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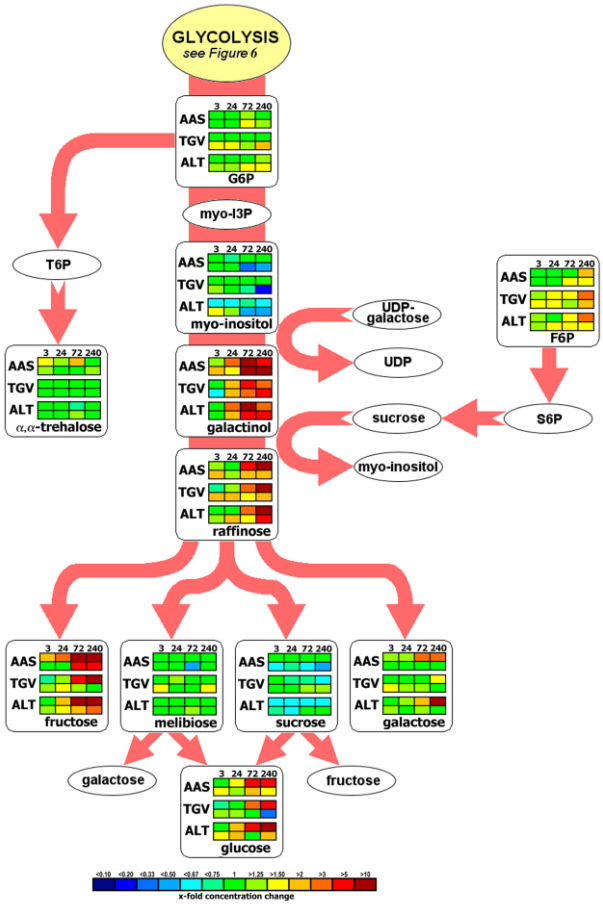
Abstract: Winter freezing damage is a crucial factor in overwintering crops such as the octoploid strawberry (Fragaria × ananassa Duch.) when grown in a perennial cultivation system. Our study aimed at assessing metabolic processes and regulatory mechanisms in the close-related diploid model woodland strawberry (Fragaria vesca L.) during a 10-days cold acclimation experiment. Based on gas chromatography/ time-of-flight-mass spectrometry (GC/TOF-MS) metabolite profiling of three F. vesca genotypes, clear distinctions could be made between leaves and non-photosynthesizing roots, underscoring the evolvement of organ-dependent cold acclimation strategies. Carbohydrate and amino acid metabolism, photosynthetic acclimation, and antioxidant and detoxification systems (ascorbate pathway) were strongly affected. Metabolic changes in F. vesca included the strong modulation of central metabolism, and induction of osmotically-active sugars (fructose, glucose), amino acids (aspartatic acid), and amines (putrescine). In contrast, a distinct impact on the amino acid proline, known to be cold-induced in other plant systems, was conspicuously absent. Levels of galactinol and raffinose, key metabolites of the cold-inducible raffinose pathway, were drastically enhanced in both leaves and roots throughout the cold acclimation period of 10 days. Furthermore, initial freezing tests and multifaceted GC/TOF-MS data processing (Venn diagrams, Independent Component Analysis, Hierarchical Clustering) showed that changes in metabolite pools of cold-acclimated F. vesca were clearly influenced by genotype.

Graphical Abstract

Metabolite profiling based on gas chromatography/ time-of-flight-mass spectrometry (GC/TOF-MS) was applied to investigate changes in central metabolism in leaves and roots of *Fragaria vesca* L. (woodland strawberry) under cold acclimation.



Highlights

- ► Metabolite pools of *F. vesca* were highly perturbated upon cold acclimation during a 10-days study, and mainly sugars, amino acids and amines were affected.
- ► Multivariate statistical analyses revealed differences between plant organs (leaf and root), genotypes (‘Ås’, ‘Tingvoll’, and ‘Alta’) and time points after onset of cold (3, 24, 72, and 240 h).
- ► Levels of galactinol and raffinose, key metabolites of the cold-inducible raffinose pathway, were drastically enhanced throughout the cold acclimation period.

Metabolite profiling reveals novel multi-level cold responses in the diploid model *Fragaria vesca* (woodland strawberry)

Running Title: Fragaria Cold Metabolome

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ABSTRACT

Winter freezing damage is a crucial factor in overwintering crops such as the octoploid strawberry (*Fragaria* × *ananassa* Duch.) when grown in a perennial cultivation system. Our study aimed at assessing metabolic processes and regulatory mechanisms in the close-related diploid model woodland strawberry (*Fragaria vesca* L.) during a 10-days cold acclimation experiment. Based on gas chromatography/ time-of-flight-mass spectrometry (GC/TOF-MS) metabolite profiling of three *F. vesca* genotypes, clear distinctions could be made between leaves and non-photosynthesizing roots, underscoring the evolvement of organ-dependent cold acclimation strategies. Carbohydrate and amino acid metabolism, photosynthetic acclimation, and antioxidant and detoxification systems (ascorbate pathway) were strongly affected. Metabolic changes in *F. vesca* included the strong modulation of central metabolism, and induction of osmotically-active sugars (fructose, glucose), amino acids (aspartatic acid), and amines (putrescine). In contrast, a distinct impact on the amino acid proline, known to be cold-induced in other plant systems, was conspicuously absent. Levels of galactinol and raffinose, key metabolites of the cold-inducible raffinose pathway, were drastically enhanced in both leaves and roots throughout the cold acclimation period of 10 days. Furthermore, initial freezing tests and multifaceted GC/TOF-MS data processing (Venn diagrams, Independent Component Analysis, Hierarchical Clustering) showed that changes in metabolite pools of cold-acclimated *F. vesca* were clearly influenced by genotype.

***Keywords*:** cold acclimation; compatible solutes; *Fragaria* × *ananassa* Duch.; *Fragaria vesca* L.; gas chromatography/ time-of-flight-mass spectrometry (GC/TOF-MS); metabolite profiling; phenotype.

1. Introduction

The complexity of plant responses to abiotic stress comprise signaling processes, which trigger transcriptional regulation and gene activation, followed by stress-induced tolerance or resistance mechanisms. Cold response and freezing tolerance of perennial crops is of major interest for breeders and farmers in temperate and cold-temperate climatic zones due to short vegetation periods and harsh growing conditions. One of the most important horticultural crops for the consumer market is the cultivated strawberry (*Fragaria* × *ananassa* Duch.). Successful production and berry yield relies significantly on plant acclimation (Rohloff et al., 2009), winter survival and rapid re-growth in spring time. Even though several *Fragaria* cultivars have been developed for cultivation under northern climates, their freezing tolerance is still rather limited (Sønsteby and Karhu, 2005; Shokaeva, 2008).

A major regulatory mechanism responsible for cold hardening and plants adaptation to low temperatures, leads to the transcriptional activation of specific C-repeat binding factors, the so-called CBF regulon (Stockinger et al., 1997; Vogel et al., 2005). Characteristic responses occur within 24 h and potentially persist for days up to several weeks and even months as a physiological memory effect of induced freezing tolerance (Kume et al., 2005; Kjellsen et al., 2010). The CBF cold response pathway has been reported to occur in many crop plants (Yang et al., 2005), among others, the strawberry (Owens et al., 2002). Following activation of the CBF regulon, the plant system undergoes many physiological and molecular changes that affect both primary and secondary metabolism. Studies in *Arabidopsis thaliana* have revealed the modularity of the metabolic cold response in short- and long-term experiments. Based on the multitude of signal and transcriptional cascades, the immediate induction of the ICE1 transcription factor is followed by activation of the CBF regulon (Lee et al., 2005). These mechanisms include the functional expression of hydrophilic and cryoprotective proteins (Alsheikh et al., 2003; Alsheikh et al., 2005), and the metabolic regulation of low-molecular

weight compounds which act as osmolytes and osmoprotectants (Cook et al., 2004; Kaplan et al., 2004; Guy et al., 2008). Beside monosaccharides, polyols, amino acids and amines, the raffinose pathway in particular has been described as an essential cold-inducible biosynthetic route in plants (Kaplan et al., 2007), leading to the formation of increased levels of the trisaccharide raffinose from galactinol and sucrose.

Metabolite levels in specific tissues and the interconnection of metabolism throughout the whole plant system, have been described through molecular approaches in the past ten years. Comparable to transcriptional and proteomic analyses, high-throughput chromatographic systems coupled with mass spectrometry (Lisec et al., 2006) are capable of describing the modularity and functionality of plant systems. Metabolite profiling has been recognized as an ideal tool for the detection of metabolic variation between genotypes and/or phytochemical changes upon stress (Rohloff and Bones, 2005; Schauer et al., 2005; Rohloff et al., 2009). Responses to environmental stresses are known to be highly evolutionary conserved throughout the plant kingdom (Ruelland et al., 2009). However, one might expect differing cold acclimation and freezing tolerance strategies, when studying different plant organs, and compare annual and perennial species. In view of the apparent limitations of biological information gained from studies in the model species *Arabidopsis thalian*, a need for new plant models has been postulated (Folta and Davis, 2006). In order to intensify breeding approaches in the cultivated octoploid strawberry, the diploid woodland strawberry *Fragaria vesca* L. has been introduced as an attractive model due to its small genome, plant size, vegetative and seed propagation, but also for its prolific fruit and seed production (Shulaev et al., 2008), and its draft genome has recently been published (Shulaev et al., 2011).

In our on-going research activities with focus on the development of molecular markers that are associated with winter survival in the cultivated strawberry, we have focused on diploid *F. vesca* genotypes in order to facilitate and expedite knowledge transfer and breeding

progress in *F. ananassa*. Due to the molecular and regulatory complexity of cold acclimation and freezing tolerance mechanisms in plants, multi-parallel gas chromatography/ time-of-flight-mass spectrometry (GC/TOF-MS)-based metabolite profiling was applied in three *F. vesca* genotypes with contrasting cold tolerance ability. The study was aimed at the (1) Identification of metabolic short- and long-term responses under cold acclimation, (2) Characterization of differences between leaf and root organs, and (3) Mapping of cold-responsive pathways and central metabolism in different *F. vesca* genotypes.

