Acclimation of photosynthetic processes and metabolic responses to elevated temperatures in cereals

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Acclimation of photosynthetic processes and metabolic responses to elevated temperatures in cereals

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The aim of the present work was to better understand the molecular mechanisms of heat acclimation processes in cereals. A large number of winter and spring wheat, barley, and oat varieties were grown under either control conditions (22/20°C) or under a mild heat stress (30°C) that induce the acclimation processes. The temperature dependence of chlorophyll *a* fluorescence induction and gas exchange parameters showed that heat acclimation increased the thermotolerance of the photosynthetic apparatus, but these changes did not differ sharply in the winter-spring type cereals. Similarly, to wheat, elevated temperature also led to increasing transpiration rate and reduced water use efficiency in barley and oat plants. A non-targeted metabolomic analysis focusing on polar metabolites in two selected barley (winter type Mv Initium and spring type Conchita) and in two oat varieties (winter type Mv Hópehely and spring type Mv Pehely) revealed substantial differences between both the two species and between the

acclimated and non-acclimated plants. Several compounds, including sugars, organic acids, amino acids and alcohols could be separated and detected. The expression level of the *CYP707*, *HSP90*, galactinol synthase, raffinose synthase, and α-galactosidase genes showed genotype-dependent changes after 1 day; however, the *CYP707* was the only one, which was still upregulated in at least some of the genotypes. Results suggest that heat acclimation itself does not require general induction of primary metabolites. However, induction of specific routes, for example the induction of the raffinose family oligosaccharides, especially the synthesis of galactinol, may also contribute the improved heat tolerance in cereals.

Introduction

Increasing temperatures cause more and more severe problems for both the natural vegetation, and the agricultural crop production, worldwide (Niu and Xiang 2018, Xu et al. 2020). Furthermore, not only the rising average temperature, but the increasing frequency of temperature extremes is responsible for the troubles (Meehl and Tebaldi 2004). The responses of plants to high temperatures have been described in many cases. These include, among others, various well-known processes such as the appearance of heat shock proteins (HSPs), activation of the antioxidant system, induction of stress hormones, such as abscisic acid (ABA), at the expense of cytokinins (Yang et al. 2019, Bheemanahalli 2020, Prerostova et al. 2020), etc. Changes in the photosynthetic machinery at high temperatures have also been intensively studied (Allakhverdiev et al. 2008, Hu et al. 2020). However, the details of thermal acclimation, also called as thermopriming, the process by which a plant exposed to a mild, sub-lethal temperature rise prepares for a subsequent more severe thermal stress, are much less known. Heat acclimation is the result of induction of different signalling routes controlling various physiological and biochemical processes, including among others, water management, and photosynthetic activity, regulating expression levels of stress-related genes, in order to help plants to prepare for a possible heat stress (Serrano et al. 2019).

Photosynthetic processes are highly sensitive to the heat-induced changes. Our earlier experiments showed that growing wheat plants at 30°C caused an induced acquired thermotolerance (Végh et al. 2018). Heat acclimation reduced chlorosis at extremely high temperatures as wel induced heat tolerance of the photosynthetic apparatus, including the function of Photosystem II (PSII), detected using the chlorophyll *a* fluorescence induction technique. The results showed that the temperature-dependent changes in each fluorescence

induction parameters, such as the actual quantum efficiency, the regulated and non-regulated non-photochemical quenching parameters in the acclimated plants started at higher temperatures than in the control, non-acclimated plants. In wheat plants, fluorescence induction parameters, in contrast to CO_2 assimilation parameters and chlorophyll content values, showed less genotype dependence, and they were more indicative of a general acclimatization processes (Végh et al. 2018). However, when the temperature-dependence of the fluorescence induction parameters has been investigated in different oat and barley genotypes, species and variety dependent differences could also be detected (Darkó et al. 2019).

Heat stress disturbs carbohydrate metabolism, too. At severe heat stress, the disturbance in metabolism inhibits the plant growth and development and finally results in a loss of grain yield. However, sugars not only serve as primary sources of carbon skeletons and energy in cells, but they also function as signalling molecules in many physiological processes, including stress responses (Finkelstein and Gibson 2002, Cho et al. 2010, Granot et al. 2013). The involvement of hexokinases in biotic and abiotic stresses has also been widely studied (Claeyssen and Rivoal 2007, Sarowar et al. 2008), and the role of fructokinases in plant responses to abiotic stresses has also been reported (Zörb et al. 2010). Furthermore, when the heat acclimation-induced metabolic changes were studied in Arabidopsis, the accumulation of certain carbohydrates, for example, sucrose or the raffinose family oligosaccharides, and the lipid metabolites showed the most significant increases (Serrano et al. 2019).

However, little is still known about the heat acclimation-induced changes at the metabolic level, especially in monocot crop plants. In the present work the following hypotheses were tested: (1) Earlier results using two spring and two winter oat and barley genotypes showed differences between winter and spring types in these species (Darkó et al. 2019). Here, we used a large number of genotypes to test whether the spring-winter type could indeed be differentiated with evaluation of the temperature curves of certain chlorophyll *a* fluorescence parameters; (2) In order to find species and/or winter-spring type differences, we also tested the responses of gas exchange parameters to heat acclimation; (3) In addition, in selected genotypes metabolic profiles, completed with gene expression studies, were investigated in non-acclimated and acclimated plants to get further information about the heat acclimation processes.

Materials and methods

Plant materials and growth conditions

Barley (*Hordeum vulgare* L. cv. Golden Promise, Jubilant, and Conchita spring and cv. MvHV0517, Mv Initium, and Meridian winter varieties), wheat (*Triticum aestivum* L. Chinese Spring spring, IKVA and Mv Nádor winter varieties) and oat (*Avena sativa* L. Mv Szellő and Mv Pehely spring, and Mv Hópehely and Mv Kincsem winter varieties) were grown and treated as described earlier (Darkó et al. 2019). Seeds were sown in plastic pots 3:1 (v:v) mixture of loamy soil and sand. Plants were grown under controlled environmental conditions in a Conviron PGV-15 growth chamber (Controlled Environments Ltd., Canada) at 22/20°C day/night temperature with 16/8 h photoperiod at 250 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), at 75% relative humidity. After 10 days, when the majority of the plants were in the two-leaf stage, the plants were dived up and part of the plants were left to grow further under the same conditions (controls), while other plants were moved to another chamber of the same type but with a temperature regime of 30/27°C (heat treatment) and left to grow for 14 days. All the other growth parameters were the same as for the controls. Plants were analysed at the end of the heat-treatment period at the 4-leaf stage. All the measurements were carried out on the youngest fully developed leaves.

