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Genotypic differences in metabolomic changes during storage induced-degreening of chrysanthemum disk florets

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ABSTRACT

Selecting chrysanthemum cultivars with long storability and vase life is a major challenge for breeders. The rate of degreening of disk florets during the postharvest phase is an important determinant of vase life. There is large genotypic variation in susceptibility to disk floret degreening. Our aim was to understand these genotypic differences at the physiological level. Carbohydrate starvation seemed to play a role, since application of sugars prevented degreening and degreening only occurred if florets had a long-term low carbohydrate content. In order to find out which metabolic processes could explain genotypic differences, we used ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy profiling, High Performance Anion Exchange Chromatography (HPAEC) and respiration measurements to compare metabolic responses of three genotypes to carbohydrate starvation. HPAEC and NMR measurements showed that carbohydrate content could not fully explain genotypic differences. A genotype with intermediate sensitivity to degreening showed similar carbohydrate content compared to an insensitive one. However, respiration rate declined faster under carbohydrate starvation in a sensitive and intermediate sensitive genotype compared to an insensitive genotype, suggesting a more abrupt constraint on the mitochondrial electron transport chain and with that oxidative stress. Changes in the metabolic profile under carbohydrate starvation were diverse and revealed candidate processes associated with disk floret degreening. Camphor content increased and correlated positively with degreening insensitivity. Phenylpropanoids and flavonoids also increased upon carbohydrate starvation and the response was genotype specific. We propose the upregulation of the phenylpropanoid metabolism as important source of nitrogen in the form of harmful ammonia during carbohydrate starvation. Our results provide a framework to identify processes associated with genotypic differences in the response to carbohydrate starvation and susceptibility to floret degreening.

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1. Introduction

The environment to which plants and their products are exposed during post-harvest life is often quite different from their original growth environment in terms of light, temperature, and availability of nutrients. During production in greenhouses, light intensity and temperature are optimized for fast growth and high yields, whereas the environment during post-harvest storage and transport is aimed at keeping the quality and appearance of the flower product as it was at harvest. Light deprivation often means absence of photosynthesis and therefore no *de novo* production of carbohydrates. However, respiration continues in the dark, especially in products that are harvested in a developmental stage characterized by fast growth and high respiration rate, like flowers and sprouts. Eventually, this leads to a state of carbohydrate starvation, which is often detrimental for plant quality, since carbohydrates are the main source for respiration in plants (Plaxton and Podestá, 2007). Carbohydrate starvation leads to oxidative stress (Couée et al., 2006; Morkunas et al., 2003), which eventually results in programmed cell death (Tiwari et al., 2002). The exact process leading from carbohydrate starvation to cell death remains largely unknown.

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Chrysanthemum (*Chrysanthemum* × *morifolium*) is an economically important cut flower, known for a relatively high postharvest performance compared to other cut flower species. However, after dry, dark, and cold storage during two weeks or more, some genotypes show quick degreening of disk florets, and eventually turn from green to yellow to brown, thereby losing market value. Yellowing is most likely due to loss of chlorophyll, while browning is likely the result of enzymatic oxidation of phenolic compounds into brown polymers, as is the case in many browning processes in plants (Ke and Saltveit, 1988; Pourcel et al., 2007). In unstressed cells, this oxidation is prevented by differential subcellular compartmentalization, but enzymes and substrates come together if membranes are leaky (Pourcel et al., 2007). Membrane damage can occur upon physical damage as in potato bruising (Bachem et al., 1994), during senescence (Thompson et al., 1998), or induced by stress, like carbohydrate starvation (Aubert et al., 1996).

Feeding flower stems with carbohydrates often increases vase life (Ichimura et al., 2005; Yakimova et al., 1996). In addition, feeding florets with sugars prevents degreening in broccoli (*Brassica oleracea* var *italica*) and *Arabidopsis thaliana* (Irving and Joyce, 1995; Trivellini et al., 2012). Finally, feeding chrysanthemum flower stems with sugars also reduces degreening (Van Geest et al., unpublished results). These results indicate that carbohydrate metabolism is very relevant for postharvest performance, specifically for problems related to degreening. There are large genotypic differences in sensitivity to degreening in chrysanthemum. Under the assumption that carbohydrate content plays a role in degreening sensitivity, these genotypes respond differently to a low availability of carbohydrates. Knowledge about genotypic variation in the response to carbohydrate starvation is generally absent. However, it has large potential to aid breeding for postharvest performance.

^{1}H nuclear magnetic resonance (NMR) allows simultaneous quantitation of both primary and secondary plant metabolites (Kim et al., 2010a), and therefore can be used to investigate a wide range of phenomena at the metabolomic level. Characterization of the carbon starved metabolome using ^{1}H NMR of *A. thaliana* cell cultures (Kim et al., 2007) and ^{31}P NMR of *Acer pseudoplatanus* cell cultures (Aubert et al., 1996), demonstrated marked increases of free amino acids, malic acid and phosphorylcholine. These observed metabolic changes are typical for carbohydrate starvation, but their amplitude or occurrence might differ strongly between genotypes that differ for sensitivity to carbohydrate starvation.

In order to investigate the genotypic differences in the metabolic response to carbohydrate starvation, we used a metabolome-wide approach to detect compounds that quantitatively vary during carbohydrate starvation. By constructing metabolomic profiles of disk florets using ^{1}H NMR and high performance anion exchange chromatography (HPAEC) at different time points in three chrysanthemum genotypes during carbohydrate starvation and feeding, we identified genotype specific and general metabolites associated with starvation. This paper discusses the differential metabolic response to carbohydrate starvation between genotypes, and proposes hypotheses that explain how a genotype can affect the tolerance to starvation of carbohydrates by altering its metabolome.

2. Materials and methods

2.1. Plant material and pre- and postharvest conditions

Three chrysanthemum genotypes were obtained from Deliflor chrysanten B.V. (Maasdijk, the Netherlands): 'DB39287', which is a single white type and sensitive to degreening of the disk florets after long storage (S), 'DB32030', a single purple type which is medium sensitive to degreening (MS), and 'DB36451', a single white type which is insensitive to degreening (I). Sensitivity was previously assessed by Deliflor Chrysanten B.V. (Maasdijk, the Netherlands) in commercial vase life tests. Plants were grown in a greenhouse in Maasdijk, the Netherlands using commercial growing practices. For initial respiration measurements, plants were grown from April to June 2014, harvested from large fields in the same greenhouse. For respiration measurements over time, plants were grown from August to November 2014 in a randomized block design. For both experiments, flowers were transported to Wageningen (the Netherlands) on the same day. For NMR, HPAEC and protein measurements, plants grown from July to September 2013 were used. Plots were planted in a randomized block design with five blocks representing five replicates. For each time point, one stem was harvested per plot. For all experiments, harvest took place in the morning, and stems were transported dry in cardboard boxes to Wageningen, the Netherlands. Except for respiration measurements, the combination of the disk florets of the upper three capitula of a flower stem was considered as one biological replicate. For all treatments, per stem the upper three capitula were cut in an angle of approximately 45°, and ray florets were removed. Disk florets sampled at day 0 were frozen in liquid nitrogen upon arrival at the lab. The cut ends of the three capitula were placed in a 35 mL pot containing 25 mg L^{-1} sodium dichloroisocyanurate (DICA) in demineralized water as bactericidal compound for the carbohydrate starvation treatment, or the same amount of DICA with 50 mM sucrose for the sugar feeding treatment. Solutions were replaced on every day on which sampling was performed. Pots with capitula were placed in a dark temperature controlled cabinet at 20 ± 1 °C. Air humidity was buffered using a 2 L saturated sodium chloride solution in the cabinet resulting in a relative humidity of approximately 75%. Samples of disk florets were taken at 0, 5, 10 and 14 days after harvest. When sampled, florets were flash frozen in liquid nitrogen and stored at −80 °C until freeze–drying.