2. Results

2.1. Fragaria genotypes differ in freezing tolerance

Woodland strawberry (*Fragaria vesca* L.) is widely distributed throughout the Northern hemisphere from sub-tropical to subarctic zones, but mostly adapted to boreal forests and found at altitudinal levels up to 3,000 m.a.s.l. Three lines (‘Ås’, ‘Tingvoll’ and ‘Alta’) from a Norwegian collection of *F. vesca* were chosen for multi-parallel GC/TOF-MS analysis, based upon their contrasting geographical origin and freezing sensitivity (Fig. 1): ‘Ås’ from South Norway (mean temperature October to March: -0.8 °C; average day length May to August: 17.3 h), ‘Tingvoll’ from the coastal area of Mid Norway (mean temperature October to March: 1.8 °C; average day length May to August: 18.1 h), and ‘Alta’ from North Norway (mean temperature October to March: -5.1 °C; average day length May to August: 21.7 h). Although all *F. vesca* genotypes were considered to be frost-tolerant, differences in acclimation strategies toward cold might be expected due to contrasting environmental conditions at their original habitats. Freezing tests with cold-acclimated and detached leaves exposed to different sub-zero temperatures, revealed that genotype ‘Alta’ showed significantly less freezing damage and better adaption toward freezing conditions at -10 °C and -15 °C (Fig. 1). ‘Alta’ demonstrated also significantly less ion leakage compared to ‘Ås’

and/or ‘Tingvoll’ at all temperatures except 0 °C. In general, leaf tissue damage and ion leakage drastically increased at -10 °C and -15 °C in all *F. vesca* lines.

2.2. Time-dependent metabolic regulation in leaves and roots during cold acclimation

Short-term (within 24 h)- and long-term (after several days) metabolic cold responses in different plant organs either surviving the winter period (roots) or those dying (leaf) were assessed. Leaf and root samples of *F. vesca* genotypes ‘Ås’, ‘Tingvoll’, and ‘Alta’, acclimated at 2 °C for 0, 3, 24, 72, and 240 h, were subjected to GC/TOF-MS-based metabolite profiling. A total of 160 compounds comprising both structurally annotated primary metabolites (129 compounds) and as yet non-identified mass spectral tags, i.e. metabolic components recognized by mass spectrum and retention index, were detected (Supplementary Table 1).

Average values of metabolites of leaf and root organs from genotypes ‘Ås’, ‘Tingvoll’, and ‘Alta’ were calculated for the 3, 24, 72, and 240 h time points. The number of unique and common increased or decreased metabolite levels (leaf or root), shared by single or groups of genotypes, are presented in Venn diagrams in Figure 2. Only those metabolites showing a ≥50% concentration increase or a ≤50% decrease were included. The total number of differentially regulated metabolites was generally higher at later time points of the cold acclimation period (72 and 240 h). Common metabolites of late responses after 72 and 240 h in leaves comprised raffinose and galactinol (also at 24 h), amino acids and amines (N-acetylserine, aspartic acid, putrescine), hexoses (fructose, glucose, sorbose), and fumaric acid. In root tissue, galactinol (also at 24 h), raffinose, and inositol conj. 4 (A300001) were commonly induced after 72 and 240 h of cold acclimation, while pentoses (mannose, lyxose) showed increases after 24 and 72 h. The amino acids norvaline (72 h) and proline (240 h) were the only leaf metabolites found to be clearly decreased in all three *F. vesca* lines at later

time points, while *myo*-inositol and 4-aminobutyric acid showed generally reduced levels in root tissue after 240 h.

The total number of metabolites with increased levels was obviously higher in the roots (89, 78, 102, and 98 at respective points) in comparison to positively affected leaf metabolites (43, 31, 81, and 64 at respective time points). On the other hand, the total number of compounds with reduced concentration levels in leaves and roots was generally lower compared to the increases. With the exception of metabolite decreases in leaf tissue, the genotype 'Alta' was overall less affected than 'Ås' and 'Tingvoll'. Genotypic relationships could be deduced from the quantity of shared compounds, which usually was higher at later time points. The number of common metabolites between pairs of genotypes was noticeably lower in 'Ås':'Tingvoll'. Moreover, the majority of shared compounds in the pairs 'Ås':'Alta' and 'Tingvoll':'Alta' was typically higher for those metabolites showing enhanced levels in leaves and roots.

Based on the total of 160 metabolites and metabolite tags, principal component analysis and definition of 5 PCs (Supplementary Table 1) was applied prior to independent component analysis. 3D-ICA diagrams depict organ-specific and genotypic differences along the time-scale of cold acclimation (Fig. 3). Changes from t0 to the 3 h and 24 h time points post treatment seemed to be less pronounced in leaves compared to the roots. However, the early time point 3 h in 'Ås' root samples strongly separated from t0, and thus emphasize the effect of simultaneous up- and down-regulation of metabolites as indicated by Venn diagrams (Fig. 2). Root metabolites of genotypes 'Tingvoll' and 'Alta' showed relatively low separation from the initial time point, while late responses (72 and 240 h) of all *F. vesca* lines could be clearly separated from t0 and 3 h. In general, genotypes definitely discriminated from each other in 3D ICA, and thus demonstrated the existence of distinct metabolic phenotypes. These results are further underscored by 2D matrix plots using both IC1, IC2,

IC3, and IC4 (Supplementary Fig. 1). Metabolites being predominantly responsible for the separation of time points and genotypes are depicted in ICA loading diagrams based on IC1 and IC2 (Fig. 4). Distinct sugars (fructose, glucose, sorbose, raffinose), amino acids (β-alanine, aspartic acid, N-acetyl-serine, 2-aminoadipic acid) and polyols (galactinol, inositol conj. 3) indicated a strong contribution to discrimination patterns of leaf samples as shown in Figure 3. In roots, amino structures (2-aminobutyric acid, 2-aminoadipic acid, histidine), mannose, tartaric acid and several unidentified metabolites were found to be highly discriminatory metabolites.

Findings from ICA visualization (Fig. 3) are resembled by hierarchical cluster (HCL) analysis of the total set of compounds (Fig. 5). Metabolite pools established 2 distinct leaf and root clusters, thus underscoring variations in metabolic regulation between different plant organs upon cold treatment. Leaf samples from early time points of cold acclimation (t0, 3, and 24 h) clearly separated from later time points (72 and 240 h). With the exception of 'Ås' (24 h), root samples formed sub-clusters for the initial time points (t0 and 3 h) and later metabolic responses after 24 h of cold treatment, and hence showed clear similarities to results visualized by ICA diagrams (Fig. 3).

2.3. Metabolic pathways are unequally affected in different F. vesca genotypes

Based on GC/TOF-MS profiling of *F. vesca* lines 'Ås', 'Tingvoll', and 'Alta', pathway maps were generated in order to visualize metabolic shifts in a functional context over time, and to identify those compounds differing and/or being equally regulated in plant organs and genotypes (Figs. 6 to 8). Figure 6 depicts biosynthetic routes of central metabolism including glycolysis/gluconeogenesis, TCA cycle, and amino acid biosynthesis. Leaf and root levels of compounds functioning as compatible solutes such as monosaccharides, phosphorylated intermediates, amino acids and amines, were partly maintained at higher levels throughout the

cold acclimation period or displayed transient increases. Leaf concentrations of fructose, glucose, N-acetyl-serine, aspartic acid, tryptophan, putrescine, fumaric and malic acid were enhanced particularly toward later time points (all genotypes). On the other hand, a rise in levels of the same compounds in roots (also including glutamine and tyrosine) was partly retained, or metabolites showed transient increases. Moreover, distinct metabolites such as sucrose, proline particularly in leaves, and homoserine, alanine, and the GABA shunt (4-aminobutyric acid, succinic acid) showed notable decreases over time. Furthermore, amino acid biosynthesis was differentially affected in the studied genotypes. Structures derived from pyruvate such as isoleucine, leucine, and valine, phenylalanine from the shikimate pathway, serine from 3PGA, but also TCA-derived amino acids such as proline, aspartic acid, threonine and methionine clearly showed decreased levels in root tissue of *F. vesca* line 'Ås' (Fig. 6). Arginine and ornithine abundance was notably increased in genotype 'Tingvoll', and vice versa, cysteine (root) and histidine levels (leaf) were decreased.

The ascorbate pathway was strongly affected (Fig. 7) resulting in raised levels of several oxidized sugars (galacturonic and ascorbic acid) towards later time points of cold acclimation, or appeared as transient increases (e.g. threonic acid in roots). In addition, monosaccharides and related phosphorylated structures such as galactose (regardless of differentiation between D- and L-isomers), mannose (only root), fructose-6-phosphate, and mannose-6-phosphate, and the sugar lactone galactonic acid-1,4-lactone (only leaf) displayed generally enhanced levels. The raffinose pathway, which is involved in cold acclimation and upon chilling stress in plants, was noticeably regulated in a time-dependent manner in both leaves and roots of all genotypes (Fig. 8). Galactinol, precursor of the trisaccharide raffinose, showed transient peaks in leaves at the 72 h time point, while compound levels in roots were increasing toward the latest time point at 240 h. Concentration levels of raffinose were generally highest in both

plant organs toward the end of the cold acclimation period. On the other hand, the abundance of the disaccharide trehalose was only sligtly affected, showing partly transient increases.

