent with earlier bud break and relatively greater losses of freezing tolerance of the former than the latter cultivar. Similarly, in both genotypes plants exposed to the warmest deacclimation treatment (16/11°C) generally showed an earlier onset of increases or decreases in significantly regulated metabolites and fatty acids than plants exposed to the lower deacclimation temperatures (13/8°C and/or 10/5°C).

In 'Ben Hope', most of the identified TCA cycle intermediates (citric, succinic and malic acids) decreased during 4-12 DOD, where after their concentrations increased. This suggests that the TCA cycle is either depleting substrates rapidly or that relatively less substrate (e.g. pyruvate) is being fed into the TCA cycle, when plants are shifted to deacclimating conditions. Accordingly, the bud respiration rate of both cultivars increased transiently, although non-significantly, during early deacclimation. In Arabidopsis, depletion of both TCA cycle and glycolytic intermediates during the first 24 h of deacclimation has also been proposed to be due to increased respiratory energy production associated with growth resumption (Pagter et al. 2017a). In 'Zusha', a similar concentration decrease in TCA cycle intermediates during deacclimation was not visible. It is possible that an increase in respiratory activity occurred before 4 DOD in this cultivar, which were more responsive to deacclimating conditions than 'Ben Hope', and therefore was not captured on a time scale of several days. After 12 DOD the flower bud respiration rate had decreased coinciding with increasing concentrations of most TCA cycle intermediates. Hence, after some days of deacclimation the need for respiratory energy production may decrease leading to less rapid substrate depletion. Increased respiratory energy production was likely fuelled by hexose sugars accumulated in cold acclimated flower primordia, as the concentrations of fructose, glucose and maltose decreased during early deacclimation in both cultivars. A general decrease in sugar content during deacclimation has also been found in other plants species (Pagter et al. 2011b, Shin et al. 2015, Zuther et al. 2015), and may be functionally related to a loss of freezing tolerance (Pagter and Arora 2013). In addition to using accumulated hexoses, the meristematic cells of the flower primordia may take up free hexoses exported from vegetative bud structures or other organs to satisfy the energy requirements of the flower primordia. Hence, under field conditions upregulation of a hexose transporter homologue in

flushing buds of blackcurrant was proposed to be associated with increased uptake of hexoses to satisfy the energy requirements of the flower bud meristems (Andersen et al. 2017). Since the sucrose concentration did not change with treatment duration, sucrose catabolism is likely not a source of energy and carbon skeletons in developing flower primordia.

Both the concentrations of TCA cycle intermediates and the bud respiration rate increased during the reacclimation treatment. Increased amounts of TCA cycle intermediates have both been associated with exposure to cold acclimating conditions (Kaplan et al. 2004, Korn et al. 2010) and bud development (Andersen et al. 2017, Dhuli et al. 2014) and it is therefore difficult to determine the reason for the increasing levels of TCA intermediates as found in this study. If the increasing respiratory rate during the reacclimation treatment was indeed a low temperature response it may be a result of metabolic cold acclimation responses, although they did not result in increased freezing tolerance. Hence, Sperling et al. (2015) recently showed that numerous tree species respond to cooling at near-freezing temperatures by significantly increasing stem respiration rates.

Increasing levels of most amino acids during late deacclimation and/or the reacclimation treatment may be the result of degradation of storage proteins as observed in cambial meristems of *Populus tremula* during reactivation in spring (Druart et al. 2007). The amino acids may be imported from other organs to supply building blocks for the synthesis of new proteins as meristems become active (Cooke and Weih 2005). Increasing levels of several amino acids has previously been observed in developing flower buds of blackcurrant grown under natural conditions (Andersen et al. 2017). Interestingly, more of the amino acids increasing in flower primordia have been associated with flower development. Hence, in *Lotus japonica*, plants with metabolic lesions reducing the glutamate content in flowers were sterile, suggesting that high concentrations of glutamate and of amino acids derived from it, such as proline, may be required for adequate development of floral tissue (Suárez et al. 2003). Correspondingly, many plants accumulate large amounts of proline during the transition to flower initiation (Kavi Kishor et al. 2015). Branched-chain amino acids have been implicated in shoot organogenesis in white spruce (Dowlatabadi et al. 2009). Thus, accumulation of valine in flower primordia of developing buds may also be associated with the development of flowers.