2.2. Color measurements

To determine the change in degreening of disk florets over time, pictures of capitula were taken in a standardized light environment created by fluorescent light scattered by a Perspex plate bent in a half cylinder. A Hitachi HV-C20 video camera with a Tamron SP 35–80 mm objective was used for imaging. The average intensity of red and green of the disk florets was quantified using ImageJ
(http://imagej.nih.gov/ij/), using a custom-made macro.

2.3. Respiration measurements

Nine capitula were placed in three 35 mL pots (three per pot) containing 25 mg L^{-1} DICA per biological replicate. For initial respiration measurements, flowers were stored dry at 4 °C overnight before excising the capitula and respiration measurements. For measurements over time, capitula were excised upon arrival in Wageningen, and placed in the 35 mL pots. Respiration measurements took place the next day. For respiration measurements, three 35 mL pots were placed in an airtight 1 L jar at 20 ± 2 °C. A 1 L jar was considered as biological replicate. Jars carried a septum and contained 40 mL saturated sodium chloride solution to buffer air humidity during the respiration measurement. Three jars per genotype were used. Jars were closed after one hour of temperature acclimation. After closure, 3 mL of air were withdrawn every 30 min for three hours. Oxygen concentration of the air sample was measured using a Dansensor CheckMate 3 headspace gas analyser (Dansensor A/S, Ringsted, Denmark). Dry weight was measured after respiration rate measurements by

weighing samples after oven-drying at 80 °C overnight. The respiration rate was calculated using the slope of the regression between time and oxygen concentration corrected by total dry weight of all capitula per jar. In order to obtain reliable constant respiration estimates, only regression lines with an R^2 higher than 99 % were considered.

2.4. Carbohydrate analysis

Carbohydrates were extracted from a 10–20 mg freeze-dried sample with 5 mL of 80% ethanol for 20 min at 80 °C. After extraction, tubes were centrifuged at 8800 × *g*. One millilitre of supernatant was vacuum dried using a vacuum centrifuge (Savant SpeedVac SPD2010, Thermo Fisher Inc., Waltham, MA USA) at 45 °C and 5.1 mbar for 150 min and re-eluted in demineralized water using an ultrasonic water bath (Branson 2200, Branson Ultrasonics, Danbury, CT, USA) at 50 Hz. The remaining pellet was stored in ethanol at −20 °C for determination of starch concentration. Glucose, fructose and sucrose were quantified from the ethanol extract using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD; Dionex ICS5000, Thermo Fisher Inc.), equipped with a CarboPac1 column (250 × 2 mm) eluted with 100 mM NaOH. Fructans were quantified as described by Verspreet et al. (2012) with some modifications. Of the extract, 150 μL were hydrolyzed with 150 μL 120 mM HCl for 90 min at 70 °C. Fructans were quantified and average degree of polymerization (DP) was determined by comparing the glucose, fructose and sucrose concentration of the hydrolysed with non-hydrolysed extract as described by Verspreet et al. (2012). All fructans were present in the 80% ethanol extract since no detectable fructose or glucose was found after hydrolysis of water extracts of the remaining pellet after ethanol extraction. For starch quantitation, the pellet was washed three times with 80% ethanol. The pellet was vacuum dried, resuspended in 2 mL 1 g L^{-1} thermostable α-amylase (SERVA Electrophoresis GmbH, Heidelberg, Germany) and incubated for 30 min at 90 °C. After that, 1 mL of 0.5 g L^{-1} amyloglucosidase from *Aspergillus niger* (Sigma 10115, Sigma, St Louis, MO, USA) in 50 mM citrate buffer (pH 4.6) was added and tubes were incubated at 60 °C for 15 min. Tubes were centrifuged at 8800 × g and glucose and fructose were quantified as described above.

2.5. Protein measurement

Proteins were extracted in an ice-cold ultrasonic water bath (Branson 2200, Branson Ultrasonics, Danbury, CT, USA; 50 Hz) for 15 min from 12 to 20 mg freeze–dried tissue with 1 mL 100 mM Tris–HCl (pH 7.6), 10 mM $MgSO_4$, 10 mM dithiothreitol and 0.1% Triton-X 100 in demineralized water (Eason et al., 1997) in a 2 mL microcentrifuge tube. After extraction, tubes were centrifuged at 12,000 × g. Of the supernatant, 500 μL were centrifuged at the same speed. Hundred microliters were added to 3 mL Bradford reagent (Bradford, 1976). Absorption was measured at 466 and 594 nm (Varian Cary 4000 UV–vis Spectrophotometer, Agilent Technologies Inc., Santa Clara, CA, USA). Protein was quantified as described by Zor and Selinger (1996) using bovine serum albumin standard series.

2.6. ^{1}NMR profiling

Freeze-dried samples were transferred to a 2 mL-micro tube to which 1 mL of the mixture of CH_3OH-d_4 and 90 mM KH_2PO_4 in D_2O (1:1) containing 0.05% (w/w) TMSP (trimethyl silyl propionic acid sodium salt, w/v) was added. The mixture was vortexed at room temperature for 30 s, ultrasonicated for 1 min, and centrifuged at 30,000 rpm at 4 °C for 20 min. Of the supernatant, 300 μL were taken into a 3 mm-NMR tube for NMR analysis.

^{1}H NMR spectra, 2D J-resolved spectra as well as ^{1}H–^{1}H homonuclear and inverse detected ^{1}H–^{13}C correlation experiments were recorded at 25 °C on a Bruker 600 MHz DMX NMR spectrometer (600.13 MHz proton frequency) equipped with TCI cryoprobe and Z-gradient system. CD_3OD was used for internal lock purposes. The parameters followed previous literature (Kim et al., 2010b) with some modifications. For ^{1}H NMR spectra a total of 32768 data points were recorded covering a spectral window of 9615 Hz. Sixty-four scans of a standard one-pulse sequence with 30° flip angle for excitation and pre-saturation during 2.0 s relaxation delay with an effective field of $\gamma B_1 = 50$ Hz for suppression of the residual H_2O signal was employed. An exponential window function with a line-broadening factor of 0.3 Hz was applied prior to Fourier transformation. The resulting spectra were manually phased and baseline corrected.

2.7. Data analysis

The ^{1}H NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to TMSP and reduced to integrated regions of equal width (0.04 ppm for ^{1}H NMR spectra; further referred to as 'bin'), corresponding to the region of δ 0.3–δ 10.00. The region of δ 4.7–δ 5.0 and δ 3.28–δ 3.34 were excluded from the analysis because of the residual signal of H_2O and CH_3OH-d_4, respectively. Missing values were imputed by half of the minimum value recorded for that bin. Intensities of NMR profiling and concentrations from HPAEC analysis were normalized by sample dry weight corrected for carbohydrate content (see Section 4):

$$c\mathrm{dw_j} = \mathrm{dw_j} - c_\mathrm{j} \tag{1}$$

$$\bar{x}_{\mathrm{ij}} = \frac{x_{\mathrm{ij}}}{c\mathrm{dw_j}} \tag{2}$$

where x is a datapoint in the ith variable and jth sample, dw represents the dry weight, c carbohydrate content and cdw carbohydrate corrected dry weight. Variables (including compounds measured with HPAEC) were selected that showed significant change in time in any treatment-genotype combination using a Kruskal–Wallis test with p-value cut-off of 0.01. This left 122 variables out of 250. A correlation matrix using Pearson correlation was calculated, and metabolites were clustered using Euclidean distance. Principal component analysis (PCA; Mardia et al., 1979) was performed with the prcomp function from the stats package in R (R Core Team, 2014). Default values were used, except for the argument 'scale.', which was set to TRUE, resulting in unit variance scaling prior to PCA. For the respiration measurements, significant differences were identified using a one-way analysis of variance followed by a Tukey's honest significant difference test. Linear relations between variables were tested using a simple linear model.