Estimation of chlorophyll content and chlorophyll *a* fluorescence induction measurements

Chlorophyll contents were estimated on the 3rd fully developed leaves using a SPAD-502 chlorophyll meter (Konica-Minolta, Japan). Chlorophyll *a* fluorescence was measured using a pulse amplitude modulated fluorometer (Imaging-PAM M-series, Germany) similarly as described earlier (Végh et al. 2018). Briefly, detached leaves were dark-adapted for 20 min after which the Fv/Fm parameter was determined. The heat-induced changes of chlorophyll fluorescence parameters - calculated according to Klughammer and Schreiber (2008) - were determined at increasing temperature using a home-made temperature regulator working with Peltier elements. Photosynthesis was activated with 100 µmol m^{-2} s^{-1} actinic light intensity. The measurements started at 25°C, and after photosynthesis was steady (15 min) the temperature of the leaves were increased from 25 to 57°C at a rate of 1.5°C min^{-1}. During the measurements saturated flashes were applied at each °C increase. After recording the Fs vs temperature curves, the critical points of Y(II), Y(NPQ) and Y (NO) vs temperature were determined. Fig. S1 demonstrates a schematic temperature-dependence curve for determination of Tc and Tp values.

Thermoluminescence measurements

Thermoluminescence measurements were performed using an apparatus and software made by Jean-Marc Ducruet (Ducruet and Miranda 1992), with a method described earlier (Janda et al. 2004). Briefly, leaf segments cut from the middle of the youngest fully developed leaves were illuminated with far-red light provided by a PAM 102-FR light source (Walz, Germany) for 30 s (setting: 11) at 2.5°C prior to the measurement. A 4 × 4 cm Peltier element (Marlow Instruments, USA) was used for temperature control. The rate of heating during measurement was 0.5°C s^{-1}. Luminescence was detected using a Hamamatsu H5701-50 photomultiplier linked to an amplifier.

Gas exchange measurements

The temperature dependence of the gas exchange parameters were determined using a Ciras 3 Portable Photosynthetic System (PP Systems USA) supplemented with a narrow leaf area chamber. The CO_2 assimilation rate (Pn), the intercellular CO_2 concentration (Ci), stomatal conductance (gs), and transpiration rate (E) were recorded at different leaf chamber temperatures (22, 30, 35 and 40°C) after reaching the steady state conditions at the given temperature. The CO_2 level was 390 ppm, and the light intensity was 500 µmol m^{-2} s^{-1}. Water use efficiency (WUE) was calculated as Pn/E.

Untargeted polar metabolite identification and quantitation by GC-MS

Measurement of polar metabolites was carried out as it was described earlier (Marček et al. 2019). Briefly, 200 mg FW of plant material was pulverised in a Qiagen (Germany) liquid nitrogen cooled TissueLyser. Sample extraction and GC-MS analysis was carried out according to Schauer et al. (2005) and Juhász et al. (2015). The analyses were carried out with a GCMS-QP2010 system (Shimadzu, Japan) using a J&W HP5ms UI 30m × 0.25mm × 0,25µm capillary column (Agilent Technologies, USA). Data analysis was carried out using Shimadzu GC-MS Solution Postrun analysis software with searching the Wiley 9th edition mass spectral database and using the Kovats retention index (RI) with a C7-40 n-alkane mixture. For identification the NIST17 Mass spectral and RI database was also used through the use of the AMDIS and MS Search v.2.3 software. Identified and quantified compounds were summarized in Table S1 with their respective retention time, retention index and their respective EIC quantitative ion or indication of TIC quantitation and reference material availability.

Gene expression analysis

Total RNA was extracted from fully developed second leaves using TRI Reagent. Samples were cleaned with Direct-Zol RNA MiniPrep Kit (Zymo Research, USA) and treated with Dnase I. RNA was quantified with a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., USA) and its quality and integrity was also checked using agarose gel. 1000 ng of total RNA was reverse transcribed with M-MLC ReverseTranscriptase (Promega Corporation, USA) and oligo(dT)18 (Thermo Fisher Scientific). Measurement was performed with CFX96 Touch Real-Time Detection System (Bio-Rad, USA), the mastermix contained 1 µl of 4-fold diluted cDNA, gene-specific primers (Table S2) and PCRBIO SyGreen Mix (PCR Biosysytems, UK). Housekeeping gene encoding Actin1 was selected as the reference gene (Kong et al. 2015). For primer design, known barley sequences were chosen. Thereafter conserved domains were identified with NCBI Conserved Domain Finder (Lu et al., 2020). Highly conversed regions were used for BLAST search in oat genome browser (www.avenagenom.org). Common regions of barley and oat sequences sharing 90% or higher homology were used to design primers with Primer3 and primers were also checked with Oligoanalyzer to avoid primer dimerisation. Amplicon size was set between 100 and 200 bp with 60°C as melting temperature. Primers with 90% or higher amplification efficiency were used for gene expression studies. Reaction specificity was checked with melt curve analysis and PCR products were also run on agarose gel to confirm the presence of a single amplicon and amplicon size. Relative transcript level was determined with $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Gene expression value was set to 1 in control treatment (calibrator), and the relative expression values of the other treatments were normalized to the control value. Normalization was performed in every case using the calibrator value of the given genotype control.

Statistical analyses

Five parallel samples were collected for each measurement. Significant differences between the treatments and the genotypes were probed using the *t*-test method. Descriptive statistical analysis (principal component analysis, PCA) was applied to segregate metabolic status of two oat and barely genotypes under optimal and heat condition. PCA was applied to the standardized data set which included 47 variables and the factor loadings were done in order to

rate the proportion of total variance with different principal components. The loadings showed associations among different principal components (PC) and variables whereby values higher than 0.75 represented as ”strong”, 0.75-0.50 ”moderate” and 0.50-0.30 considered as ”weak” correlation (Liu et al. 2003). For gene expression analyses, all reactions were performed in triplicate using three biological and three technical repetitions. For statistical evaluation one-way ANOVA followed by Tukey's *post-hoc* test was carried out with SPSS 16.0 software.