3. Discussion

Changes in the plant environment from optimal to low temperatures lead to the induction of multiple regulatory mechanisms and homeostatic control systems and thereby maintain essential biological functions. Generally, the plant system as a whole is affected both under chilling conditions and cold acclimation. The latter process constitutes an adaptive and necessary survival strategy in the life cycle of biennial and perennial species, and plays a natural role in cold hardening of significant agricultural crops under temperate and boreal climates. In this context, the diploid woodland strawberry (*Fragaria vesca*) has been adopted as one of the most important Rosaceae model species within *Fragaria* and closely related genera. cDNA libraries have been generated from cold-, heat- and salt-stressed *F. vesca* (Shulaev et al., 2008). Ploidy might play a role in cold-adaptive processes in diploid and octoploid *Fragaria* species due to potentially increased cell size with ploidy level (Walker et al., 2008), differential expression of cold-induced genes (Limin et al., 1995), and altered photosynthetic characteristics (Chandra and Dubey, 2009). However, a high degree of genome colinearity between diploid and octoploid *Fragaria* sp. exists (Rousseau-Gueutin et al., 2008), and plant biological processes including metabolic regulation under low temperature conditions were likely to be highly similar in both species.

In our approach, we chose to focus on initial short- (within 24 h) and long-term metabolic cold responses (after several days) in leaf and root organs of the model *F. vesca*. The raffinose pathway in particular establishes a highly conserved cold-inducible mechanism in plants (Nishizawa et al., 2008), and was expected to be differentially regulated in genotypes originating from contrasting environments. One might expect unequal effects of cold

acclimation temperatures on whole plants when comparing leaves exposed to air vs. roots growing in watered soil substrate with apparently higher thermal capacity and conductivity. However, sponataneous initial metabolic responses in roots already after 3 h of cold acclimation as displayed in Figures 6 to 8, generally disproved this point at issue.

3.1. Short- and long-term regulation of soluble sugars and carbohydrate metabolism

According to the categorization by Shinozaki and Yamaguchi-Shinozaki (2006), homeostatic processes in plants upon low temperatures comprise the induction of regulatory and functional proteins, the latter being involved in the biosynthesis of compatible solutes and osmoprotectants (Kurz, 2008), membrane transport mechanisms (Lundmark et al., 2006), detoxification and macromolecule protection (Ruelland et al., 2009). Due to the nature of the adopted profiling approach, metabolic changes are basically discussed with regard to primary metabolism. Important sugars found in our study, which concentration range was drastically altered (>2.5-fold), comprised pentoses (xylose and lyxose) and hexoses (fructose, glucose, galactose, and mannose) together with their corresponding hexose-phosphates. Our results thus confirm earlier findings in the model *Arabidopsis* (Kaplan et al., 2004; Usadel et al., 2008), cereal crops (Livingstone et al., 2006), and legumes (Hekneby et al., 2006). However, a cold period of 10 days at above-zero temperatures as applied in our study or even shorter in others investigations, might only serve to describe the earlier adaptive responses, and will not be sufficient to indicate metabolic shifts occuring in nature after initiation of cold acclimation following long-lasting exposure to sub-zero temperatures. Long-term studies in oat (*Avena sativa* L.) and rye (*Secale cereale* L.) (Livingstone et al., 2006; 30 days), and close relatives of the Rosaceae family, raspberry (*Rubus idaeus* L.) (Palonen et al., 2000; 10 weeks) and peach (*Prunus persica* (L.) Batsch) (Yooyongwech et al., 2009; 7 months) clearly

demonstrated transient increases of soluble sugars, which were strongly genotype-dependent as discussed later in the context of raffinose pathway regulation.

An expected initial strong up-regulation of disaccharides such as the osmolyte trehalose (Cook et al., 2004) was not confirmed in our experiments, and only minor transient increases in leaves and roots of genotype 'Ås' were displayed. Moreover, the decrease in sucrose abundance in leaf and root samples (except 'Tingvoll'), in combination with highly increased phosphorylated sugars (Figs. 6 to 8), corroborate starch breakdown patterns described by Kaplan et al., (2007), leading to rapidly increasing hexose-phosphate pools and finally, fructose and glucose levels. However, an initial increase of sucrose levels as described by these authors and in other reports (reviewed by Ruelland et al., 2009), was absent in our experiment, and emphasize a consecutive starch and sucrose breakdown in *F. vesca* during cold acclimation. Seen in a different context, the long-term cryopreservation of *Fragaria* meristems (Caswell and Kartha, 2009), glucose and sucrose have shown their suitability in vitrification solutions (Vysotskaya et al., 1999; Suzuki et al., 2008) to prevent freezing damage of plant tissue. In our study, hexoses were found to be the *in planta*-favoured soluble sugars highly up-regulated in leaf and root tissues during the 10-days cold period. Sucrose has been shown to be transiently up-regulated after 48 h (Kaplan et al., 2007), and is known to be involved in the regulation of cold acclimation in *Arabidopsis* during diurnal dark periods (Rekarte-Cowie et al., 2008). The non-CBF regulation of sucrose synthases underlies clock-gene regulation, or even other mechanisms as shown in roots (Hekneby et al., 2006), which was reflected by transitional changes of soluble sugars in *F. vesca* in non-photosynthesizing roots compared to the leaves.

3.2. Cold-induced metabolic shifts in roots are retained or transient comparing to leaves and involve different metabolites

Metabolic responses in roots regarding soluble sugars (Figs. 6 and 8) seemed to be decelerated probably due to the potential higher heat storage capacity of the soil substrate used in the plant experiment. However, this did not explain instantaneous and strongly induced metabolite changes in roots already occuring at 3 h after onset of cold when compared to the leaves. Such early induced metabolites included both amino acids (Fig. 6) and ascorbate pathway-related compounds (Fig. 7), which were clearly differently affected in roots. In accordance with earlier low-temperature experiments in the model *Arabidopsis* (Cook et al., 2004; Kaplan et al., 2004) levels of distinct amino acids and polyamines were clearly transiently increased in at least one or several *F. vesca* genotypes (Fig. 6), without obvious preference of biosynthetic route or side chain polarity. Tissue concentrations of proline, a potential osmolyte which is thought to be initially up-regulated upon cold treatment (Kaplan et al., 2007), or even kept at elevated levels for weeks and months (Bandurska et al., 2009), showed minor but anyhow stronger induction in roots of genotypes 'Tingvoll' and 'Alta'. However, its functioning in plant cold stress and freezing tolerance are still not clearly defined as pointed out by Korn et al. (2008). Considering the detected levels in our study, a minor role of this amino acid in low-temperature acclimation in *F. vesca* is suggested. Furthermore, glutamine, induced after 72 h of cold acclimation (Usadel et al., 2008), also showed enhanced levels in *Fragaria* samples being more pronounced in the roots.

The aromatic amino acid tyrosine, the precursors arginine and ornithine and its product, the diamine putrescine, were obviously differently regulated in root organs and between genotypes. The latter metabolite has been reported to serve in several biological functions related to cold stress as compatible solute (Kaplan et al., 2004), modulation of antioxidant systems (Zhang et al., 2009), and signaling and the control of ABA levels (Cuevas et al.,

2009). Since putrescine concentrations were drastically enhanced in leaves, the transient increase in 'Alta' or even decrease in 'Ås' in root tissue indicated other functioning roles. The aspartate – β-alanine route displayed co-ordinately increased levels of both metabolites in leaves and transiently in roots in 'Tingvoll' and 'Alta' genotypes (Fig. 6; Supplementary Table 1), which is in accordance with previous reports in *Arabidopsis* (Cook et al., 2004; Allan et al., 2008).

The branch of amino acid biosynthesis leading from pyruvate to the amino acids isoleucine, leucine and valine, has had little attention in terms of cold-regulated metabolism. Reasons might be their relatively low molecular weight and potential in osmolytic functioning due to their nonpolar side-chains and lower ability to retain water. Interestingly, their metabolic shifts during the 10-days cold period followed strict genotype-dependent patterns of up- or down-regulation in leaf and root tissues (Fig. 6). These findings here indicated a central metabolic regulator or switch, probably the functioning of the branched-chain aminotransferase 4 (BCAT4) both acting in biosynthesis and degradation of these amino acids. Furthermore, organ-dependent differences were clearly displayed in the acetyl-serine – cysteine route (Fig. 6). Instantaneous metabolite increases in roots could be detected in genotypes 'Ås' and 'Alta', and thus, point towards unequally regulated gene expression, metabolic demand and acclimation strategies in different plant organs upon cold treatment.

3.3. Differences in raffinose pathway regulation reveal potential metabolic phenotypes

The raffinose pathway (Fig. 8; Supplementary Table 1) was clearly affected resulting in raised levels of galactinol and raffinose in leaf and root tissues. Cold-induced changes in metabolite pools of soluble sugars in leaves have been described in previous reports (Mattana et al., 2005; Yano et al., 2005; Korn et al., 2008), and the significance of both phosphorylated sugars (Kaplan et al., 2004; Gray and Heath, 2005) and the raffinose pathway (Cook et al.,

2004) has been stressed. Similar metabolic shifts of soluble sugars have also been reported in below-ground tissues (Equiza et al., 2001; Bourion et al., 2003; Hekneby et al., 2006). Recently, galactinol and raffinose have been shown to be involved in plant protection upon oxidative stress (Nishizawa et al., 2008). Moreover, drastically increased levels of hexose phosphates as found in our data, are associated with a targeted biosynthesis of compatible solutes, since these compounds exert a higher ROS scavenging capacity (F6P > fructose) compared to non-phosphorylated sugars as recently reported (Spasojević et al., 2009).

To the best of our knowledge, this is the first approach toward the simultaneous assessment of cold responses in leaf and root tissue using multiparallel metabolite profiling. Only few studies have so far highlighted organ-dependent differences as in the study on water stress in perennial ryegrass (*Lolium perenne* L.) (Foito et al., 2009) and differentially affected amino acid and carbohydrate metabolism in leaf and root tissues of salt-stressed *Arabidopsis* (Renault et al., 2010), *Lotus japonicus* (Regel) K. Larsen (Sanchez et al., 2008b), and rice (*Oryza sativa* L.) (Narsai et al., 2010). Global transcriptional analysis (ESTs) in the close relative species, apple (*Malus* × *domestica* 'Royal Gala') (Wisniewski et al., 2008), exposed to water deficiency, revealed high dissimilarity between the number of differentially expressed genes in various plant organs (leaf > root). These findings are supported by tissue-specific transcriptional profiling in *Arabidopsis* and *Oryza sativa* (Narsai et al., 2010), which showed that gene expression in roots at the functional level seems to be more conserved compared to leaves, flowers and seeds.