Fatty acid desaturation and changes in lipid composition are broadly linked to acclimation to both chilling and freezing temperatures (Angelcheva et al. 2014), and although little is known about lipid changes during deacclimation, the few existing studies indicate that membrane lipid changes during deacclimation are largely a reversal of those observed during cold acclimation (Yoshida 1986, Iivonen et al. 2004). Hence, it might be expected that long-chain polyunsaturated fatty acids would disperse during deacclimation. In keeping with this expectation, the levels of ɣ-linolenic (c18:3 n6), linoleic (c18:2) and eicosadionic (c20:2) acids decreased and the level of oleic acid (c18:1) increased during late deacclimation and the reacclimation treatment, when flower primordia lost further freezing tolerance, indicating that lipid saturation is reversed during loss of freezing tolerance. Coordination between these fatty acids during loss of freezing tolerance was further supported by the strong

correlations between their relative levels in 'Zusha' and to a lesser extent in 'Ben Hope'. The strong negative correlation between ɣ-linolenic (c18:3) and α-linolenic (c18:3) acids in 'Zusha' and a negative, but non-significant correlation (P = 0.10) in 'Ben Hope' suggests that these isomers are functionally different in blackcurrant. The contents of α- and ɣ-linolenic acids have previously been shown to correlate negatively in the wild species *Ribes spicatum* (Johansson et al. 2000). In addition to their key roles in signal perception and transmission, plant plasma membranes are themselves an important source of signaling molecules, many of which are derived from fatty acids. The substrate of jasmonate (JA) biosynthesis is α-linolenic acid (Wasternack and Hause 2013, Li et al. 2016). Free α-linolenic acid (18:3) is oxygenated by lipoxygenase enzymes (LOX) and then converted to 12-oxo-phytodienoic acid (OPDA) through the combined action of allene oxide synthase (AOS) and allene oxide cyclase (AOC). OPDA is reduced by OPDA REDUCTASE3 (OPR3) and converted to JA by three cycles of β-oxidation (Wasternack and Hause 2013). In Arabidopsis, both *LOX1*, *AOC* and *OPR3* were significantly up-regulated when cold acclimated plants were transferred to warm conditions (Pagter et al. 2017a), indicating that deacclimation is associated with increased endogenous JA levels. Hence, accumulation of α-linolenic acid in blackcurrant flower buds may be associated with increased JA synthesis during deacclimation. JA is known to regulate a wide spectrum of plant processes, such as growth and development, as well as stress defense. Recently, very-long-chain fatty acids (> 18 carbons) have also been proposed to play a signaling role in floral organ differentiation. Thus, in Arabidopsis the two transcription factors CRC and AP1, which are key regulators of floral organ differentiation, are positive regulators of a number of fatty acids, including arachidic acid (c20) (Han et al. 2012). Decreasing levels of saturated arachidic acid (c20) in deacclimating blackcurrant flower buds may therefore not be entirely related to a change in membrane fluidity. Palmitic acid (c16), which quantitatively was one of the most important fatty acids, does not seem to play an important role in transitions in freezing tolerance in floral buds of blackcurrant, as it did not change with treatment duration although it increased with increasing deacclimation temperature. Similarly the relative amount of palmitic acid in needles of *Picea obovate* changed little during cold acclimation (Angelcheva et al. 2014).