Results

Winter-spring type does not directly affect the temperature dependence of chlorophyll *a* fluorescence induction parameters in cereals

In the first set of experiments the effects of mild heat stress on the temperature dependence of certain chlorophyll *a* fluorescence induction parameters were tested in various oat, barley, and wheat genotypes, including both winter and spring types. The Fv/Fm parameter, representing the maximum quantum efficiency of PSII was determined for every sample before the heating started. The very moderate or no change of Fv/Fm in the heat acclimated plants compared to the controls suggests that the applied heat acclimation did not cause severe stress (Table S3). Increasing temperature leads to a progressive decrease of the Y(II) parameter above a critical temperature (Y(II)Tc) (Fig. 1, S1). This threshold temperature, where the temperature dependence curve tend to decrease, demonstrates the temperature sensitivity of PSII. Generally, the decrease in Y(II) started at 3-4°C higher temperatures in plants growing at 30°C than in those grown at control temperature (22°C) (Fig. 1; Table 1). Among the investigated genotypes, oat varieties usually reacted at lower temperatures than wheat and barley plants. Interestingly, the spring type wheat Chinese Spring was the worst among the control plants, but after heat priming, it performed better than the oat varieties. However, direct relationship between the spring-winter type and the heat stability of Y(II) could not be observed: neither the critical temperatures for the inflexion points, nor the shape of the curves were separated according to the winter-spring types. When temperature increases, the regulated heat dissipation processes, determined by Y(NPQ), start to increase above a critical temperature, Y(NPQ)Tc, and then they reach a plateau (Tp) (Figs 2, S1). Both the Tc and Tp of NPQ were higher in the heat-acclimated plants (Fig. 2, Table 1) than in the controls grown at 22°C. Comparing the genotypes, similarly to Y(II), the barley variety Meridian performed best of the different varieties both under control and heat-primed conditions. Although heat-priming was also effective in oat genotypes, they usually were the first to react to the increasing temperatures. The non-regulated heat dissipation,

Y(NO) also increases above a threshold temperature, but the Tc values of Y(NO) were generally higher than Y(II)Tc or Y(NPQ)Tc (Fig. 3, Table 1), indicating that NO is less sensitive to the temperature rise than Y(II) or Y(NPQ). Although the differences between the control and heat acclimated wheat, barley, and oat cereal species in the temperature dependence of chlorophyll fluorescence curves or in the Tc and Tp values were obvious, the spring genotypes did not show marked separation from the winter ones in any of the fluorescence induction parameters (Table 1).

For better understanding the acclimation of the photosynthetic membranes to the elevated temperatures, thermoluminescence investigations were carried out in control and heat-acclimated oat leaves. Illumination of leaf segments with far-red light at low, but non-freezing temperatures before the measurements induces the B-band, reflecting a charge recombination between the S2/S3 states of the water splitting system and Q_B^-, the secondary quinone acceptor of PS II, and the AG thermoluminescence band, which is related to the cyclic electron pathway providing back electron transfer towards PS II centres (Ducruet 2003, García-Calderón et al. 2019). However, although the plants were grown under controlled conditions, the leaves of the same type also showed a large variance, so no significant differences were detected as a result of heat acclimation (data not shown).

Temperature dependence of gas exchange parameters in cereals

Gas exchange measurements were also carried out focusing on the responses of certain photosynthetic processes to elevated temperatures with or without heat acclimation at 30°C for 2 weeks in wheat, oat, and barley varieties. With a few exceptions (mild heat stress slightly reduced Pn in Golden promise barley and Mv Pehely spring oat), there were no significant differences in Pn at 22°C between the control and heat-primed plants (Fig. S2). When the temperature increased, Pn tended to decrease in all the cases. However, growing at 30°C provided protection against the heat-induced decrease in Pn in the case of the Mv Initium barley variety, in which the most pronounced decrease occurred in the non-acclimated plants. In spite of the fact that the decrease in Pn was often partly accompanied with a decrease in the stomatal conductivity (gs), the transpiration rate (E) showed a marked increase in all the genotypes, parallel with the temperature rise. Interestingly, growing at 30°C already caused higher E and Ci levels mainly in barley varieties. Measured at 22°C, the water use efficiency (WUE) was generally higher in plants growing at control temperatures than in the heat-acclimated ones. The

elevated temperature-induced increase in E and decrease of Pn also resulted in a substantial decrease in WUE in all the tested genotypes. However, similarly to the fluorescence induction parameters, winter/spring type did not substantially affect the behaviour of gas exchange parameters.

Heat acclimation modifies the metabolomic profiles in barley and oat plants

In the next part of the experiments two selected barley (the winter type Mv Initium and the spring type Conchita) and two oat varieties (winter type Mv Hópehely and spring type Mv Pehely), which have been investigated in one of our earlier studies (Darkó et al. 2019), were further investigated. A non-targeted metabolomic analysis focusing on polar metabolite identification and quantitation revealed substantial differences between these species and the primed and non-primed plants. Using this method, several compounds, including sugars, organic acids, amino acids and alcohols could be separated and detected. The effects of heat treatment on average values of the metabolite levels can be seen in Table S4. Furthermore, the heatmap which enables the comparison of the relative abundance of the metabolites and the difference between the genotypes and treatments (primed and non-primed plants) also shows certain species-, variety-, and heat treatment-dependent changes in the different metabolite levels (Fig. 4A).

Comparing the genotypes growing at control temperature (22°C), it was found that the most abundant metabolite, sucrose was predominantly higher in oat than in barley plants, and the winter genotypes contained higher sucrose level than the spring ones. In addition, the levels of malic acid, citric acid, and isocitric acid were more elevated in barley than in oat, while the isocitric acid was under the detection limit in oat plants. However, the opposite was observed in the case of *cis*-aconitic acid, which was detectable in oat, but not in the barley genotypes. Furthermore, the levels of 2 non-identified sugars (called sugar1 and sugar2), fructose, phosphoric acid, D-ribose, aspartic acid, and malonic acid were much higher in barley, while the levels of tetronic acid and shikimic acid were higher in oat plants, although the amount of these metabolites was lower than previous ones. Even if there were differences between the varieties within the same species, these differences were lower than the differences between the species, or between the control and heat-primed plants.