3. Results

3.1. Color and carbohydrate metabolism

We tested whether isolated flower heads in water showed the same degreening compared to commercial vase life trials as executed by Deliflor Chrysanten B.V. Our system revealed the expected genotypic differences (Fig. 1). The increase in red/green (R/G) intensity represented visually observable degreening in the disk florets (Fig. 2). Significant increase in R/G occurred at day 10 in the sensitive genotype, and at day 14 in the MS genotype. It did not occur during the experiment (stopped after 14 days) in the

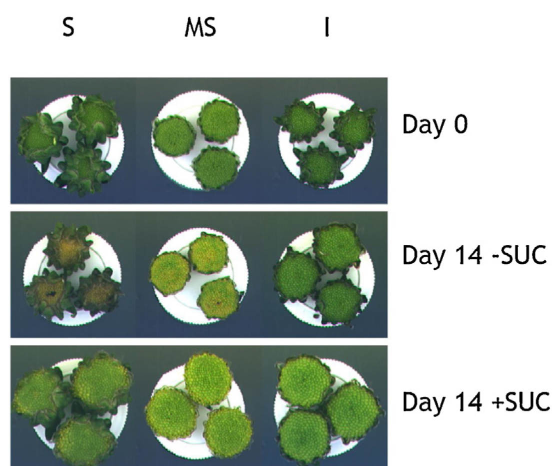

Fig. 1. Appearance of flower heads of three genotypes before and at the end of two treatments. First column: sensitive (S), second column: medium sensitive (MS) and third column: insensitive (I) genotype. -SUC: carbohydrate starvation, +SUC: treatment with 50 mM sucrose. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

insensitive genotype. Application of sugar to the medium in which the flower heads were kept prevented degreening in all genotypes and facilitated development of disk florets towards maturation (Fig. 1).

Carbohydrates provided a significant part of the total dry weight, and carbohydrate content varied strongly during sucrose feeding and carbohydrate starvation (Fig. 3A). Therefore, we normalized measured variables by the carbohydrate corrected dry weight (Eqs. (1) and (2); Figure S1). Sucrose feeding increased carbohydrate corrected dry weight, whereas during starvation carbohydrate corrected dry weight remained stable initially, and eventually declined.

Sucrose feeding resulted in an altered carbohydrate content and distribution between mono-, oligo-, and polysaccharides. This distribution was also highly dependent on genotype. The relative amount of fructans was much higher in the MS genotype compared to the S and I genotypes. The S genotype contained 73% less

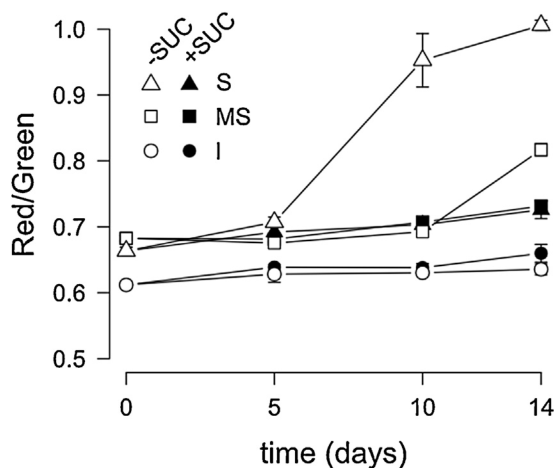

Fig. 2. Average intensity of red divided by average intensity of green over time of disk florets with (closed symbols) or without (open symbols) 50 mM sucrose feeding for three genotypes: sensitive (S; triangles), medium sensitive (MS; squares) and insensitive (I; circles). Error bars represent standard errors of the mean, $n = 5$.

carbohydrates at harvest compared to the other genotypes (Fig. 3). Starch was not very abundant; it made up at most 5% of total carbohydrates and declined quickly within the first five days irrespective of sucrose treatment. Sucrose, glucose, and fructose (Fig. 3B–D respectively) content decreased only upon carbohydrate starvation. The decrease in glucose was faster than that of fructose. Fructose and glucose increased strongly with addition of sucrose, whereas sucrose increased slightly. Similar to the effect of sucrose on starch content, sucrose treatment only slightly increased fructan content. Protein loss only occurred after major losses of carbohydrates, and occurred concomitantly with degreening (Figure S2).

Despite having a similar carbohydrate content, respiration rate was highest in the MS genotype and lowest in the I genotype at harvest (Figure S3). Respiration rate decreased during the carbohydrate starvation and increased if sucrose was fed (Figure S4A). Within genotype there was a strongly significant correlation between carbohydrate content and respiration rate in the S and MS genotype ($p < 0.001$) and a weak but significant ($p = 0.02$) correlation in the I genotype (Figure S4B). The MS and S genotype had a much stronger decrease in respiration rate with lower carbohydrate contents compared to the I genotype.

3.2. Multivariate analysis of the metabolic response

Carbohydrate starvation drastically changed the disk floret metabolome. All carbohydrates measured with HPAEC changed significantly over time for at least one genotype-treatment combination according to a Kruskal–Wallis test at $p = 0.01$ (Fig. 4). NMR spectral intensities were binned in integrated regions of 0.04 ppm. From these bins, 116 out of 253 changed significantly over time. Under sugar feeding the metabolome remained rather stable, with eleven, twelve, and one bin for the I, S and MS genotype respectively changing significantly over time (not shown). Most bins that changed over time changed only in the carbohydrate starvation treatment.

1D and 2D based NMR allowed identification of 21 compounds (Table 1). Based on correlation in samples, we identified two main groups of metabolites using Euclidean distance (Fig. 4). The first, group A, contained the carbohydrates, in which the soluble carbohydrates were highly correlated with each other. A larger second group, group B, generally correlated negatively with group A. Group B was metabolically diverse. It contained most identified amino acids, some organic acids, and molecules containing aromatic rings among which flavonoids, phenylpropanoids, and aromatic amino acids. Of the identified compounds, the strongest negative correlation with group A was found for asparagine (Fig. 4).

We conducted a principal component analysis (PCA) in order to visualize maximum variation between samples and identify compounds that explain this variation. The first three components explained 67% of the variation. The score plot (Fig. 5) of the two components explaining most variation separated carbohydrate starvation from sugar feeding at the first component, with low carbohydrate content on the right, and high carbohydrate content on the left side. This separation was also characterized by high amounts of asparagine and an unidentified compound characterized by a bin at δ 7.32 for sugar-starved samples. The second component separated mainly genotypes. Bins characterizing luteolin-7-*O*-glycoside, chlorogenic acid and bins at δ 2.12 and δ 2.48, which likely characterize glutamine content, had large influence on this separation. These compounds were particularly high in the MS genotype. They were low in the sensitive genotype and intermediate in the insensitive genotype. Separation between genotypes was explained in the opposite direction by a bin at δ 0.84 and threonine, which were high in the S genotype, low in the MS and intermediate in the I genotype.