Apart from organ differences, a possible impact of plant origin on modulation of the raffinose pathway and the potential development of metabolic phenotypes might be considered based on our study in the model *F. vesca*. The factor winter temperature and probably also summer temperature at the plant accession sites might explain some of the metabolic differences of genotype 'Tingvoll' compared to (Fig. 8 and Supplementary

Table 1). Moreover, also the parameter latitude, implying highly varying day length conditions during the growth season with 24 h daylight durign summer time for the most Northern genotype ‘Alta’ might have an impact on metabolic regulation under cold acclimation. *F. vesca* genotypes ‘Ås’ and ‘Alta’ had earlier been reported to behave quite differently in terms of genetically-determined flowering control in overwintering studies in the field (Heide and Sønsteby, 2007), with ‘Alta’ being the latest (and most northern) of all investigated clones. Moreover, ‘Alta’ did not produce inflorescences at all under any artificially applied combination of light (short- and long-day) and temperature conditions (9, 15, and 21 °C) (Heide and Sønsteby, 2007). Thus, the effect of environmental parameters such as temperature, light and day length on genotypic variation and the potential development of metabolic phenotypes needs to be further addressed in future cold acclimation studies with the model *F. vesca*.

4. Conclusions

Metabolite pools in the model *F. vesca* were highly perturbated during cold acclimation, and concentration changes of compatible solutes (sugars, amino acids, amines) occured in a time-dependent and coordinate fashion. The osmolyte proline was shown to play a minor role, whereas our study clearly emphasized significant changes of amines (putrescine), aspartic acid, N-acetyl-serine, also suggesting possible roles of branched-chain amino acids (leucine, isoleucine, and valine). Single metabolites from the raffinose pathway, amino acids, amines, and oxidized sugars might be considered as candidates for potential biomarkers in the further validation of *F. vesca* crosses and *F. x ananassa* breeding lines. In general, phenotypic variation has to be considered when interpreting results of cold-induced metabolic responses in plants. Annual species such as *Arabidopsis thaliana* have developed mechanisms of cold acclimation and metabolic regulation which apparently differ compared to biennial or

perennial species. Since most studies with *Arabidopsis* were carried out at the vegetative stage, gained results indicate the plants' needs to keep up with the negative effect of low temperature stress in leaves, in order to potentially provide enough photosynthetic assimilates for flowering, seed set and a successful reproduction. Perennials such as the diploid *F. vesca* undergo cold acclimation as a natural and necessary process, which the plants are genetically and phenotypically adapted to. Thus, they have established partially different strategies to prepare for long-term freezing temperatures, also involving deacclimation mechanisms (dehardening) after winter. Our study has gained new insights into cold acclimation processes of plants by broadening our understanding of single biological processes at the tissue and organ level, and has opened up the use of a new plant model toward breeding and crop research.

5. Materials and Methods

5.1. Plant experiment and cold acclimation

Eight weeks old runner-propagated *Fragaria vesca* L. plants from 3 wild accessions of Norwegian populations, designated as 'Ås' (59°40'N 10°45'E) from South Norway (AAS), 'Tingvoll' (62°51'N 08°18'E) from a coastal area in Mid Norway (TGV), and 'Alta' (69°55'N 23°0'E) from North Norway (ALT), were investigated. Voucher specimens are held in a living collection of *F. vesca* accessions at Bioforsk Grassland and Landscape Division, Kvithamar, Stjørdal, Norway. Plants were grown on fertilized soil (P-Jord; Emmaljunga Torvmull AB, Sweden) in 18 cell plug trays in a greenhouse at 18 ± 2 °C under natural light and long-day conditions. Then, plants were short-day adapted for 1 week at 12 °C under artificial light (fluorescent tubes, ~90 µmol m^{-2} sec^{-1}) in a conditioning room prior to transfer to a cold room at 2 °C under artificial light (fluorescent tubes, ~90 µmol m^{-2} sec^{-1}) and relative humidity at average of 80%. Plant sampling was carried out at the following time

points: 0 (t0), 3, 24, 72, and 240 h after onset of the cold treatment. Control samples (t0) were harvested prior to the transfer to the cold room. Plant material from leaves and roots of three plants per replicate (n=5), genotype and time point was flash-frozen in liquid N_2 and stored at -80 °C before sample processing and subsequent GC/TOF-MS analysis.

5.2. Evaluation of freezing tolerance

Freezing tests with detached leaves of genotypes *F. vesca* 'Ås', 'Tingvoll' and 'Alta' were based on plant material harvested after 10 days of cold acclimation at 2 °C (see above). Tests were carried out to determine tissue damage and electrolyte leakage, following a temperature-modified protocol described by Houde et al. (2004). The following sub-zero temperatures were applied: -1, -5, -10, -15, and -20 °C.

5.3. Sample extraction and metabolite profiling

Homogenized leaf and root samples (120 mg f.w.) of genotypes 'Ås', 'Tingvoll' and 'Alta' were transferred into round-bottomed 1.5 mL microtubes. 360 µL of pre-cooled methanol was added containing ribitol as internal standard for the correction of volume errors. Samples were extracted at 70 °C for 15 min. After cooling to room temperature, 200 µl $CHCl_3$ was added to the tubes, which were then agitated at 37 °C for 5 min. Finally, 400 µl H_2O was added in order to induce liquid phase separation. Samples were vortexed prior to centrifugation at 13,000 rpm for 5 min. 80 µl of the upper polar phase containing the primary metabolite fraction were transferred into a 1.5 mL tapered microtube, dried in a SpeedVac vacuum concentrator overnight without heating, and stored dry at -80 °C. Chemical derivatization, i.e. methoxyamination and trimethylsilylation, and subsequent gas chromatography/ time-of-flight-mass spectrometry-based metabolite profiling (GC/TOF-MS) was as described by Sanchez et al. (2008a).

5.4. Metabolite data processing and analysis

Chromatographic data sets from GC/TOF-MS were aligned and baseline corrected using the MetAlign software (Lommen, 2009). TagFinder software v.4.0 (Luedemann et al., 2008) was used for subsequent non-targeted, multi-parallel chromatography data processing, data matrix generation and metabolite identification, using authenticated reference spectra from the *Golm Metabolome Database* (Kopka et al., 2005; Hummel et al., 2010). Numerical analyses were based on peak height values (response) which were corrected for fresh weight variation using the internal standard ribitol (normalized response).

Prior to statistical assessment, log2(n)-transformed response ratios were calculated for each of the 160 identified metabolites and non-identified mass spectral tags of leaf and root metabolite profiles (GC/TOF-MS) (Supplementary Table 1). Venn diagrams were drawn with Micrososft® Word, and only those metabolites showing a ≥50% increase or ≤50% decrease in concentration, compared to the initial time point t0 of individual genotypes, were considered. Log ratios calculated on the basis of the median of t0 from individual genotypes, were used for independent component analysis (ICA) according to Scholz et al., (2004). Metabolic pathway maps were drawn based on the x-fold change of metabolite concentration changes compared to the t0 time point of individual genotypes (leaf or root). Hierarchical clustering (HCL) using the distance measure, Pearson's correlation, and complete linkage was performed with the MultiExperiment Viewer software v.4.4 (Saeed et al., 2003). Log2(n) ratio values for HCL were re-calculated, and based on the median metabolite concentration from t0 time points of all genotypes (leaf and root) in order to emphasize genotypic variation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:

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FIGURE LEGENDS

Figure 1. Freezing tests with *Fragaria vesca.* Single detached leaves of 3 genotypes (AAS: Ås; TGV: Tingvoll; ALT: Alta) were exposed to different freezing temperatures after cold-acclimation (240 h). The upper graph (**A**) shows tissue damage based on visual scores (1: <10% damaged; 2: 10-25% damaged; 3: 25-50% damaged; 4: 50-75% damaged; 5: >75% damaged), the lower graph (**B**) represents data from ion leakage measurements (in %) of the same samples. Different letters indicate significant differences among means at different sub-zero temperatures ($p \leq 0.05$).

Figure 2. Venn diagrams showing the co-ordinate up and down-regulation of metabolism. Diagrams are based on a set of 160 identified metabolites and non-identified mass spectral tags of cold-acclimation time points 3, 24, 72, and 240 h. Similarities in number of increased (UP) and decreased metabolites (DOWN) in and between the studied *F. vesca* genotypes ('Ås', 'Tingvoll', and 'Alta') are shown. Only those metabolites showing a ≥50%- increase or a ≤50%-decrease were included, based on the detected concentration levels compared to the initial time point (t0, n=5) of each genotype.

Figure 3. Independent Component Analysis (ICA). 3D ICA based on components IC1, IC2, and IC3 from metabolite profiles (160 identified metabolites and non-identified mass spectral tags). Segregation patterns of leaf and root samples of *F. vesca* (AAS: Ås; TGV: Tingvoll; ALT: Alta) harvested at different time points after onset (t0) of cold acclimation (3, 24, 72, 240 h) are shown. IC values were calculated from log2(n) values based on the median of t0 time points of individual genotypes for either leaf or root samples. See also Supplementary Figure 1.

Figure 4. Loading plots of independent components. ICA loading plots of leaf (**A**) and root variables (**B**) are based on the two independent components IC1 and IC2. Metabolites showing highest discrimination are tagged in the graphs (black-filled symbols).

Figure 5. Hierarchical clustering (HCL) of metabolite pools. Hierarchical trees (Pearson correlation) were drawn, based on 160 identified metabolites and non-identified mass spectral tags, from leaves and roots of *F. vesca* (AAS: Ås; TGV: Tingvoll; ALT: Alta) sampled at different time points upon cold acclimation (0, 3, 24, 72, and 240 h). Genotype×time points are depicted in single columns, while distinct metabolites are represented by rows. Heat map visualization of differences in metabolite pools are based on log2(n) ratio amended concentration levels to the median concentration from t0 time points of all genotypes (leaf and root). Bluish colours indicate decreased concentration levels of metabolites, yellow-reddish colours increased metabolite levels (see colour scale).