Exposure to a mild heat stress also modified the metabolic composition of barley and oat. The heat map (Fig. 4A) demonstrates that most of the metabolites were down-regulated after the

heat treatment, except raffinose, 2 non-identified sugars (sugar5 and sugar6), myo-inosytol, and galactinol, which were accumulated during the heat acclimation. Metabolite data were also subjected to principal component analysis (PCA) (Fig. 4B, C). The factor loadings present the ratio of total variance explained by different principal components (PC) and their correlations with variables (Table S5) under control and heat treatment. PCA yielded four significant components contributing 88.75% of total variation. The cumulative contribution of PC1 and PC2 was 65.77%. The first component (PC1) was characterised with strong negative loadings for malonic, phosphoric, maleic, fumaric, D-malic, glutamic, citric and isocitric acid, proline, ribose, serine and undetermined disaccharide (S2) while glycerol, *cis*-aconitic acid and undetermined disaccharide (S4) had strong positive correlations in the same principal component. The second component (PC2) showed strong negative loadings for glycine, succinic and tetronic acid, galactose, glucose, sucrose and undetermined disaccharide (S3) while galactinol had positive loadings. The third component (PC3) was largely determined by positive loadings for L-threonine and negative correlation for oxalic acid. The amino acids isoleucine, valine and phenylalanine had negative loadings in the fourth component (PC4).

PCA of the thermoregulated metabolites grouped the different varieties of the same species together. The score plot distinguished four groups (Fig. 4C). Cluster analyses revealed a species-specific metabolic profile. *Cis*-aconitic acid was not detected in barley genotypes while iso-citric acid was absent both in control and heat-treated oat plants. First two clusters belong to unstressed plants. Oat genotypes (Mv Hópehely and Mv Pehely) (cluster 1) showed low amount of organic acids (malonic, maleic, glutamic, citric, fumaric, and phosphoric acid), ribose, undetermined sugars (S2, S4) and proline accumulation but higher glycerol than in barley genotypes (Conchita and Mv Initium) segregated in cluster 2. Cluster 3 included heat-stressed barley genotypes while oat genotypes were grouped into cluster 4. Both species under heat had similar pattern in metabolic response like their corresponding control genotypes. According to strong associations presented in correlation matrix (Table S5), heat treated cereals were separated from controls based on decreased sugar (sucrose, glucose, fructose, galactose, glycine), amino acids (serine, glycine, alanine, GABA) and proline contents.

Comparison between the varieties indicated that the metabolic activities of these plants within the same species were highly similar. However, the control and the heat-treated groups differed from each other in the case of both species. This means that both the species and the treatment with heat had more significant effects on the metabolome than the variety.

According to correlation matrix only galactinol showed strong positive correlation with PC2. Myo-inositol showed moderate positive loadings. Raffinose had weak correlation pattern in all PCs. However, comparing the different metabolites in the PCA analysis, these 3 compounds, namely myo-inosytol, galactinol and raffinose were grouped together separately from the others. These are the metabolites, which were mainly upregulated by heat treatment (Fig. 4).

Mild heat stress mainly affected gene expression levels at short-term treatment

In order to better understand the acclimation processes and the metabolic changes, the expression levels of certain genes, including *CYP707*, *HSP90*, galactinol synthase, raffinose synthase, and α-galactosidase have also been measured after 1 day and 2 weeks of heat-treatment (Fig. 5). In general, while all the genes showed genotype-dependent changes in the expression level after 1 day, the *CYP707* was the only one, which was still upregulated in at least some of the genotypes, mainly in barley, after 2 weeks. The other transcripts showed difference only after short-term treatment. After 1 day, the raffinose synthase declined in Mv Initium and Mv Hópehely, it did not change in Mv Pehely, but increased in Conchita. In contrast to this, the expression level of raffinose synthase substantially increased in Mv Initium, and, in less extent, in Conchita, but did not change in Mv Hópehely and Mv Pehely. The relative expression of α-galactosidase was barely influenced by the treatments in the investigated genotypes, although a slight increase in Mv Pehely could be detected. The *HSP90* gene was mainly induced after 1 day in Mv Initium and Mv Pehely, and it did not change in Mv Hópehely.

Discussion

Increasing temperatures may decrease the actual quantum yield, Y(II)), and increase the Y(NPQ) and Y(NO) fluorescence induction parameters. Our earlier results using different winter wheat varieties demonstrated that the critical temperatures, in which the changes in these chlorophyll *a* fluorescence induction parameters start can be increased by growing plants at elevated, but sub-lethal, acclimating temperatures (Végh et al. 2018). However, direct relationship between heat tolerance of the varieties and the changes in the critical temperature values could not be detected in wheat plants. In another study, where different barley and oat genotypes were compared, results suggested that the temperature dependence could differ in spring and winter varieties of these species (Darkó et al. 2019). However, since that result was

only obtained using 1-1 spring and winter genotypes in both species, a confirmation was necessary in order to generalise the differences between the spring and winter varieties.

The photosynthetic apparatus is sensitive to the increase of temperature. Both the CO_2 assimilation capacity and electron transport processes including the PS II activity showed a decline above a critical temperature value. It has also been demonstrated previously, that heat acclimation alters both the light and dark reactions of photosynthesis (Jing et al. 2019). However, even if the changes contained common elements, they varied depending on genotypes. In the present work several wheat, barley and oat genotypes were tested to reveal whether the temperature dependent chlorophyll *a* fluorescence or the CO_2 assimilation measurements are suitable to separate the heat acclimation processes in spring or winter varieties. As observed previously (Végh et al. 2018, Darkó et al. 2019) while heat acclimation at 30°C did not cause any injury demonstrated by the Fv/Fm parameters in any genotypes, it induced an increased thermotolerance of the photosynthetic apparatus, which manifested in the special characteristics of temperature dependent changes in Y(II), Y(NPQ) and Y(NO). The critical points of these parameters and the slope of the curves indicates the heat stability of the photosynthetic apparatus. It may also happen, that plants with the same, or even with higher Tc values can be characterised with relatively lower heat tolerance of the photosynthetic membranes, when the slope of the heat dependence is sharp, and reaches the 2nd Tc value in the case of Y(II) and the Tp in Y(NPQ) earlier than another genotype with the same, or even lower Tc value. It means, that the complex analysis of the temperature dependence curves may indicate the temperature dependence of the membrane stability. Comparing the genotypes, the results showed some, not very sharp differences in critical points of fluorescence parameters between the species; however, the differences were not correlated with the winter/spring type of genotypes.

Regulation of gas exchange can also be important part of acclimation to elevated temperatures. Using wheat varieties with different levels of heat tolerance it has been shown that heat tolerant genotypes are able to close their stomata faster when they are exposed to severe high temperatures. Furthermore, some of the genotypes were able to increase their transpiration, probably to induce a kind of cooling effect in the shoots (Végh et al. 2018). In the present study, heat-acclimation did not generally increase the Pn values in barley or oat plants. However, in accordance of earlier findings, elevation in E parallel with the increasing temperatures was also observed. Especially, in the case of barley, this was often more pronounced in the heat-acclimated plants then in the controls. In oat this difference could not be observed. These results

confirm the observation, that different strategies can be activated to avoid the damaging effects of high temperatures in these species (Darkó et al. 2019), and it seems, that these differences can also be manifested as regulation of the photosynthetic processes. However, the changes of Pn, gs, Ci and E were not correlated with the winter/spring types of genotypes.