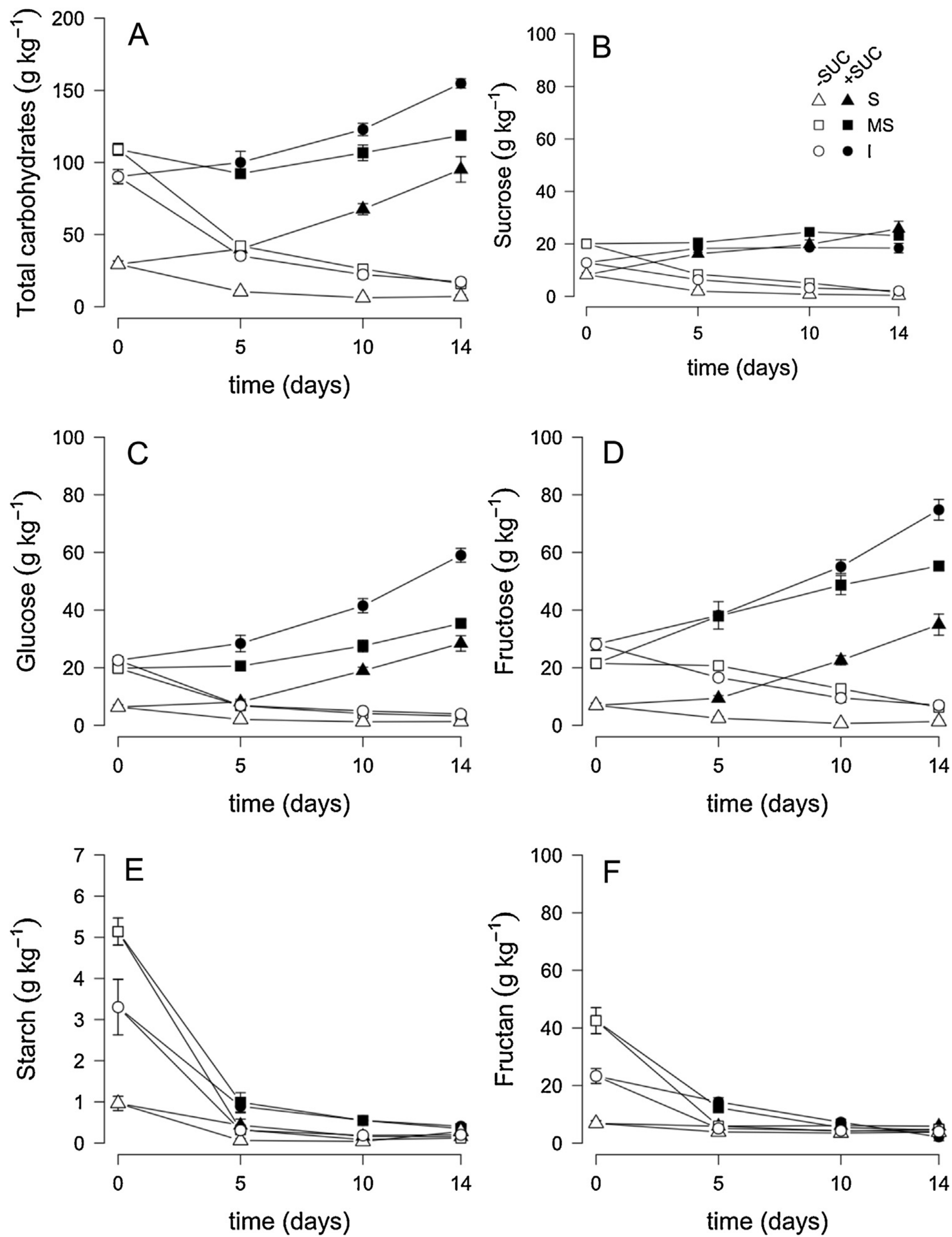

Fig. 3. Disk floret total soluble carbohydrate (A), sucrose (B), glucose (C), fructose (D), starch (E) and fructan (F) content over time as measured by HPAEC of disk florets with (closed symbols) or without (open symbols) 50 mM sucrose feeding for three genotypes: sensitive (S; triangles), medium sensitive (MS; squares) and insensitive (I; circles). Error bars represent standard error of the mean, $n = 5$.

In the PCA score plot with the first and third component (Fig. 5), degreened sugar starved disk florets from both the sensitive and MS genotype clustered together. Degreening only occurred if carbohydrates were low, in combination with presence or absence of compounds explaining variation along the third component. Degreened and brown flower heads were low in camphor. 3,5-dicaffeyol quinic acid and peaks within the bin at δ 4.44, which was too small for identification, were high in degreening tissue.

Four identified amino acids quantified with NMR explaining major variation in the PCA score plots were selected for further inquiry (Fig. 6). Arginine, asparagine, and valine (Fig. 6A–C respectively) increased in the first days of carbohydrate starvation and their concentration remained constant with sugar feeding. Threonine specifically increased only under carbohydrate starvation in the S genotype, whereas arginine did not change much at carbohydrate starvation in the S genotype (Fig. 6D).

The phenolic compounds chlorogenic acid, 3,5-dicaffeoyl quinic acid and luteolin-7-*O*-glycoside generally increased upon carbohydrate starvation (Fig. 4; Fig. 7A–C respectively). They are substrates for polyphenol oxidases and peroxidases, and therefore precursors of brown polymers. The MS genotype showed a stronger increase in chlorogenic acid and 3,5-dicaffeyol quinic acid upon carbohydrate starvation compared to the other two genotypes (Fig. 7A and B). At harvest, it had much higher levels of luteolin-7-*O*-glycoside (Fig. 7C), but the compound did not increase upon carbohydrate starvation, whereas it did for other genotypes. The terpenoid camphor was associated with remaining green at carbohydrate starvation (Fig. 5). It increased in the MS and I genotype, but not in