In conclusion, our results document that despite differences in the progression of deacclimation, the capacity of ecodormant blackcurrant flower buds to reharden in late winter is non-existing or very low, indicating that increasingly irregular temperature patterns during late winter and spring is critical for blackcurrant fruit yield. Deacclimating conditions both promote loss of freezing tolerance and bud break of blackcurrant. Growth resumption and development of flower primordia is associated with a transient increase in respiration and is likely fuelled by catabolism and interconversion of sugars and proteins accumulated during cold acclimation. Decreasing levels of sugars and desaturation of membrane lipids, which likely decreases membrane fluidity, may cause loss of freezing tolerance. However, changes in membrane fatty acids may also reflect biosynthesis of signalling molecules stimulating growth and floral organ differentiation.

Author contributions

K.H.K. and M.P. designed and performed the experiment; J.W. and K.K.P. performed the fatty acid analysis, K.H.K. and C.C.Y. performed the HR-MAS NMR analysis; K.H.K. and M.P. analyzed the data and wrote the manuscript; all authors read the manuscript, edited and commented on it before submission. All authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1. Variables factor map of the PCA based on 22 metabolites in flower primordia.

Fig. S2. Variables factor map of the PCA based on 8 lipids in flower buds.

Table S1. Outcome of the analysis of metabolite concentrations identified from the HR-MAS NMR spectra using a three-way ANOVA.

Table S2. Outcome of the analysis of relative fatty acid values using a three-way ANOVA.

Table S3. Spearman correlation matrix with the *P*-values for all pairwise correlations between relative fatty acid levels in flower buds.

Figure legends

Fig. 1. Percentage bud break measured for the five uppermost positioned lateral buds of 10 replicate plants of *Ribes nigrum* 'Ben Hope' (circles) and 'Zusha' (triangles). Cold acclimated plants were deacclimated for 12 days at 10/5°C (black), 13/8°C (grey) or 16/11°C day/night (white) and then reacclimated for 12 days at 4°C. Bud stages were arranged in two classes, where 0 = no bud development and 1 = visible signs of bud break (green tip or one or more leaves unfolded) respectively.

Fig. 2. Dark respiration rates of detached buds from *Ribes nigrum* 'Ben Hope' and 'Zusha'. Cold acclimated plants were deacclimated for 12 days at 10/5, 13/8 or 16/11°C day/night and then reacclimated for 12 days at 4°C. The values are means of 24 measurements ± SE. Different letters indicate significant differences ($P \leq 0.05$) between treatment durations.

Fig. 3. Score plot from principal component analysis (PCA) applied to a NMR metabolite dataset from flower meristems of *Ribes nigrum* 'Zusha' (closed symbols) and 'Ben Hope' (symbols with a cross). Cold acclimated plants (CA) were deacclimated for 12 days at 10/5°C day/night (circles) or 16/11°C day/night (squares) and then reacclimated for 12 days at constant 4°C (n = 4-6). Colours illustrate cold acclimated samples and the different sampling days from 4-12 days of deacclimation (DOD) and 4-12 days of reacclimation (DOR).

Fig. 4. Overview of the dynamic changes in primary metabolism in flower primordia of *Ribes nigrum* 'Zusha' (Z) and 'Ben Hope' (BH). Cold acclimated plants (CA) were deacclimated at 10/5°C (10) or 16/11°C (16) for 4, 8 or 12 days (DOD) and then reacclimated at 4°C for 4, 8 or 12 days (DOR, n = 4-6). Metabolite concentrations normalized against the concentration determined in flower primordia of cold acclimated plants were clustered using Pearson correlation and average linkage. The panel shows normalized metabolite intensities as indicated by the different colors.

Fig. 5. Score plot from principal component analysis (PCA) of relative fatty acid values in flower buds of *Ribes nigrum* 'Zusha' (closed symbols) and 'Ben Hope' (symbols with a cross). Cold acclimated plants (CA) were deacclimated for 4, 8 or 12 days at 10/5°C day/night (circles), 13/8°C (triangles) or 16/11°C day/night (squares) followed by 4, 8 or 12 days of reacclimation at 4°C (n = 3-6). Colours illustrate cold acclimated samples and the different sampling days from 4-12 days of deacclimation (DOD) and 4-12 days of reacclimation (DOR).