In order to better understand the heat responses of the acclimation processes, gene expression and metabolomic analyses have also been carried out. The early results of the effects of heat stress on a carbohydrate level are controversial. An increased carbohydrate content with elevated temperatures has been reported in creeping bentgrass (Duff and Beard 1974). However, other results reported an opposite trend. Heat stress caused substantial decrease in glucose and sucrose levels especially in the shoots and to lesser extent in roots of other bentgrass varieties (Liu and Huang 2000). Recent results on heat stress in Arabidopsis plants indicated that the changes in the metabolomic profile highly depend on the severity of the treatment. While mild heat stress or short-term priming hardly affected or down-regulated the accumulation of metabolites, long-term priming or severe heat stress induced the accumulation of various compounds (Serrano et al. 2019). Under the present environmental conditions, the Fv/Fm chlorophyll *a* fluorescence induction parameter also indicated that growing at 30°C did not cause severe stress, but could induce processes providing protection against severe heat stress in every tested genotype.

In one of our recent works, we have partly characterised the responses of winter and spring barley varieties Mv Initium and Conchita and the winter and spring varieties Mv Hópehely and Mv Pehely (Darkó et al. 2019). We have shown that both overlapping and different strategies may operate to avoid the damaging effects of high temperatures in these plant species. The involvement of certain antioxidant enzymes, the polyamine metabolism, and the changes in certain hormone levels have been described in these genotypes (Darkó et al. 2019). Therefore, we chose these varieties in the detailed metabolome analyses of control and heat-treated plants. We analysed 51 metabolites, which were mainly related to the tricarboxylic acid (TCA) cycle and associated pathways (Fig. 6). Interestingly, majority of these metabolites were down-regulated by growing at elevated temperatures. This can be explained by an enhanced consumption under moderate heat conditions. Similar effect was described by Ren et al. (2019) when the effects of heat stress on *Populus tomentosa* plants were studied. Heat stress also enhanced glycolysis and retarded the TCA cycle in poplar trees.

The PCA analyses revealed that heat priming induced the synthesis of galactinol in parallel with an increase in the level of myo-inositol, which is one of the precursors of galactinol synthesis.

Similar results have been found in chickpea plants, where galactinol and raffinose contents increased in response to elevated temperatures, due to the overexpression of the galactinol synthase genes (Salvi et al. 2017). Interestingly, genes related to galactinol or raffinose synthesis, namely galactinol synthase, raffinose synthase, and α-galactosidase, were mainly induced after short-term, 1 day heat treatment, and their expression levels were down-regulated after long term, 2 weeks heat exposure. It was suggested that galactinol may improve the heat tolerance in Arabidopsis plants (Salvi et al. 2017). Our results also suggest that galactinol may play an important role during the heat acclimation period in oat and barley plants.

Heat shock factors are transcriptional regulators of the heat shock response. They mainly regulate genes encoding heat shock proteins (HSPs), which may provide protection against cytotoxic effects (Panikulangara et al. 2004). Busch et al. (2005) have identified certain heat shock factor HsfA1a/1b-dependent genes and pathways, which contribute to the development of stress tolerance in Arabidopsis plants. Earlier results suggest that the galactinol synthase gene *GolS1* is a heat shock factor target gene, which is responsible for heat stress-dependent synthesis of oligosaccharides galactinol and raffinose (Panikulangara et al. 2004). In agreement with these findings, it has been demonstrated that one of the two heat-inducible galactinol synthase genes, *GolS1* was activated via HsfA1a/1b (Busch et al. 2005). *HSP90* genes are involved in various signalling processes both in plants and animals. They also mediate various abiotic stress signal pathways; however, the mechanisms behind are still unclear (Xu et al. 2012). Under the present experimental conditions, growing at 30°C induced its expression in both barley and oat in a genotype dependent manner. However, after 2 weeks, this induction could no longer be detected. It suggests that this gene is probably important in the regulation of the early events of the heat acclimation in these plants. Other findings also suggest that raffinose and galactinol may scavenge hydroxyl radicals reducing the oxidative damage occurring with various environmental stresses (Nishizawa et al. 2008).

An interaction between ABA and sugars has been described, as ABA may regulate the expression of certain genes encoding components of the sugar metabolism and transport (Gibson et al. 2004). However, although both ABA and sugars regulate the response of plants to abiotic stressors, only few studies have focused on how the interactions between ABA and sugars influence heat tolerance in plants (Islam et al. 2018). *CYP707* gene, a members of the cytochrome P450 (CYP) super-family, encodes an ABA 8′-hydroxylase protein, one of the key enzymes of the ABA8′-hydroxylation reaction, which may play central role in regulating ABA level (Zheng et al. 2012). ABA is mainly oxidized to 8′-hydroxy-ABA catalysed by ABA 8′-

hydroxylase, which is unstable, and can be converted to phaseic acid, and finally reduced to dihydrophaseic acid (Nambara and Marion-Poll 2005). Earlier we have demonstrated that 2 weeks of heat acclimation induced ABA accumulation in oat plants, but not in barley; and the levels of phaseic acid and dihydrophaseic acid were much lower in oat than in barley (Darkó et al. 2019). Present results also showed an induction, already after one day of heat treatment, of the *CYP707* gene in barley, but not in oat, and this induction was maintained for 2 weeks. Species-dependent differences between barley and oat plants have also been confirmed in the present paper. However, as it was suggested by the PCA results, that the differences between the species were more pronounced under control conditions than after heat priming, so these changes probably cannot be considered as a general ABA response.

Conclusions

According to the temperature dependence of chlorophyll *a* fluorescence induction and gas exchange measurements, the heat acclimation caused an increase in thermotolerance of the photosynthetic apparatus in all the tested wheat, barley, and oat varieties. However, we refuted the hypothesis that there was a strong correlation between the winter/spring type and the temperature dependency of the chlorophyll *a* fluorescence induction parameters. Furthermore, we confirmed that similar to wheat, elevated temperature may lead to an increasing transpiration rate and reduces the water use efficiency in barley and oat. Heat priming itself does not require general induction of primary metabolites. However, induction of specific routes, for example the synthesis of galactinol, may contribute the improved heat tolerance in barley and oat leaves.