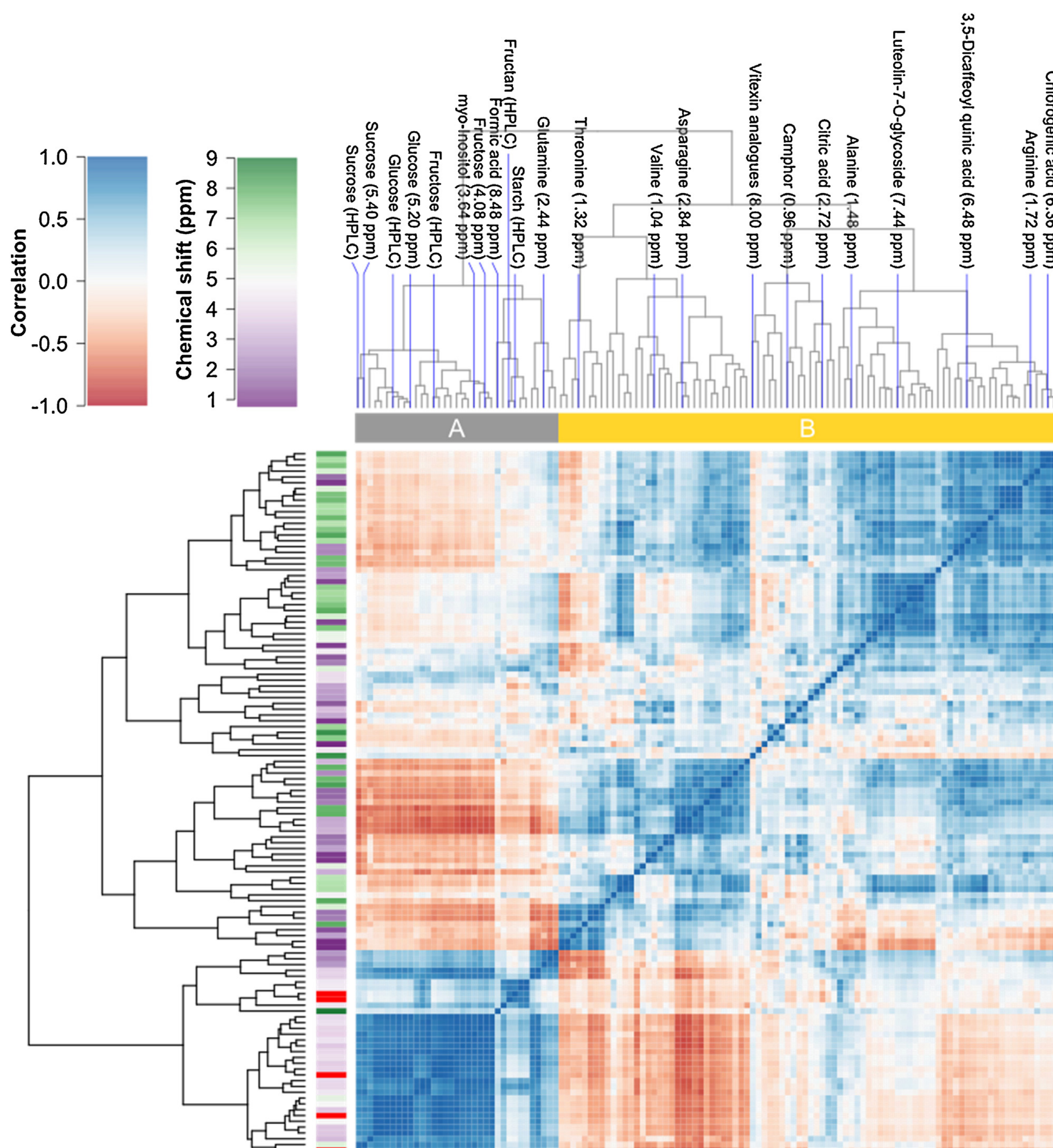

Fig. 4. Heatmap of Pearson correlation matrix of 128 variables; 122 variables derived from NMR spectroscopy and six variables derived from HPAEC that significantly changed normalized intensity over time in at least one genotype in one of the treatments according to a Kruskal–Wallis test at $p = 0.01$. The order of compounds is based on clustering by Euclidian distance and indicated by the dendrograms. On the vertical color bar, chemical shift of the bins are depicted as color, the rows with compounds measured with HPAEC are shown in red. On the horizontal axis, two distinct groups are depicted, and 22 compounds identified with HPAEC and/or NMR are indicated (blue lines). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the S genotype. At harvest, it was most abundant in the I and MS genotype but increased more strongly in the I genotype compared to the MS genotype during carbohydrate starvation (Fig. 7D).

4. Discussion

Although different from commercial vase life trials, our method revealed the same degreening and genotypic differences in susceptibility. By isolating capitula, we could investigate disk floret degreening on a simplified level, without effects of metabolite transport from other parts of the plant. In this section, we bring observations in a broader context, by first defining the type of stress we presume to play a role, than by discussing genotypic differences in carbohydrate metabolism and its effect on susceptibility to degreening, and last by discussing the genotype specific and common responses to carbohydrate starvation.

Table 1
Identified compounds using NMR and HPAEC-PAD.

Metabolite	Chemical shift (NMR)	Retention time (HPAEC-PAD) minutes	Significant change?[a]
Polysaccharides[b]			
Starch	–	–	Yes
Fructans	–	–	Yes
Sugars			
Glucose	δ 5.20	5.067	Yes
Sucrose	δ 5.40	10.033	Yes
Fructose	δ 4.08	5.650	Yes
Amino acids			
Valine	δ 1.04	–	yes
GABA	δ 3.04	–	No
Glutamine	δ 2.44	–	Yes
Arginine	δ 1.72	–	Yes
Threonine	δ 1.32	–	Yes
Adenine	δ 8.20	–	No
Asparagine	δ 2.84	–	Yes
Alanine	δ 1.48	–	Yes
Small organic acids			
Citric acid	δ 2.72	–	Yes
Formic acid	δ 8.48	–	Yes
Phenylpropanoids			
Chlorogenic acid	δ 6.36	–	Yes
3,5-Dicaffeoyl quinic acid	δ 6.48	–	Yes
Flavonoids			
Luteolin-7-*O*-glycoside	δ 7.44	–	Yes
Apigenin-7-*O*-glycoside	δ 7.92	–	No
Vitexin analogues	δ 8.00	–	Yes
Other			
Choline	δ 3.20	–	No
myo-Inositol	δ 3.64	–	Yes
Camphor	δ 0.96	–	Yes

[a] Significant change over time in at least one treatment-genotype combination according to a Kruskal–Wallis test at $p = 0.01$.
[b] See Section 2 for quantitation method.

4.1. Sugar feeding effects on appearance

It is very likely that degreening of disk florets is the result of carbohydrate starvation. In this experiment, application of 50 mM sucrose to isolated flower heads prevented degreening entirely. Other types of soluble sugar like glucose and fructose could also prevent degreening, but not the non-metabolisable sugar analog *myo*-inositol (not shown). This process is similar to broccoli (*B. oleracea* var. *italica*) and *A. thaliana*, where sucrose feeding strongly reduces sepal yellowing (Irving and Joyce, 1995; Trivellini et al., 2012).

Despite the fact that similar processes occur during carbohydrate starvation, dark-induced deterioration and senescence, there are also substantial differences. These differences are present in crucial pathways related to amongst others amino acid metabolism, lipid catabolism, and flavonoid biosynthesis (Buchanan-Wollaston et al., 2005; Trivellini et al., 2012; van der Graaff et al., 2006). The word 'senescence' is derived from the Latin *senex*, which means 'aged' or 'matured', and is defined as 'the growth phase in a plant or plant part (as a leaf) from full maturity to death' (Merriam-Webster Online, 2015). Since disk florets are far from reaching full maturation when degreening occurs, it can best be described and treated as a stress-induced deterioration. This concept should also apply to other instances of premature deterioration of plant organs.

4.2. Normalization by carbohydrate corrected dry weight

Soluble sugars made up a very large part of the dry weight in chrysanthemum disk florets. At harvest, up to 10.4% of the dry weight consisted of carbohydrates. Under carbohydrate starvation, it is evident that most of these carbohydrates disappear. Normalization by dry weight would result in a substantial artificial increase of other metabolites. Other researchers have solved this issue by expressing a compound per organ, such as per flower (Eason et al., 1997) or per root tip (Brouquisse et al., 1992). This approach does not solve the problem if multiple genotypes are used. As demonstrated in our results, there can be major genotypic differences in carbohydrate content at harvest. In addition, organ size may differ between genotypes, making it impossible to compare genotypes if corrected by organ. In order to enable comparison between genotypes and time points, we attempted to correct for the change in dry weight over time by subtracting the total carbohydrate weight from the dry weight. Other metabolites change and will have effect on the dry weight as well, but their contribution to total dry weight is probably negligible compared to the effect of carbohydrates.

4.3. Polysaccharide breakdown is independent of carbohydrate content

In contrast to starch, fructans form by far the most abundant polysaccharides in chrysanthemum disk florets. They make up 24 to 40% of the total amount of carbohydrates. This is different from whole inflorescences, where starch is the most abundant polysaccharide (Trusty and Miller, 1991). Fructans are ubiquitous in the plant kingdom, including the agricultural relevant liliaceae, poaceae and asteraceae (Hendry, 1993). They seem to be an important source of monosaccharides in the form of fructose