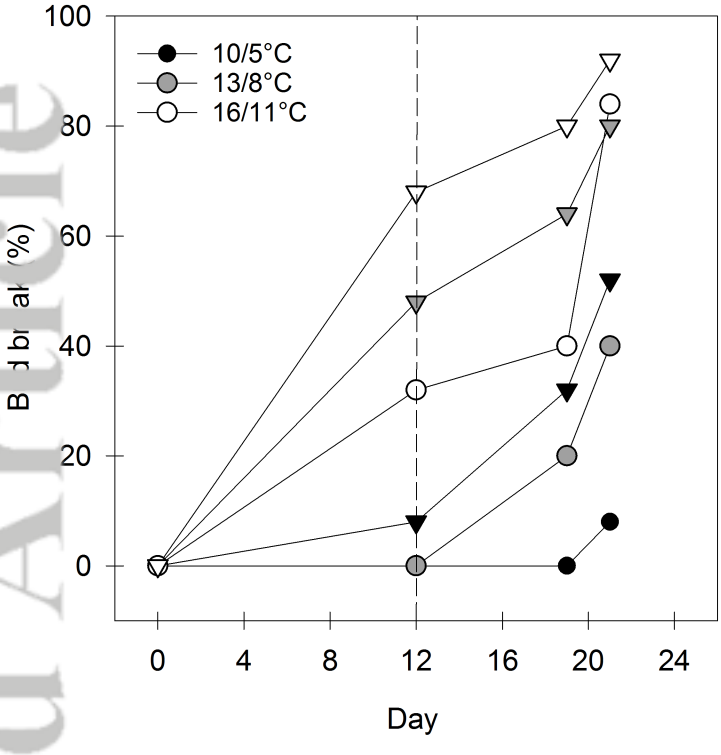
Fig. 6. Overview of the dynamic changes in palmitic, oleic, linoleic and arachidic acids in flower buds of *Ribes nigrum* 'Ben Hope' (left) and 'Zusha' (right). Cold acclimated plants (CA) were deacclimated for 4, 8 or 12 days at 10/5, 13/8 or 16/11°C day/night and then reacclimated at 4°C for 4, 8 or 12 days (n = 3-6).

Fig. 7. Overview of the dynamic changes in ɣ-linolenic, α-linolenic, eicosadionic and behenic acids in flower buds of *Ribes nigrum* 'Ben Hope' (left) and 'Zusha' (right). Cold acclimated plants (CA) were deacclimated for 4, 8 or 12 days at 10/5, 13/8 or 16/11°C day/night and then reacclimated at 4°C for 4, 8 or 12 days (n = 3-6).

Fig. 8. Correlation coefficients between relative fatty acid levels in *Ribes nigrum* 'Ben Hope' (top panel) and 'Zusha' (lower panel). Cold acclimated plants were deacclimated for 4, 8 or 12 days at 10/5, 13/8 or 16/11°C day/night and then reacclimated at constant 4°C for 4, 8 or 12 days. $P \leq 0.05$ (yellow), $P \leq 0.01$ (orange), $P \leq 0.001$ (red). The numerical *P*-values can be found in Table S3.

Table 1. Freezing tolerance expressed as freezing temperature (°C) was estimated as the temperature where 50% of the buds contained dead flower primordia (LT_{50}) of buds of *Ribes nigrum* 'Ben Hope' and 'Zusha' subjected to 12 days of deacclimation (DOD) at either 16/11, 13/8 or 10/5°C day/night followed by 12 days of reacclimation (DOR) at constant 4°C. LT_{50} values [mean ± SE (°C)] are shown for six plants tested at eight temperatures. Within cultivars different letters indicate significant differences between LT_{50} values. Differences between LT_{50} values were regarded as significant, if the 95% confidence intervals did not overlap.