Author contributions

T.J. and É.D. conceptualized and supervised the work. É.D. and T.M. were responsible for the fluorescence induction experiments. B.I. carried out the gas exchange measurements. J.T performed the gene expression works, K.Á.H. performed the analytical chemical works, T.M. helped in the PCA analyses, G.Sz. and M.P. helped in analytical work and the discussion. T.J. wrote the manuscript, É.D., E.D.Z., G.Sz., and M.P. critically revised and corrected the manuscript proof. All authors read and approved the manuscript.

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Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Figure legends:

Fig. 1. Temperature dependence of Y(II) in different oat (green lines), barley (red lines), and wheat (black lines) varieties in control (22°C; A) and heat-primed (30°C; B) plants. Solid and dotted lines belong to winter and spring type varieties, respectively.

Fig. 2. Temperature dependence of Y(NPQ) in different oat (green lines), barley (red lines), and wheat (black lines) varieties in control (22°C; A) and heat-primed (30°C; B) plants. Solid and dotted lines belong to winter and spring type varieties, respectively.

Fig. 3. Temperature dependence of Y(NO) in different oat (green lines), barley (red lines), and wheat (black lines) varieties in control (22°C; A) and heat-primed (30°C; B) plants. Solid and dotted lines belong to winter and spring type varieties, respectively.

Fig. 4. (A) Heatmap showing concentration of metabolites in control and heat-treated (30°C, 2 weeks) barley (Mv Initium winter, Conchita spring) and oat (Mv Hópehely winter, Mv Pehely spring) varieties. Sugar1, 2, and 8 represent unidentified sugar compounds. Columns represent the different samples, 5 biological replicates per sample. Rows are grouped according to the abundance of the different compounds. Absolute values can be seen in Table S4. (B, C) Results of the principal component analysis (PCA) for the separation of the different metabolites (B) Red colour: sugars; green: organic acids; blue: amino acid; violet: alcohols), and showing groups of different varieties with (red letters) or without (black letters) heat priming (C).

Fig. 5. Effects of 1-day (HP-1d) and 14-day heat priming (HP-14d) on relative expression levels of *CYP707* (A, B), raffinose synthase (C, D), galactinol synthase (E, F), α-galactosidase (G, H) and *HSP90* (I, J) in the leaves of different barley and oat genotypes. The relative gene expressions were determined with 2^{-ddCt} method. Different letters indicate significant differences at $P \leq 0.05$ level.

Fig. 6. Associated pathway analysis of metabolites in control (C) and in heat-acclimated (H) winter barley Mv Initium (INI), spring barley Conchita (CON), winter oat Mv Hópehely (HP), and spring oat Mv Pehely (P) plants. Different colours indicate concentration of metabolites in different ranges.

Supporting information:

Fig. S1. Schematic demonstration of the temperature dependence of certain chlorophyll-a fluorescence induction curves. The heat-induced changes of chlorophyll fluorescence parameters are determined at increasing temperatures. The measurement starts at normal temperature followed by heating the leaves until Y(NO) and Y(NPQ) reach maximum levels. During the measurements saturated flashes are applied at each °C. After recording the fluorescence vs temperature curves, the critical points of Y(II), Y(NPQ) and Y(NO) vs temperature can be determined.

Fig. S2. Temperature dependence of of gas exchange parameters, Ci (µmol CO_2 mol^{-1}), gs (mmol m^{-2} s^{-1}), Pn (µmol m^{-2} s^{-1}), E (mmol m^{-2} s^{-1}), and WUE in control, grown at 22°C (blue, solid lines) and heat-acclimated (red, dotted lines) wheat (A), barley (B) and oat (C) varieties. * represents significant differences between the control and heat-acclimated plants at $p<0.05$ level.

Table S1. Peak name, quantitation TIC/EIC ion, retention time in minutes, n-Alkane Kovats Retention Index for 5% phenyl semi-standard non-polar column (RI), reference material (Yes) or tentative identification according to mass spectral and RI match (No).

Table S2. Primer sequences used for RT-PCR measurements.

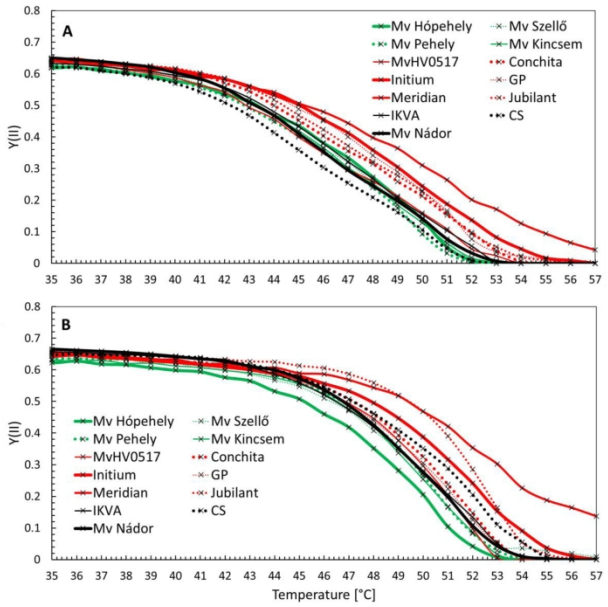
Table S3. The Fv/Fm values in control and heat-treated (30°C for 10 days) wheat, barley, and oat varieties. * and ** represent significant differences between the control and heat-treated plants at $p\leq0.05$ and 0.01 levels, respectively.

Table S4. Mean±SD values for metabolites in control and heat-treated (30°C, 14 days) barley (Mv Initium winter, Conchita spring) and oat (Mv Hópehely winter, Mv Pehely spring) varieties. Sugar1, 2, and 8 represent unidentified sugar compounds. Compounds are grouped according to the range of the order of magnitude of the mean values. Mean and SD values were calculated from 5 independent samples. Yellow cell background of the SD cell indicates significant difference between the control and heat-treated plants at 0.05 level.

Table S5. Factor loadings of metabolites of two oat and barely genotypes. (PC-principal components)

Table 1. Tc, Tcz (Tc to zero), and Tp values (°C) of the temperature-FI parameter curves, namely Y(II), Y(NPQ), and Y(NO) calculated from the average graphs demonstrated in Figs. 1-3. Y(II)Tc and Y(NPQ)Tc/Y(NO)Tc values represent the critical temperatures were the temperature dependence curve tend to decrease and increase, respectively. Y(II)Tcz represents the critical temperature value in which it tends to reach zero. Y(NPQ)Tp values represent the critical temperatures in which the Y(II) temperature dependence curve tends to reach a plateau. The determination of the Tc and Tp values is demonstrated in Fig. S1. Values for control plants/heat-acclimated plants are presented.