Figure 6. Functional regulation of glycolysis, citric acid cycle (TCA), and amino acid biosynthesis are depicted as pathway maps. Leaf and root samples of *F. vesca* (AAS: Ås; TGV: Tingvoll; ALT: Alta) were harvested at different time points after cold acclimation (0, 3, 24, 72, and 240 h). Colours in metabolite arrays represent x-fold change of metabolite concentration changes compared to the t0 time point of individual genotypes (leaf or root). The upper row from paired rows of each genotype represents leaf samples, the lower row the roots. Bluish colours indicate decreased concentration levels of metabolites, yellow-reddish colours increased metabolite levels (see colour scale). Abbreviations: 3PGA = 3-phosphoglyceraldehyde; F6P = fructose-6-phosphate; G6P = glucose-6-phosphate; GABA = 4-aminobutyric acid; PEP = phosphoenolpyruvate. See also comprehensive metabolite information in Supplementary Table 1.

Figure 7. Functional regulation of ascorbate metabolism. For further details regarding pathway map, experiments and colour settings, see Figure 6. Abbreviations: F6P = fructose-6-phosphate; G1,4L = galactonic acid-1,4-lactone; G1P = galactose-1-phosphate; M1P = mannose-1-phosphate; M6P = mannose-6-phosphate; UDP = uridine-diphosphate. See also comprehensive metabolite information in Supplementary Table 1.

Figure 8. Functional regulation of raffinose biosynthesis. For further details regarding pathway map, experiments and colour settings, see Figure 6. Abbreviations: F6P = fructose-6-phosphate; G6P = glucose-6-phosphate; myo-I3P = *myo*-inositol-3-phosphate; S6P = sucrose-6-phosphate; T6P = trehalose-6-phosphate; UDP = uridine-diphosphate. See also comprehensive metabolite information in Supplementary Table 1.

Supplementary Table 1. Data from GC/TOF-MS metabolite profiling of *Fragaria vesca* based on 160 identified metabolites and non-identified mass spectral tags. Data of leaf and root tissue (genotypes 'Ås', 'Tingvoll', and 'Alta') comprise log2(n) ratios of metabolite levels in relation to the median concentration from t0 time points of individual genotypes. Further chemical information include metabolite IDs (Golm Metabolome Database, KEGG, and CAS), chemical structure, derivatization products and levels, and retention indices. In addition, statistical data on Principle Components (PC), Independent Components (IC), and *p*-values and *F*-ratios from 2-way ANOVA are presented.

Supplementary Figure 1. Matrix plot of individual components. 2D ICA based on components IC1, IC2, IC3 and IC4 from metabolite profiles (160 identified metabolites and non-identified mass spectral tags) (see also Supplementary Table 1). Segregation patterns of

leaf and root samples of *F. vesca* (AAS: Ås; TGV: Tingvoll; ALT: Alta) harvested at different time points after onset (t0) of cold acclimation (3, 24, 72, 240 h) are shown. IC values were calculated from log2(n) values based on the median of t0 time points of individual genotypes for either leaf or root samples.

Figure 1

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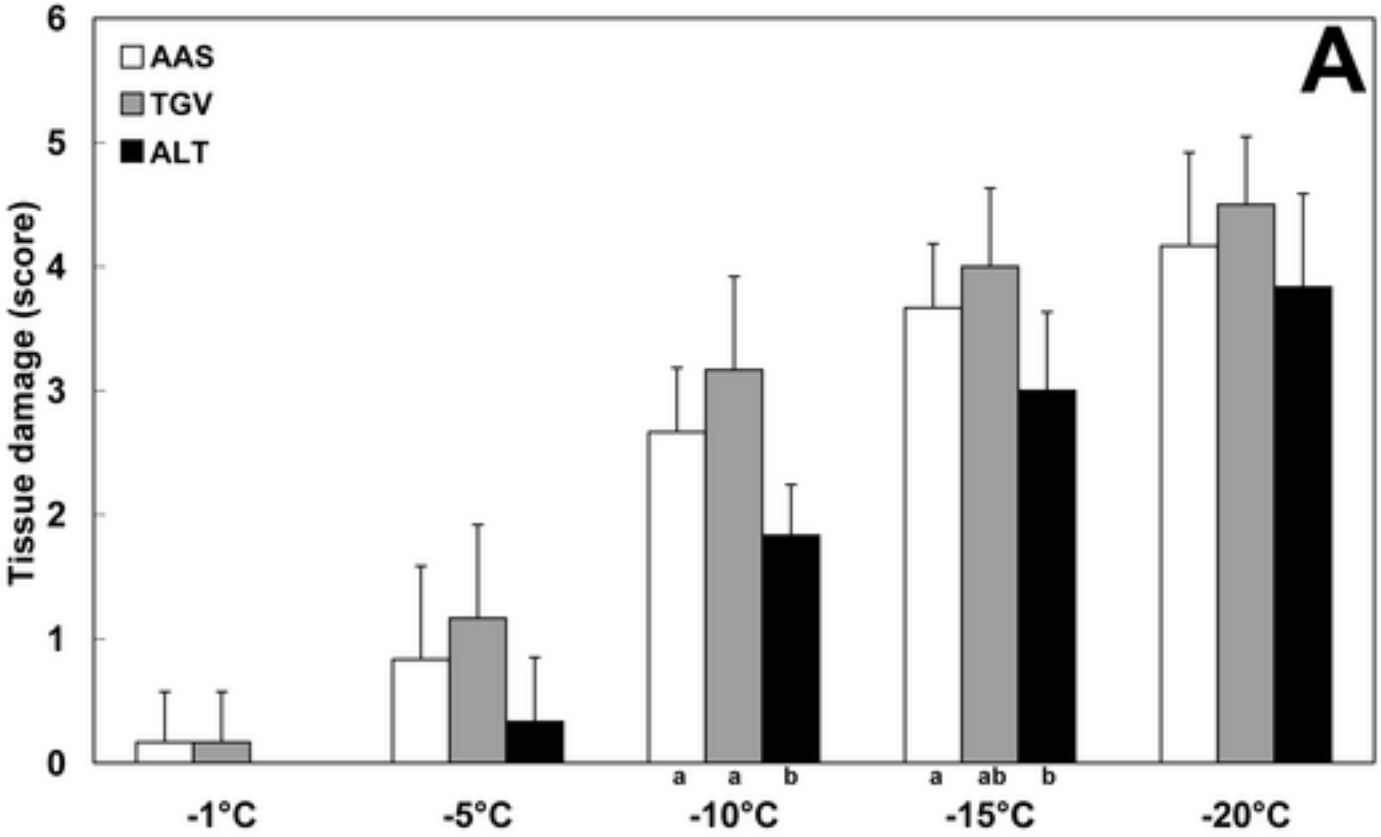