Cultivar	0 DOD	Deacclimation temperature	12 DOD	12 DOR
			LT_{50} (°C) ± SE	
'Ben Hope'	-39.1 ± 2.6^{e}	16/11°C	-20.5 ± 0.7^{b}	-19.2 ± 1.0^{ab}
		13/8°C	-28.9 ± 4.4^{d}	-19.8 ± 0.0^{a}
		10/5°C	-25.0 ± 2.2^{cd}	-22.1 ± 0.7^{bc}
'Zusha'	-24.1 ± 0.6^{c}	16/11°C	-9.5 ± 2.2^{a}	< -10
		13/8°C	-11.6 ± 1.9^{a}	< -10
		10/5°C	-19.8 ± 0.1^{b}	< -10



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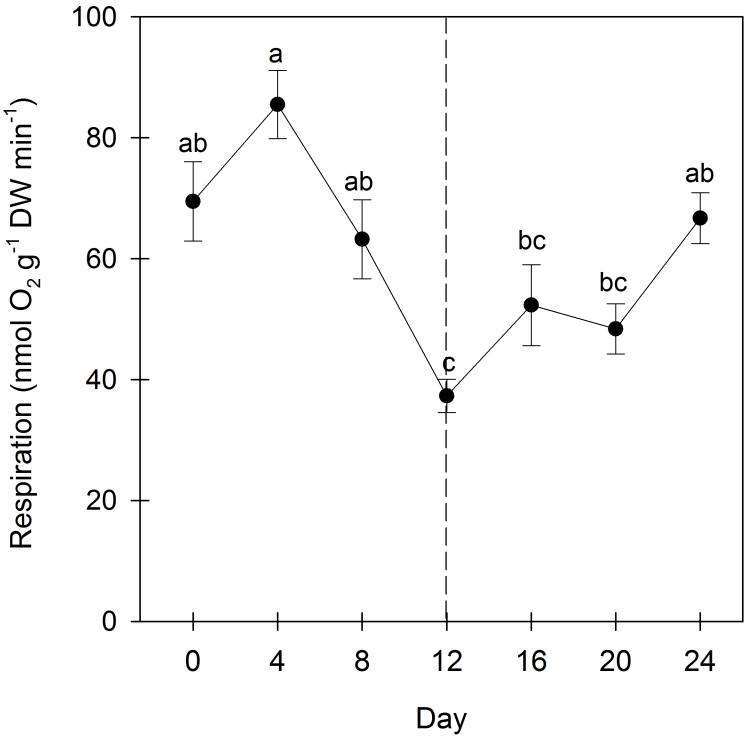

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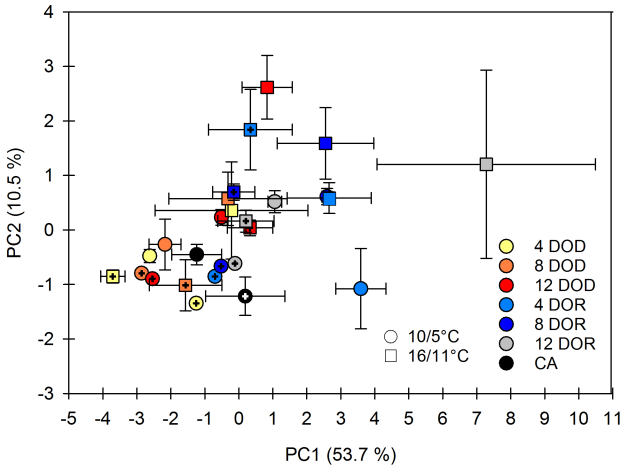