	Y(II)Tc	Y(II)Tcz	Y(NPQ)Tc	Y(NPQ)Tp	Y(NO)Tc
Wheat					
Spring:					
Chinese Spring	40.7/45.8	52.2/55.0	41.2/45.0	48.1/53.2	48.7/50.4
Winter:					
IKVA	42.3/45.3	53.0/53.8	42.4/45.6	51.7/51.4	50.0/51.3
Mv Nádor	41.5/45.5	52.6/53.7	42.0/45.7	48.8/51.3	48.5/51.0
Barley					
Spring:					
Conchita	41.8/47.1	54.5/53.6	41.8/46.2	50.3/52.0	50.3/51.3
Golden Promise	43.5/46.3	54.3/53.8	42.2/45.4	51.5/52.1	49.9/50.8
Jubilant	42.5/49.3	54.4/54.4	42.2/49.1	50.8/53.1	48.8/52.2
Winter:					
MvHV0517	42.0/46.5	53.1/53.1	41.2/46.7	49.0/51.9	49.6/50.9
Mv Initium	44.0/47.5	54.6/55.0	43.5/46.8	51.7/53.6	50.1/50.3
Meridian	44.4/48.3	54.8/58.0	43.8/48.1	52.8/54.1	51.6/52.6
Oat					
Spring:					
Mv Szellő	42.1/45.6	52.2/54.0	41.7/45.2	49.4/50.9	49.6/50.8
Mv Pehely	42.3/46.4	52.0/52.8	41.7/46.0	49.0/50.7	49.6/50.4
Winter:					
Mv Hópehely	42.9/44.8	52.6/53.2	42.2/45.0	49.8/50.0	49.6/49.7
Mv Kincsem	42.5/46.4	52.3/53	42.9/46.1	48.4/50.4	49.6/50.6



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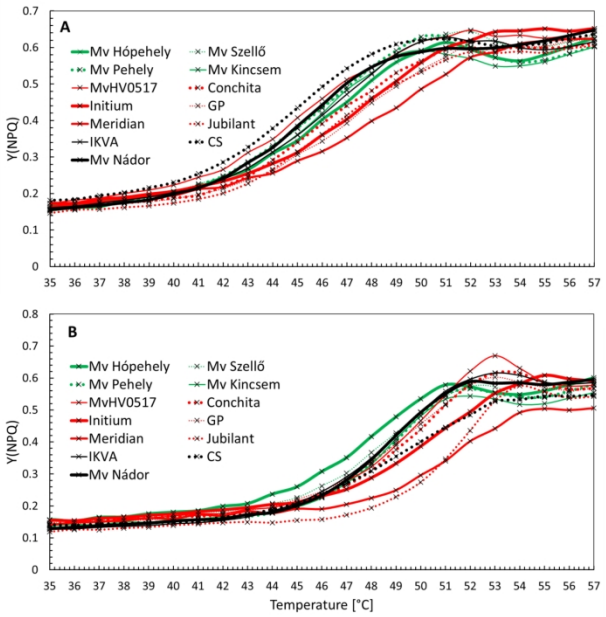

Fig. 2. Temperature dependence of Y(NPQ) in different oat (green lines), barley (red lines), and wheat (black lines) varieties in control (22°C; A) and heat-primed (30°C; B) plants. Solid and dotted lines belong to winter and spring type varieties, respectively.

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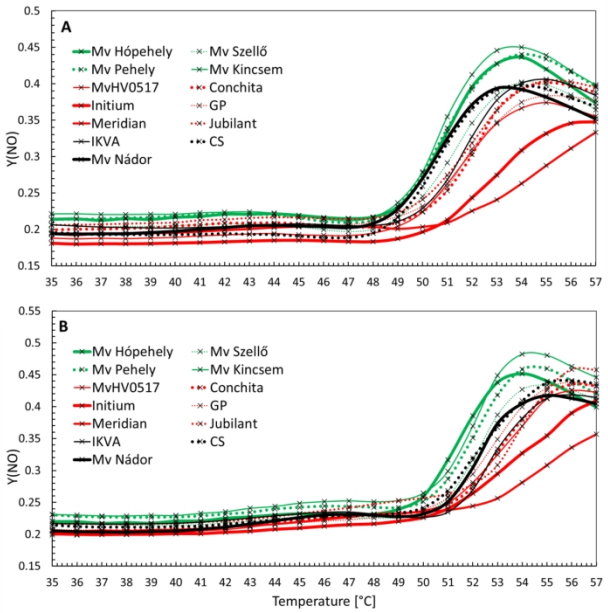

Fig. 3. Temperature dependence of Y(NO) in different oat (green lines), barley (red lines), and wheat (black lines) varieties in control (22°C; A) and heat-primed (30°C; B) plants. Solid and dotted lines belong to winter and spring type varieties, respectively.

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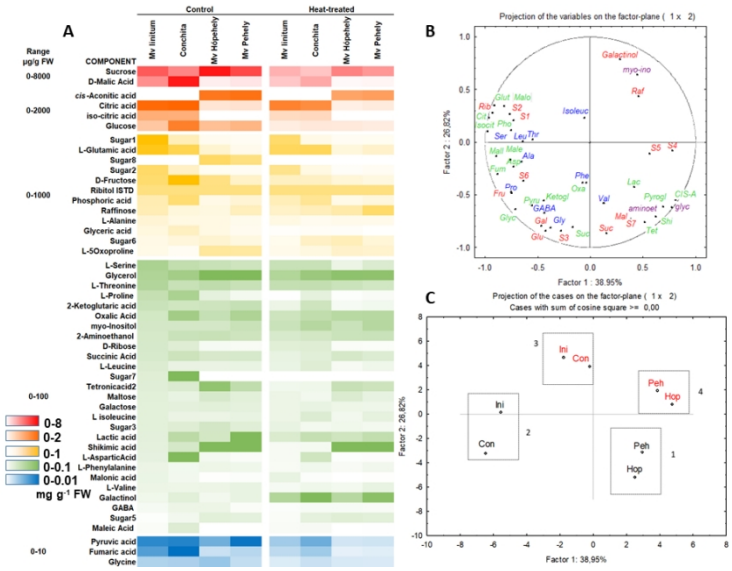