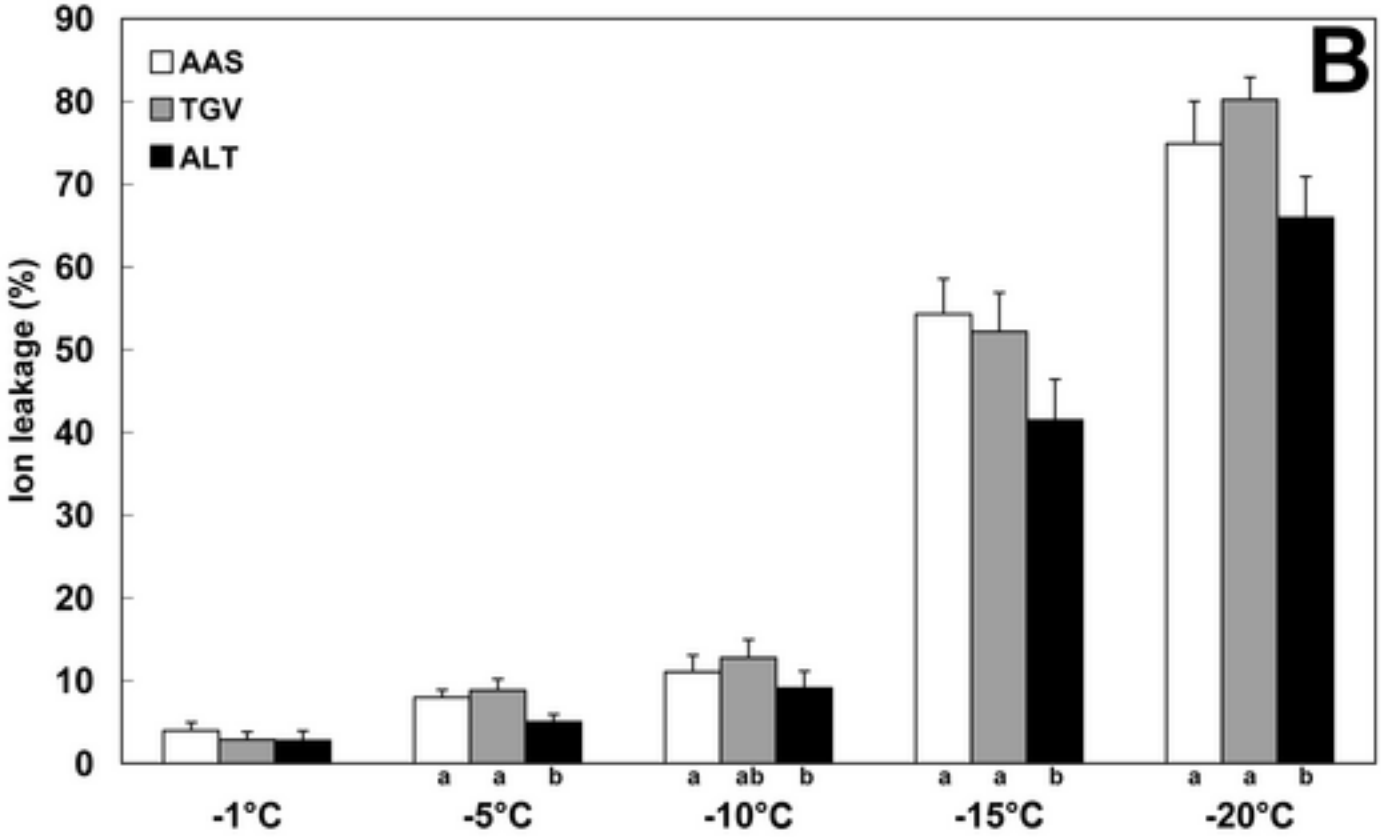

Figure 2

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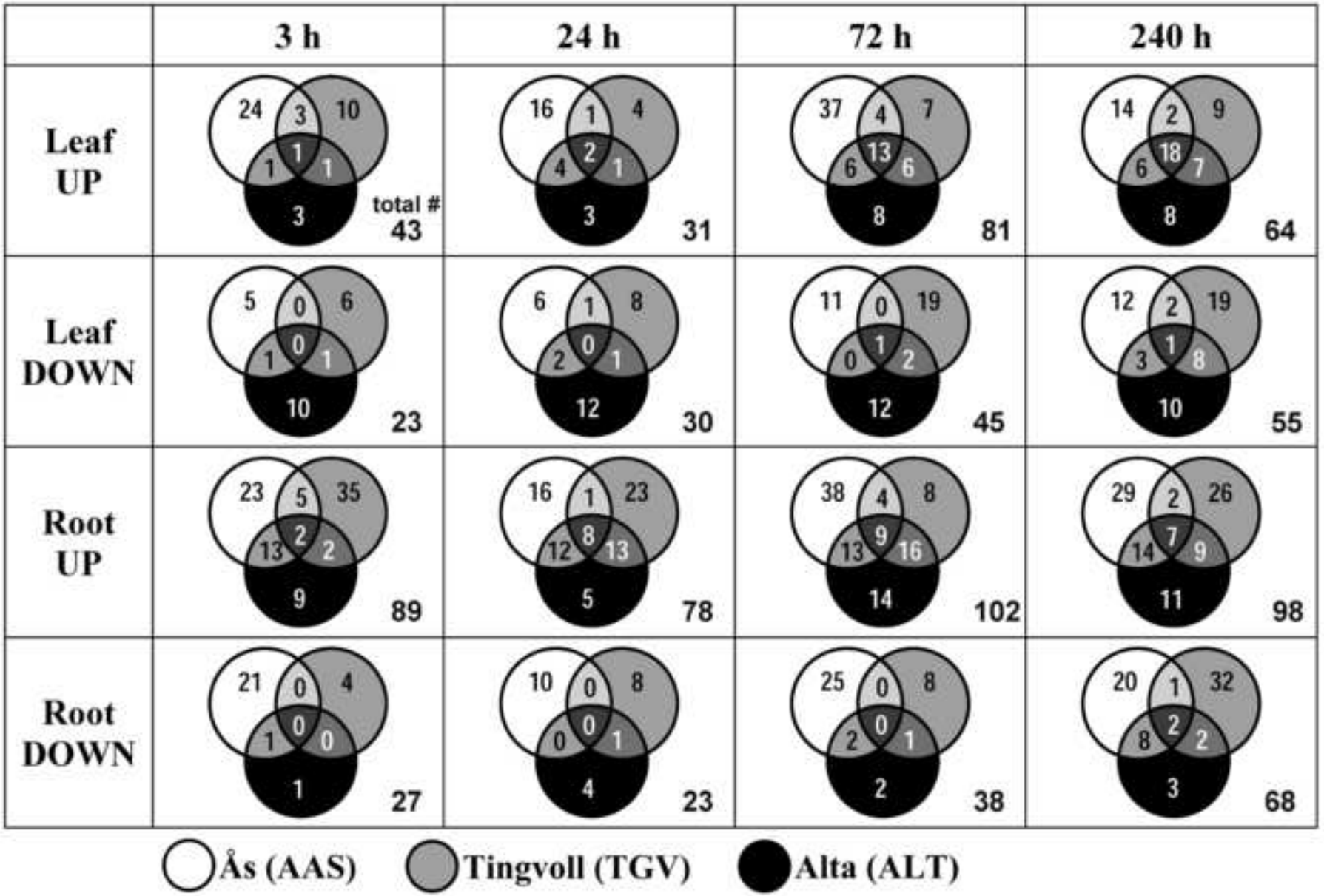

Figure 3

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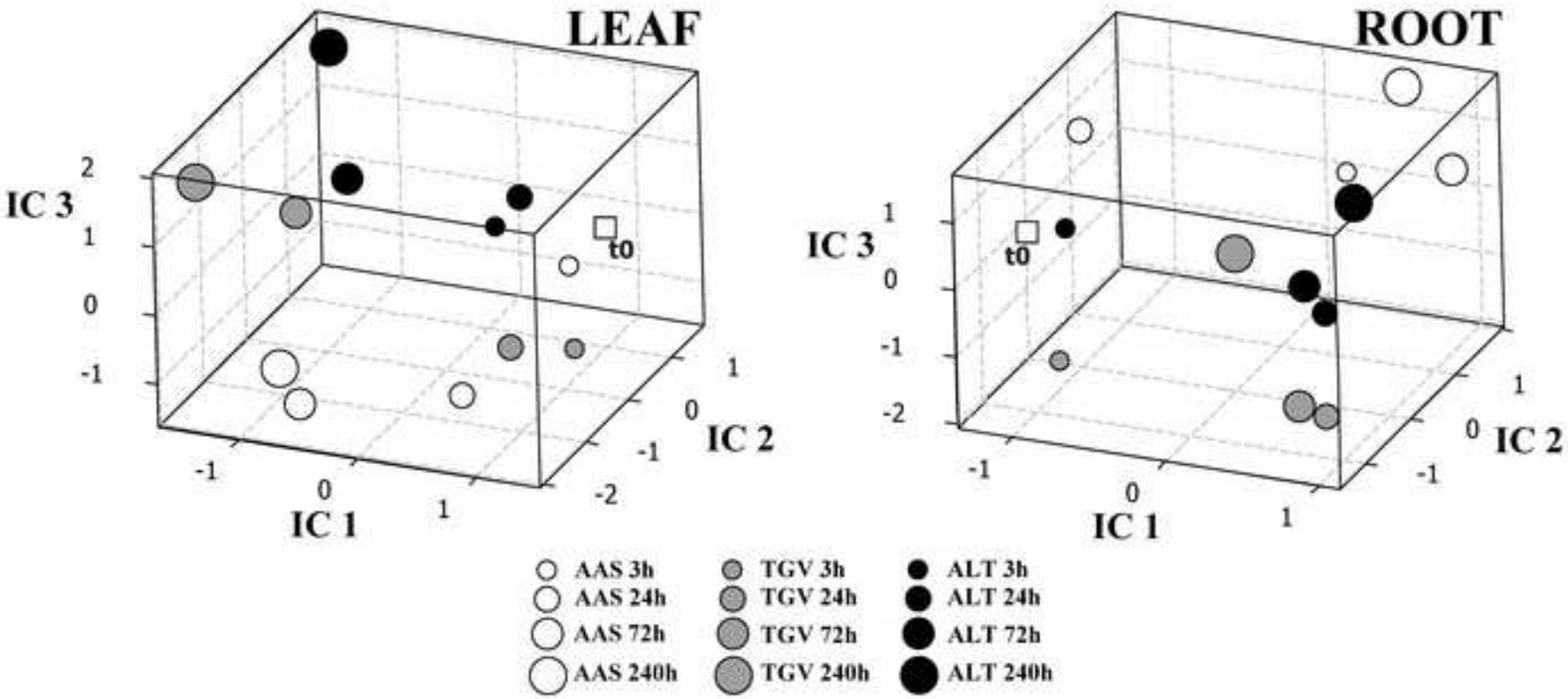