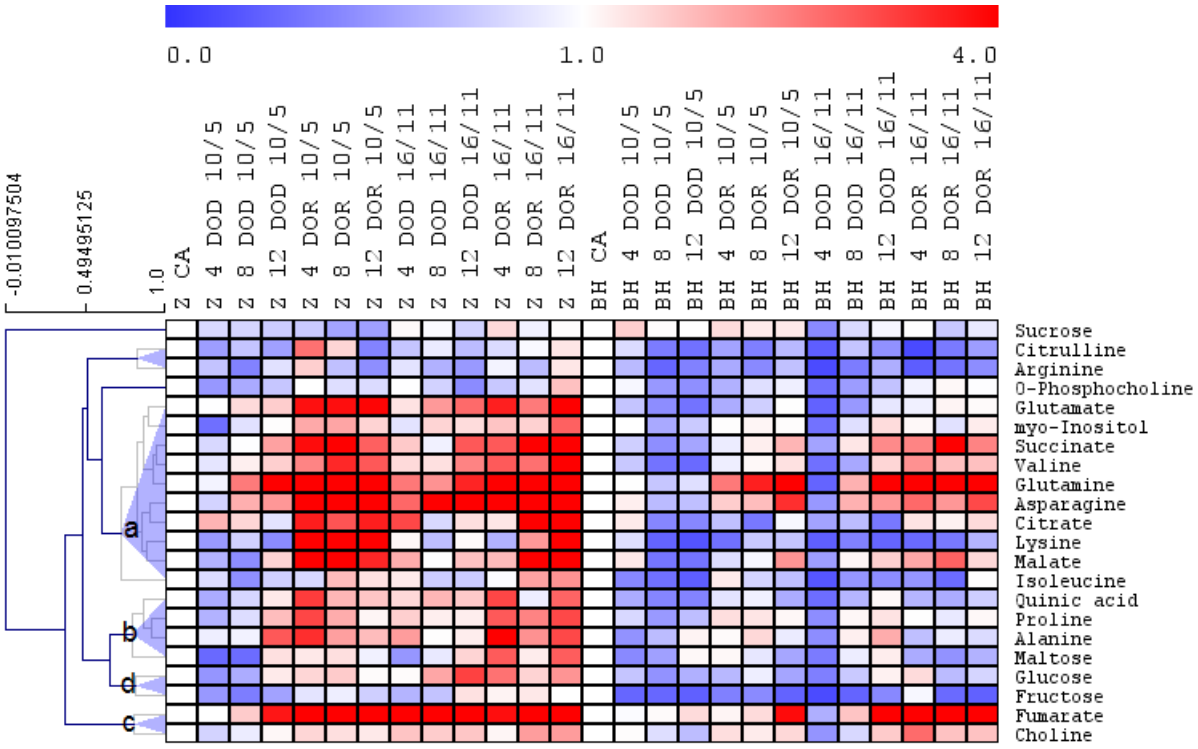

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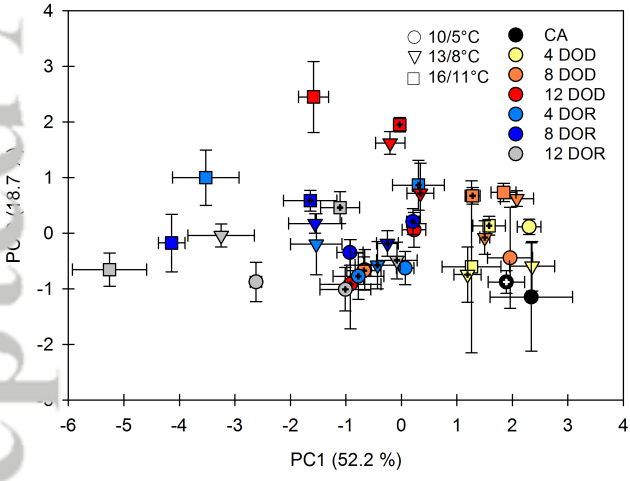