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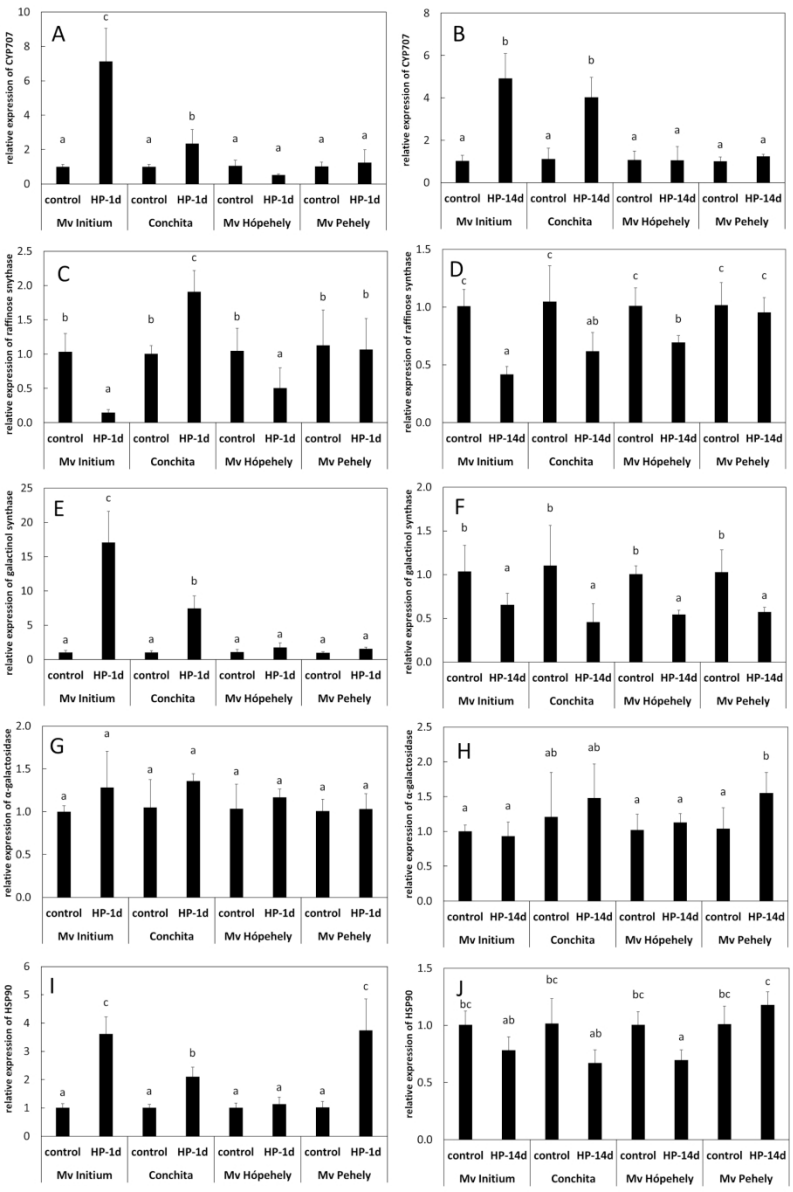

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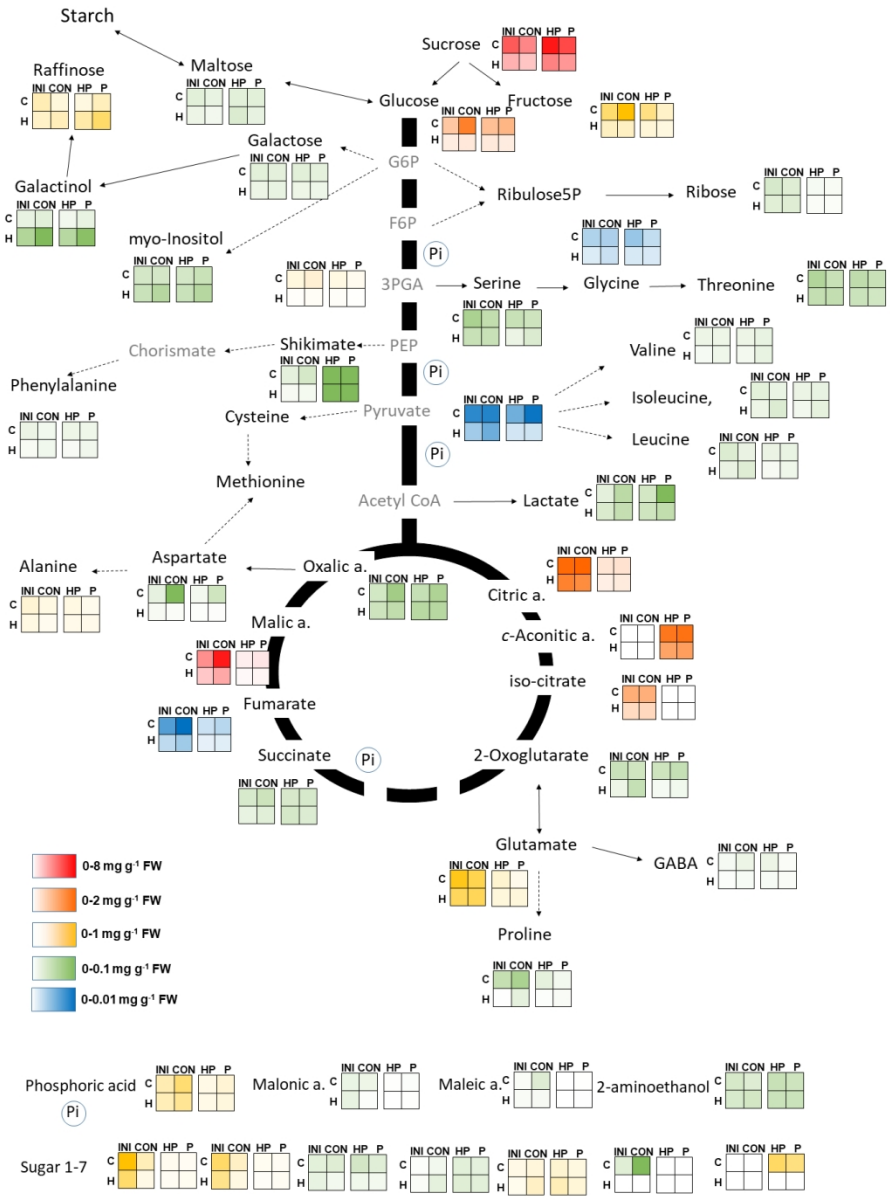

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365x487mm (96 x 96 DPI)