Figure 4

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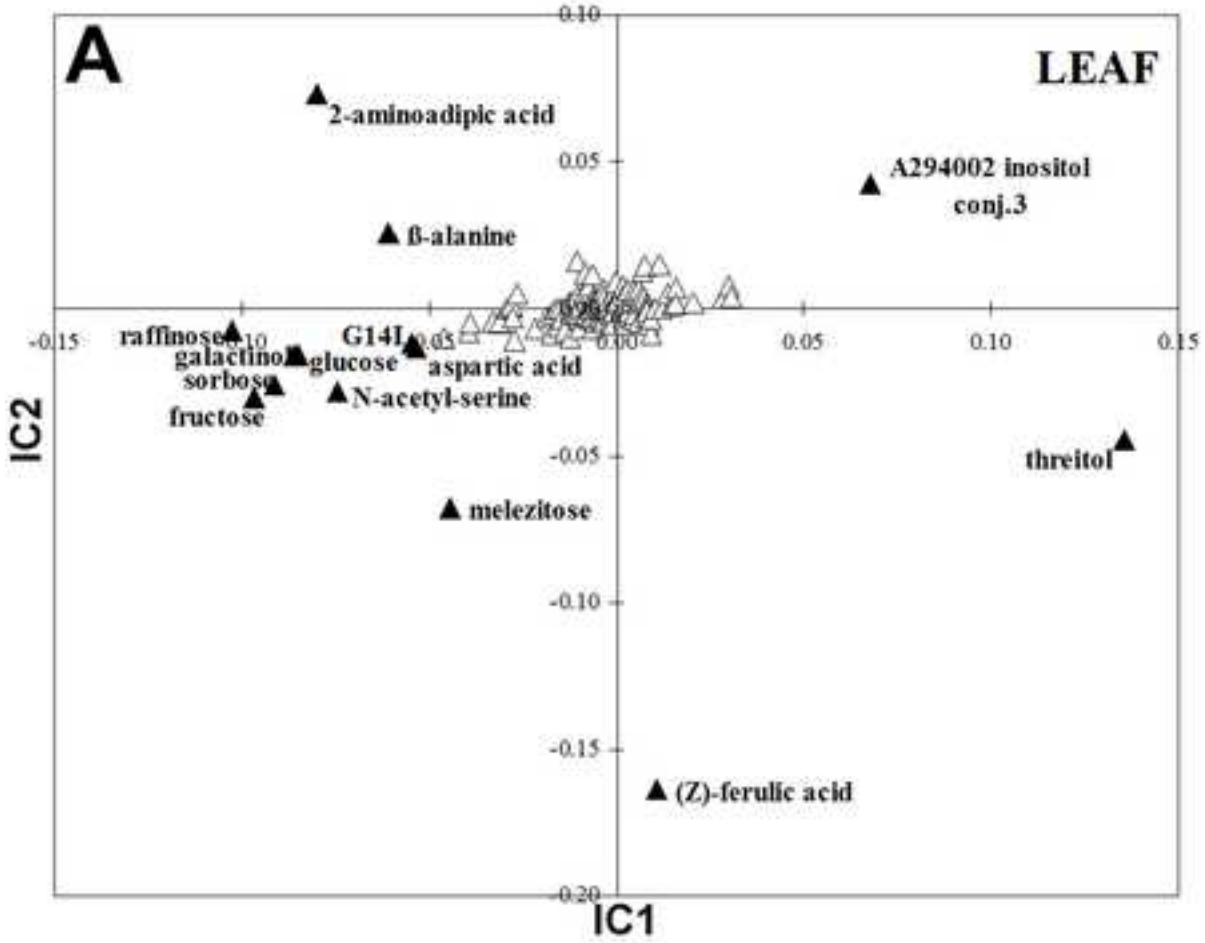

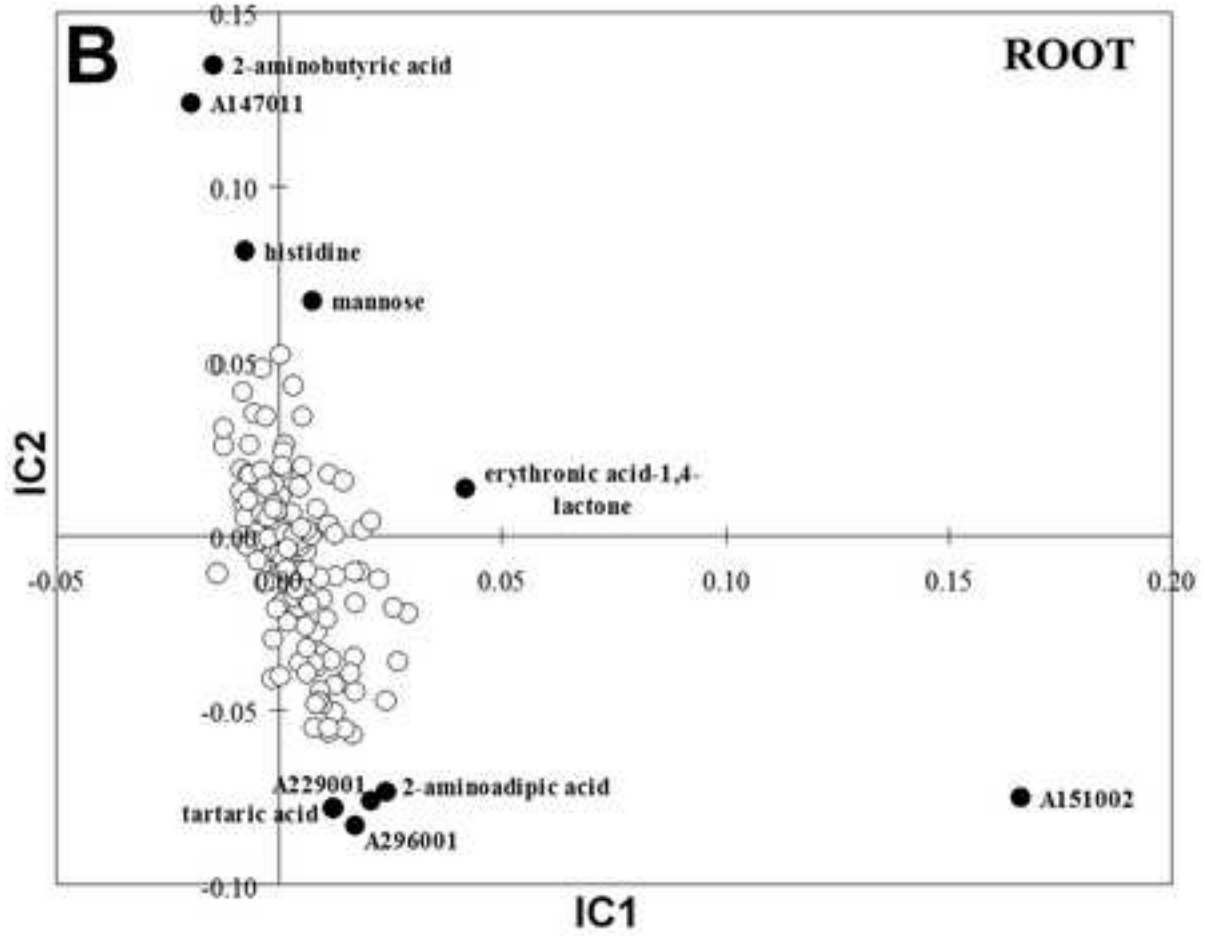

Figure 5

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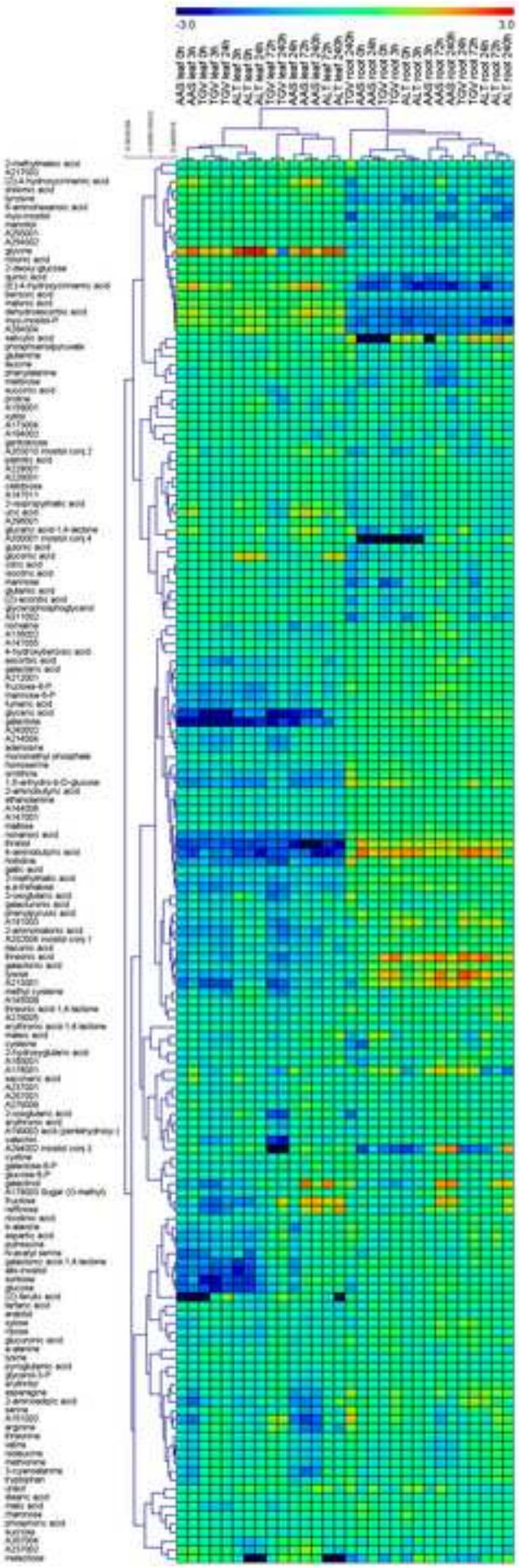