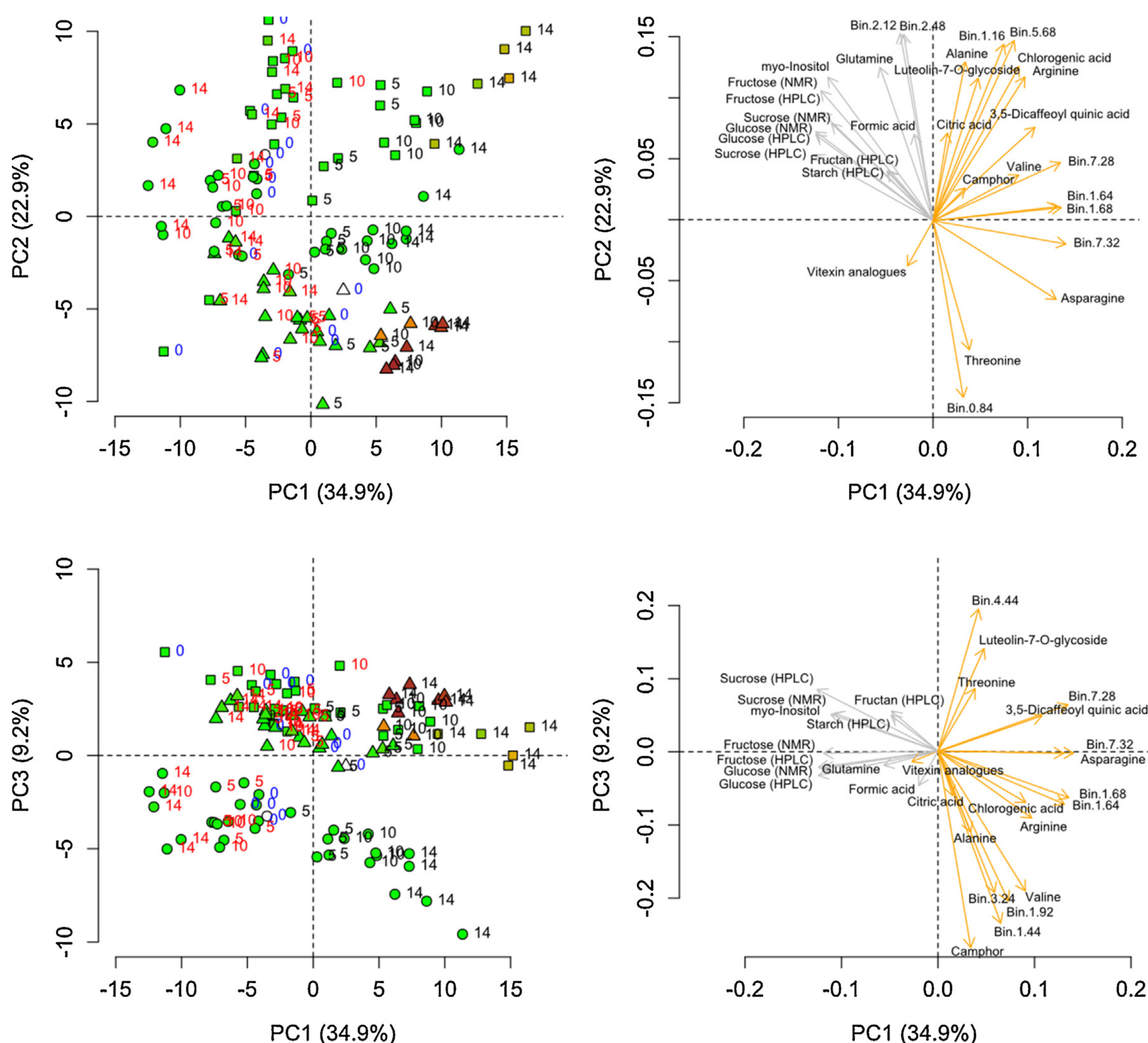

Fig. 5. PCA score plots (left) and loading plots (right), of principal component 1 and 2 (upper) and component 1 and 3 (lower). Genotypes: Insensitive (○), medium sensitive (□) and sensitive (△). Numbers at symbols in score plots represent day after harvest and their colours represent treatments, which are: directly after harvest (blue), sugar feeding (red) and carbohydrate starvation (black). The color of the symbols represents the measured color of the disk florets going from green to brown. In the loading plots, the identified compounds, and five compounds with largest absolute loading along each component were plotted. The color of the arrows corresponds to the color of the groups depicted in Fig. 4, where grey represents group A and orange group B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

during the first days of carbohydrate starvation, since during fructan breakdown in the first five days, fructose content remained stable, whereas glucose content decreased strongly.

Breakdown of polysaccharides in disk florets was independent of carbohydrate content. Fructans are synthesized from sucrose by sucrose:sucrose 1-fructosyltransferase and fructan:fructan 1-fructosyltransferase (Ritsema and Smeekens, 2003). Starch is synthesized from glucose through ADP-glucose by starch synthase (Preiss, 2009). Although sucrose and glucose concentrations increased upon sucrose feeding, disk florets were unable to synthesize polysaccharides. Fructans are water-soluble and can be synthesized elsewhere in the plant. Chrysanthemum stems have high fructan concentrations (Adachi et al., 1999), and could therefore be the source of fructans in the disk florets. Developing flowers often show breakdown of polysaccharides (Bieleski, 1993; van Doorn and Van Meeteren, 2003; Vergauwen et al., 2000). Fructan and starch breakdown in disk florets could therefore be part of a developmental process. Since we kept flower heads in the dark, polysaccharide breakdown might also be induced by light deprivation. However, light did not have a positive effect on starch content during the post-harvest phase in lettuce (Zhan et al., 2013).

4.4. Genotypic differences in carbohydrate metabolism

Carbohydrate content could only partly explain genotypic differences in degreening susceptibility. Despite this, our results show that occurrence of degreening within genotypes is clearly related to absence of carbohydrates. Although the S genotype showed by far the lowest carbohydrate content at harvest, the total carbohydrate content between the MS and I genotype was not different during carbohydrate starvation. The MS genotype even had a higher carbohydrate content in many cases, although having a higher respiration rate at harvest. Since carbohydrate starvation likely causes degreening, the MS and I genotype probably have a different response to low carbohydrate content. This difference in response is also indicated by a different response in respiration rate to carbohydrate starvation. Whereas respiration rate is strongly reduced upon carbohydrate starvation in the MS genotype, respiration rate of the I genotype is much less reduced. This more abrupt reduction in respiration rate in the MS genotype might be an indication of an earlier constraint on the mitochondrial electron transport chain (mtETC). Carbohydrate starvation leads to reduced ADP contents (Brouquisse et al., 1991), which will impair ATP