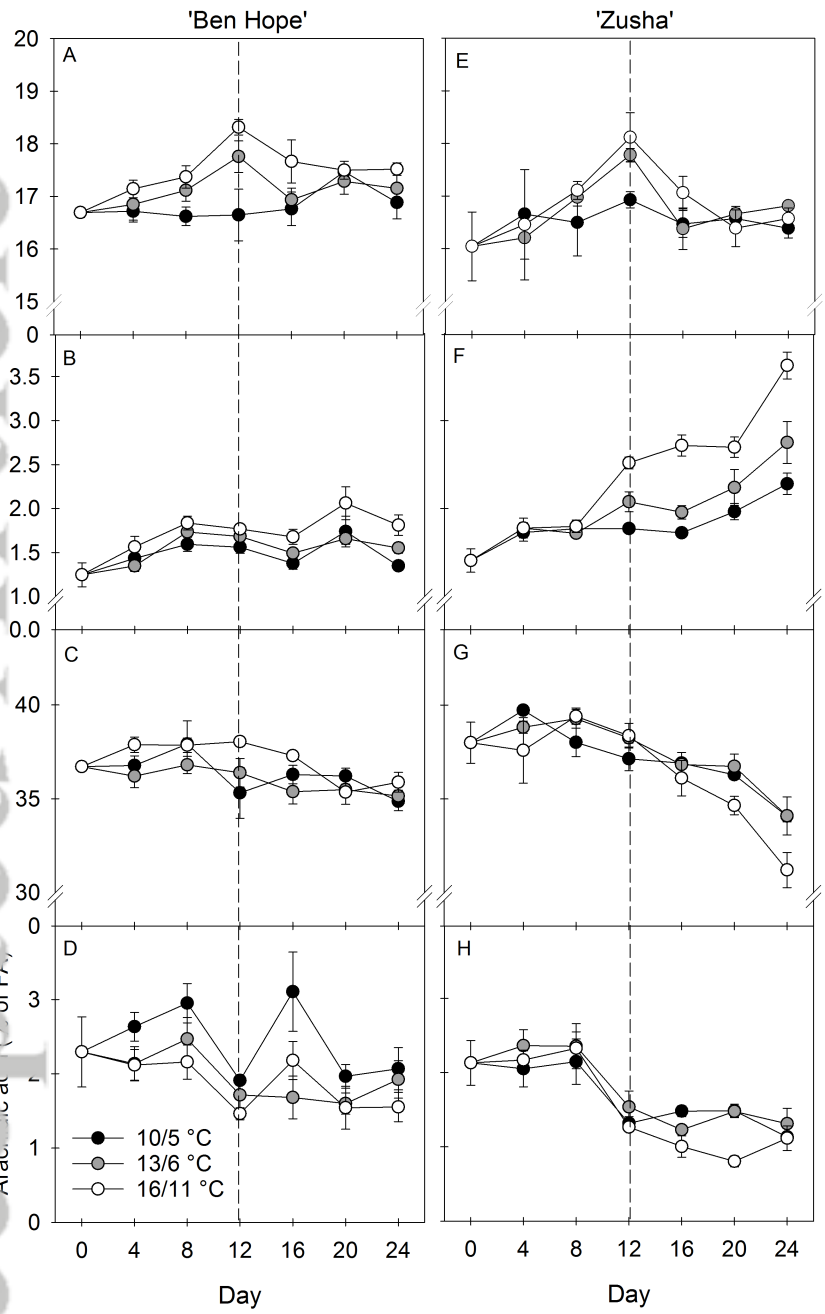

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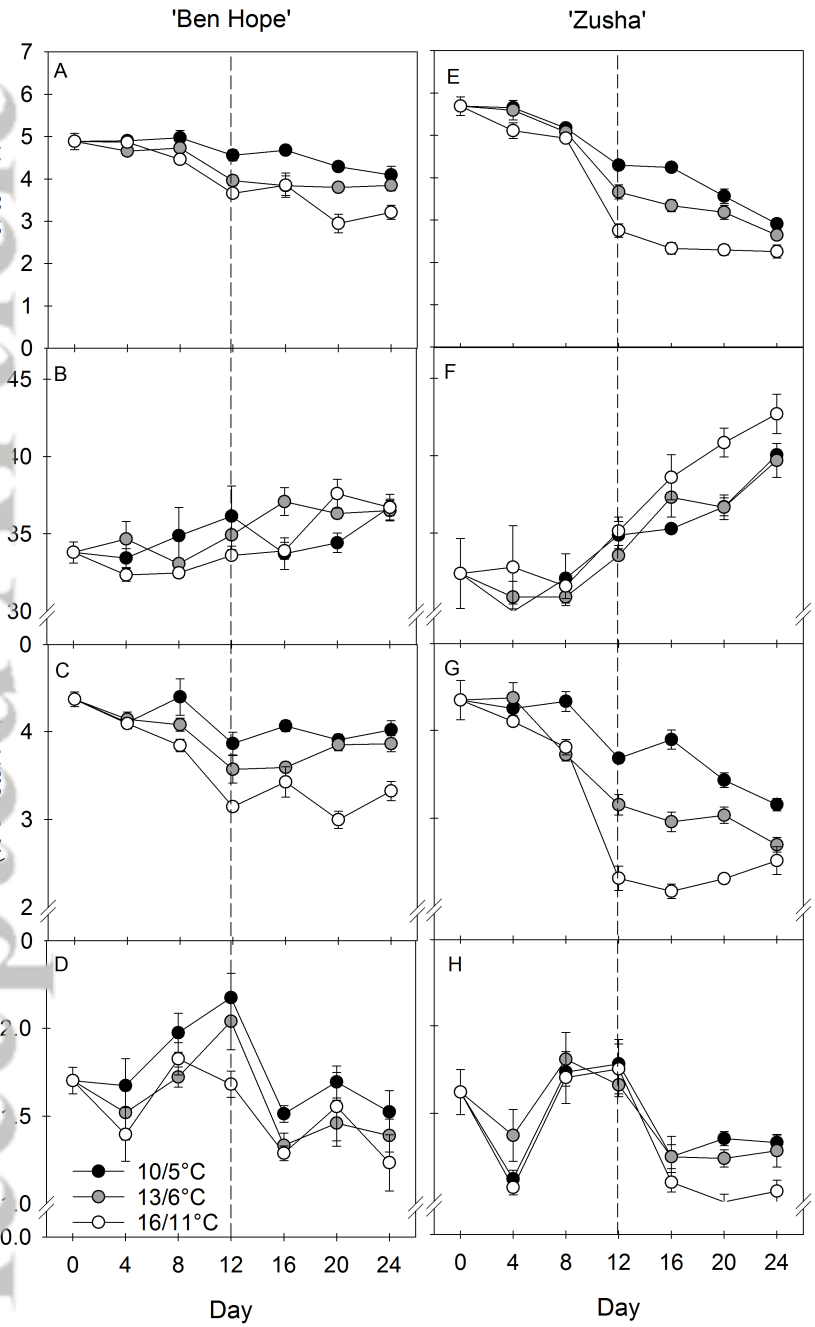

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	Palmitic acid	Oleic acid	Linoleic acid	Arachidic acid	ɣ-linoleic acid	α-linoleic acid	Eicosadionic acid	Behenic acid
Palmitic acid		0.65		-0.58	-0.83		-0.81	
Oleic acid					-0.59		-0.72	
Linoleic acid		-0.62				-0.73		
Arachidic acid		-0.70	0.75		0.76		0.76	
ɣ-linoleic acid		-0.87	0.78	0.87			0.93	
α-linoleic acid		0.74	-0.95	-0.85	-0.92			
Eicosadionic acid		-0.88	0.62	0.85	0.92	-0.78		
Behenic acid								

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