Figure 6

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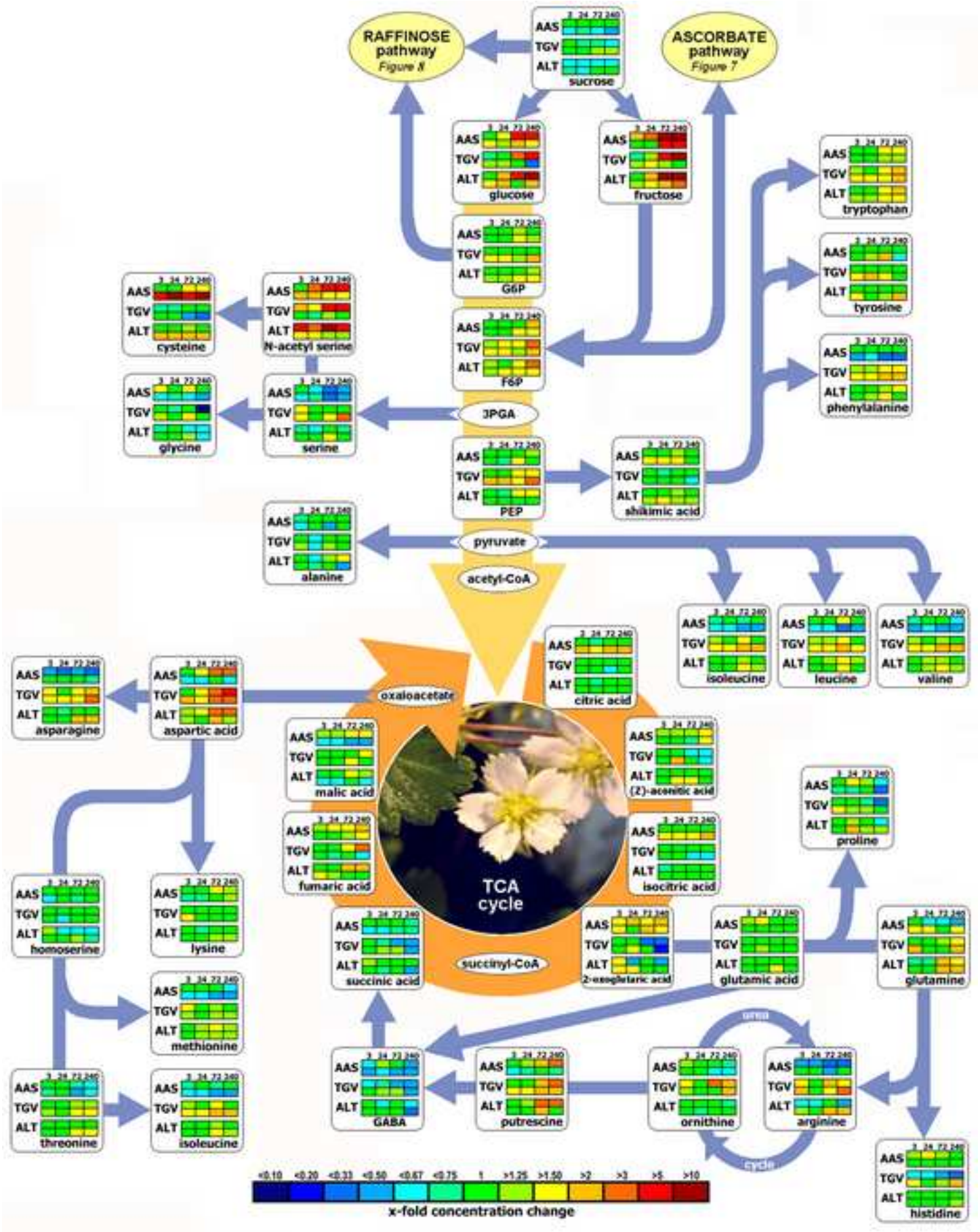

Figure 7

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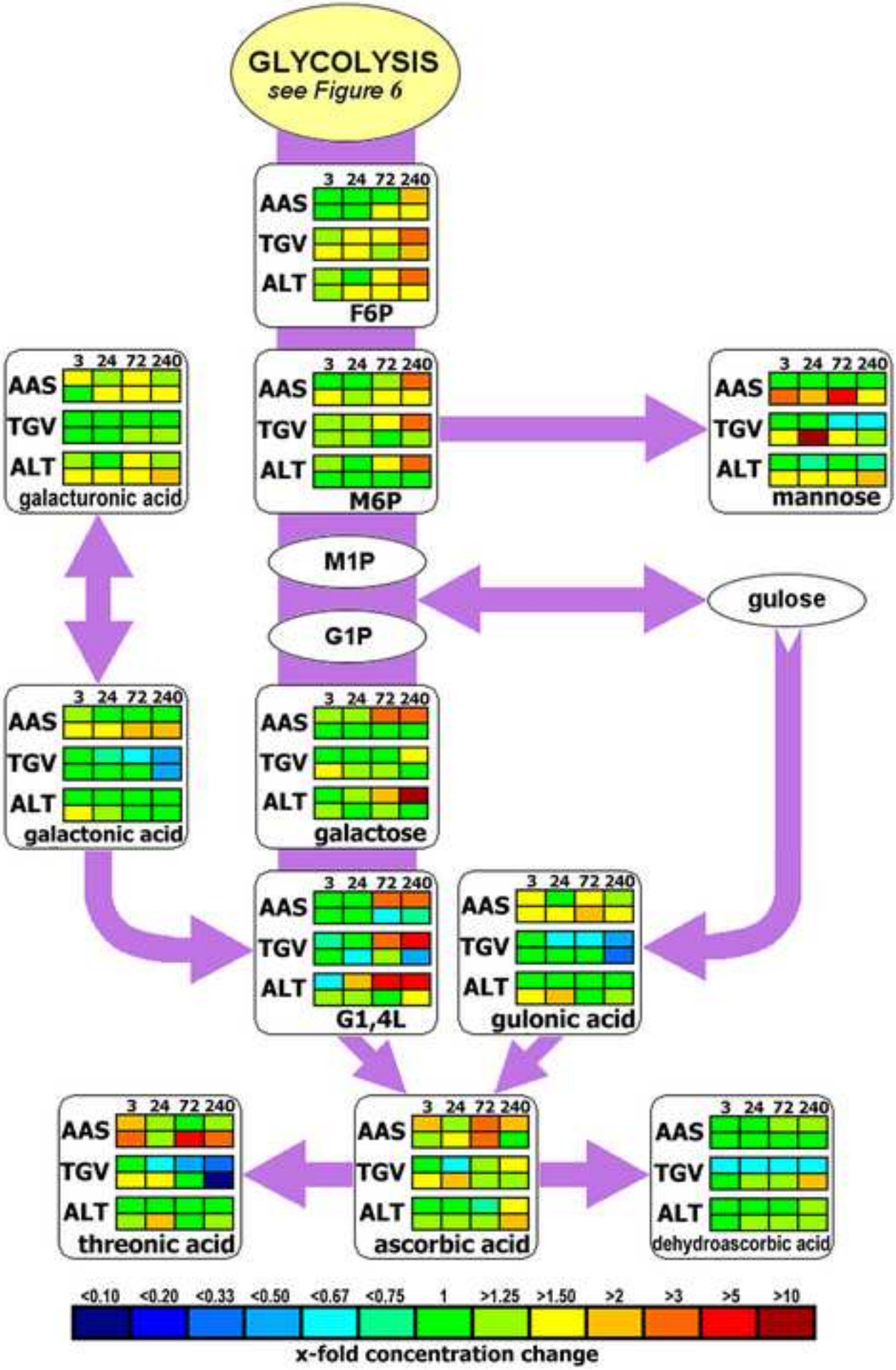

Figure 8

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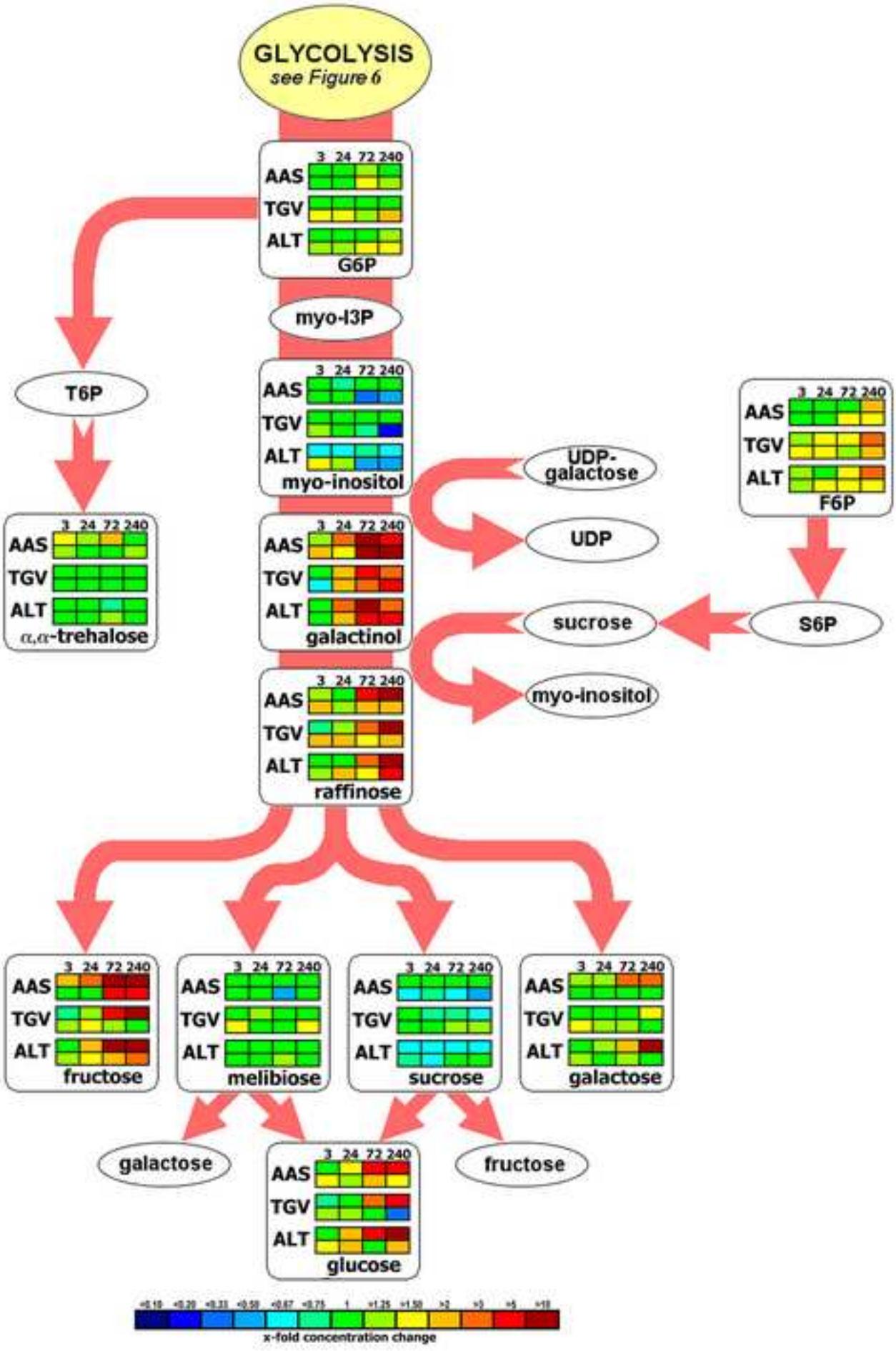

Supplementary Table 1

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Supplementary Figure 1

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