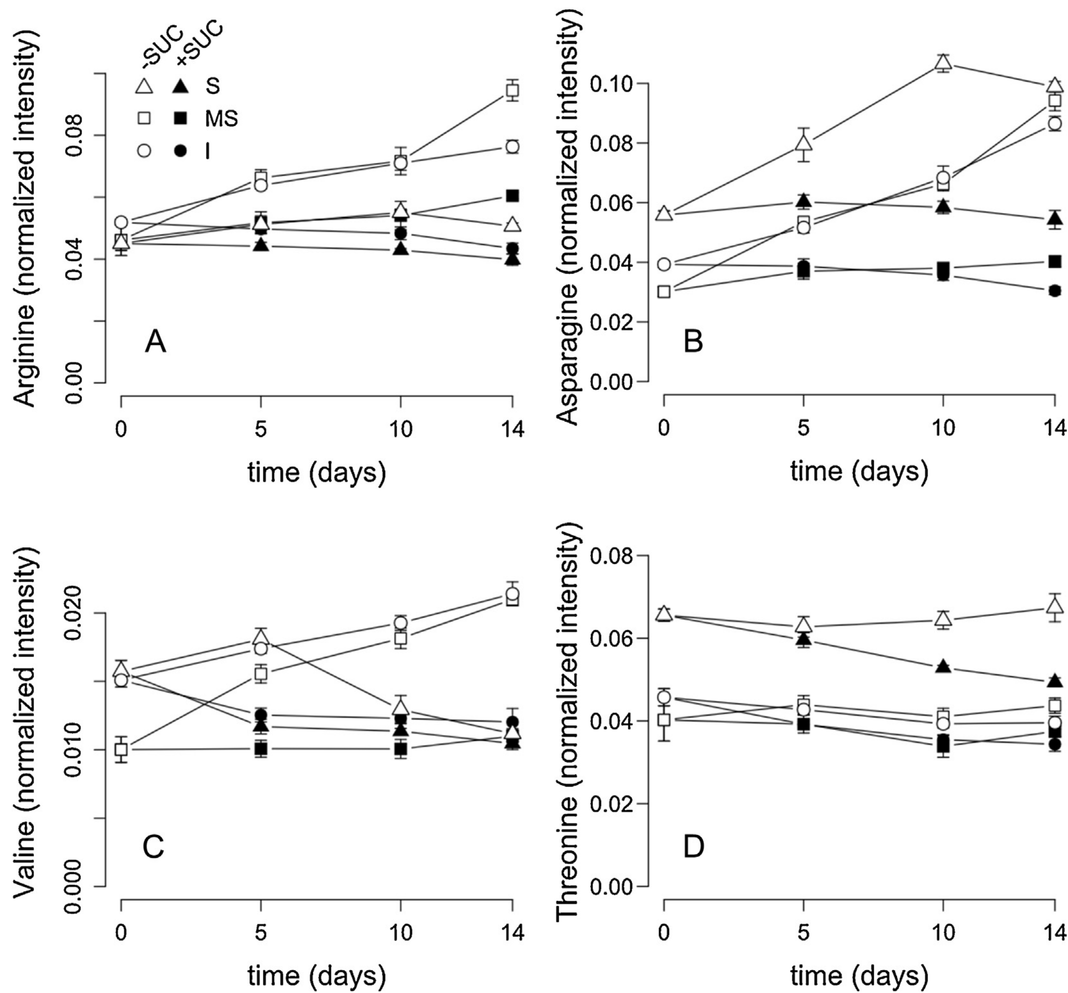

Fig. 6. The normalized bin intensity of arginine (A), asparagine (B), valine (C) and threonine (D) in disk florets with (closed symbols) or without (open symbols) 50 mM sucrose feeding for three genotypes: sensitive (S; triangles), medium sensitive (MS; squares) and insensitive (I; circles). Error bars represent standard error of the mean, $n = 5$.

synthase (Gout et al., 2014). Blockage of ATP synthase will lead to over reduction of the mtETC and therefore oxidative stress (Vanlerberghe, 2013). As indicated by the stronger reduction in respiration rate, the MS and S genotypes might have earlier blockage of the mtETC leading to oxidative stress, resulting in cell damage (Rhoads et al., 2006) and earlier degreening. Differences in ADP availability, alternative oxidase activity or other protective mechanisms might cause these differences.

4.5. Protein is degraded when degreening starts

As soon as degreening occurred, disk florets lost proteins. Protein degradation often occurs concomitant to loss of quality in stored fresh produce (Collier, 1997; Downs et al., 1997; King et al., 1990) and in sugar starved tissue (Aubert et al., 1996; Brouquisse et al., 1992; Devaux et al., 2003). Proteins, next to lipids, can provide energy as substrate during carbohydrate starvation (reviewed by Araújo et al., 2011). It seems that protein breakdown only occurs if carbohydrate content is low for five days or more, since significant protein breakdown was only observable at day 14 in the MS and I genotype and at day 10 in the S genotype, whereas carbohydrates were already very low at day 10 and 5 respectively. Loss of protein could connect carbohydrate starvation to degreening. Proteins are indispensable for cell survival. If a significant part of viable processes is lost due to breakdown of proteins in essential enzymatic steps, it is inevitable that cells lose membrane integrity and eventually die. At formation of the brown polyphenols, proteins can be incorporated in the complex polymeric structure and become insoluble (Stevens and Davelaar, 1996). In addition, a decline of protein content was mainly observed when degreening occurs, and not before. It is therefore more likely that the protein loss is an effect of the reduction of membrane integrity and not a cause.

4.6. Metabolic response to carbohydrate starvation is diverse

Treatment duration and sugar feeding affected a large part of the metabolome of chrysanthemum disk florets. In total, 116 out of 253 bins changed significantly over time for at least one of the genotype-treatment combinations, showing that the response to carbohydrate content is very diverse. Most of the metabolites having a change in concentration over time changed only significantly in the sucrose starvation treatment. The response to carbohydrate starvation was therefore more drastic than the response to sucrose feeding.

4.7. Phenolic compounds and flavonoids

Oxidation of flavonoids and phenylpropanoids most likely caused browning at the last step of the degreening process. They are oxidized if they come in contact with polyphenol oxidases (PPO) and peroxidases upon membrane damage (Ke and Saltveit,

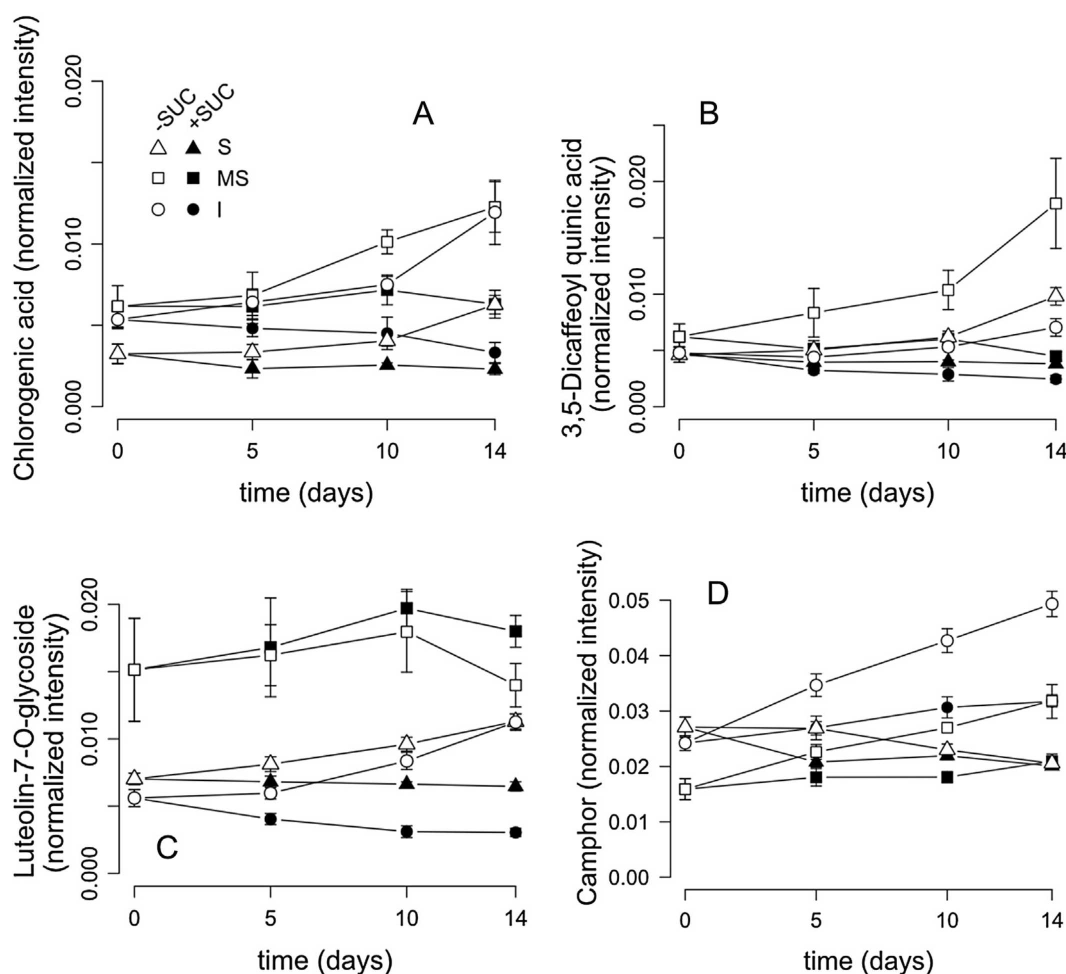

Fig. 7. Normalized bin intensity of chlorogenic acid (A), 3,5-dicaffeoyl quinic acid (B) luteolin-7-*O*-glycoside (C) and camphor (D) in disk florets with (closed symbols) or without (open symbols) 50 mM sucrose feeding for three genotypes: sensitive (S; triangles), medium sensitive (MS; squares) and insensitive (I; circles). Error bars represent standard error of the mean, $n = 5$.

1988; Pourcel et al., 2007). Membrane damage most likely occurs at late stages of carbohydrate starvation. Chlorogenic acid, 3,5-dicaffeoyl quinic acid and luteolin-7-*O*-glycoside increase early during carbohydrate starvation, and therefore most likely before membrane damage occurs. This means that carbohydrate starvation not only results in increased access of PPO to its substrates, but also to an earlier increase in synthesis of those substrates.

Varying classes of phenylpropanoids increase during different kinds of stress (Dixon and Paiva, 1995). Genes involved in flavonoid biosynthesis are strongly upregulated during sugar starvation in *A. thaliana*, and the type of response differs substantially from leaf senescence (Buchanan-Wollaston et al., 2005). Some genes of the flavonoid biosynthesis pathway are specifically regulated by the sugar starvation signalling pathway (Baena-González et al., 2007). Among those is flavanone 3-hydroxylase, which catalyses the synthesis of flavonols from flavanones. This suggests a specific role for flavonoids during carbohydrate starvation. Since flavonoids are potent anti-oxidants (Agati et al., 2012), this role could be in the protection against oxidative stress. Luteolin-7-*O*-glycoside increased in the I and MS genotype during carbohydrate starvation, whereas apigenin-7-*O*-glycoside did not change significantly, resulting in an increased ratio between luteolin and apigenin. This increased ratio is also observed during UV-B light stress (Markham et al., 1998), and was suggested to result in larger anti-oxidative capacity (Agati et al., 2012). However, the reaction in which luteolin is synthesized from apigenin is catalysed by flavonoid 3'-hydroxylase, which is specifically upregulated during leaf senescence and not carbohydrate starvation (Buchanan-Wollaston et al., 2005). Our observed increase in luteolin might therefore result from an alternative pathway, but it is more likely that flavonoid 3'-hydroxylase activity increases during carbohydrate starvation in chrysanthemum disk florets as opposed to the sugar-starved *Arabidopsis* cell cultures investigated by Buchanan-Wollaston et al. (2005).

Phenylpropanoid and flavonoid content was genotype dependent, but did not correspond to browning sensitivity. However, since they are important precursors of the brown polymers, their quantity might be important for the intensity of browning that occurs after the loss of green color. This would suggest the MS genotype would have a higher browning intensity compared to the S and I genotype. We did not measure browning intensity in this study, but visual observations of commercial vase life trials seem to substantiate this statement (results not shown).

4.8. Amino acid and nitrogen metabolism

If changed, the amino acids arginine, asparagine and valine increased over time during carbohydrate starvation. In maize root tips, there is a strong increase of amino acids at 24–48 h of carbohydrate starvation and cell damage became irreversible when amino acid levels declined back to the initial level (Brouquisse et al., 1992). This dramatic transient change was not

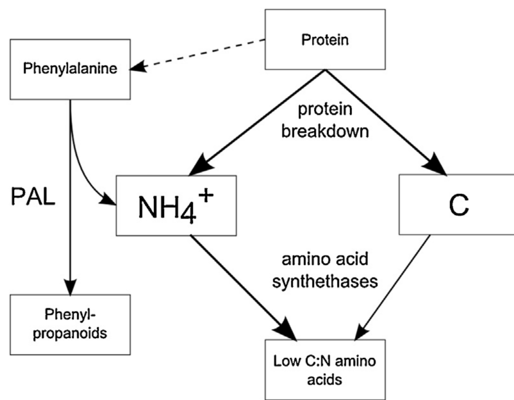

Fig. 8. Concept map of ammonium metabolism during carbohydrate starvation. Arrow size represents relative flow, the dotted arrow represents a relation of which significance is unknown. PAL: phenylalanine ammonia lyase; C: carbon. Protein can serve as carbon source during carbohydrate starvation, and thereby releases ammonium. Ammonium is scavenged by synthesizing high C:N amino acids like arginine and asparagine, resulting in a net synthesis of free carbon. However, protein breakdown is only significant late during carbohydrate starvation. A second source of ammonium is PAL, which catalyzes a rate limiting step for phenylpropanoid biosynthesis. This process leads to net loss of free carbon.

present in our material, and a decline in amino acid level was only observed in the S genotype for valine and slightly for asparagine. The type of tissue probably causes the difference in timing of these events between chrysanthemum disk florets and maize root tips; root tips have most likely much lower carbohydrate reserves compared to disk florets and are therefore much earlier exhausted of carbohydrates. Since there was no decrease in measured amino acid levels in the MS and I genotype, they probably did not reach the threshold at which cell damage becomes irreversible during the experiment, but the S genotype might.

Arginine and asparagine have a relatively high ratio between nitrogen and carbon. It is suggested they function as scavengers for toxic ammonium (Brouquisse et al., 1992). The use of protein as alternative substrate for respiration is generally suggested as main cause for the ammonium increase during carbohydrate starvation (Brouquisse et al., 1992; Devaux et al., 2003; Gary et al., 2003; Gaufichon et al., 2010). However, higher activity of the key enzyme in phenylpropanoid metabolism, phenylalanine ammonia lyase (PAL), would also result in production of ammonium. Despite a strong increase in asparagine, protein content did not decrease at the first days of carbohydrate starvation. However, compounds synthesized through the phenylpropanoid pathway increased in the first days of carbohydrate starvation. Therefore, upregulation of the phenylpropanoid metabolism through higher activity of PAL might be responsible for release of ammonium and therefore increase in asparagine and arginine at the cost of carbon. This would suggest that upregulation of the phenylpropanoid metabolism is more important for the alteration of nitrogen metabolism during carbohydrate starvation than protein breakdown, especially during early stages of starvation. These hypotheses and their relations are visualized in Fig. 8.

4.9. Possible roles for camphor

Camphor was the main compound that could explain genotypic differences in the response to carbohydrate starvation in terms of degreening. It is a terpenoid and occurs in several specific plant families. It is the main component in essential oils extracted from flower heads of several species in the chrysanthemum genus, like *Chrysanthemum indicum* (Shunying et al., 2005) and *Chrysanthemum coronarium* (Alvarez-Castellanos et al., 2001; Basta et al., 2007).

The I genotype showed the strongest increase in camphor upon carbohydrate starvation and largest absolute concentration. However, these differences only explained genotypic differences under carbohydrate starvation; the camphor content at harvest did not correspond to susceptibility to degreening. How camphor could play a role in preventing degreening remains unknown. It might be easily oxidized and therefore serve as an anti-oxidant under starvation stress.

Chrysanthemum essential oils have antifungal and antibacterial activity (Alvarez-Castellanos et al., 2001; Kim et al., 2003; Shunying et al., 2005). The antibacterial activity can be specifically addressed to camphor (Lee et al., 2011). The increase of camphor in carbohydrate starved disk florets might be the result of an abiotic stress-induced pathogen defence mechanism, like drought stress-induced increase of defence metabolites in tomato (English-Loeb et al., 1997). Our observed increase in phenylpropanoids likely also adds to this pathogen defence (Dixon et al., 2002). These types of responses could be very relevant for postharvest quality, since large amounts of fresh produce are being lost because of infection of microorganisms.

5. Conclusions

Carbohydrate metabolism plays an important role in postharvest performance. Our results show that carbohydrate starvation increases the level of secondary metabolites with biologically and agriculturally relevant functions. Among those are camphor, phenylpropanoids, and flavonoids. Genotypes respond differently in primary and secondary metabolism to carbohydrate starvation. Understanding this differential response between genotypes on a physiological level helps to identify processes causative for genotypic differences in their response to carbohydrate starvation and susceptibility to degreening. This paper poses hypotheses for causative relations between metabolic events and degreening susceptibility. We are currently testing these hypotheses in a broader set of genotypes. Once causative processes are identified, QTL mapping based on specific phenotypes (at the gene expression, metabolomic, and phenomic level) together with characterization of causative allelic variation based on candidate genes become much more feasible.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.postharvbio.2015.12.008.

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