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

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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 (aspartic 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.

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

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mechanisms include the functional expression of hydrophilic and cryoprotective proteins (Alsheikh et al., 2003, 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 10 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 evolutionarily 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 comparing annual and perennial species. In view of the apparent limitations of biological information gained from studies in the model species *A. thaliana*, 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 (*F. 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 3000 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 and −15 °C (Fig. 1). 'Alta' demonstrated also significantly less ion leakage compared to 'Ås' and/or 'Tingvoll' at all temperatures except 0 °C. *F. vesca* 'Tingvoll', in comparison, tended to be the least freezing-tolerant line regarding tissue discoloration and ion leakage data. However, the southern genotype 'Aas' showed signs of enhanced tissue damage at 0 and −15 °C compared to 'Tingvoll' (Fig. 1B). In general, leaf tissue damage and ion leakage drastically increased at −10 and −15 °C in all *F. vesca* lines.

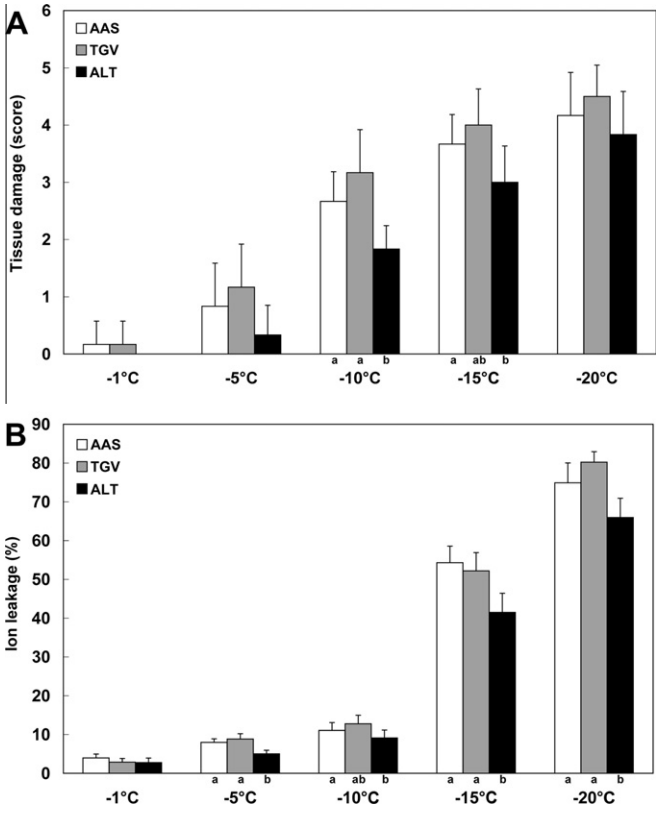

Fig. 1. Freezing tests with *Fragaria vesca*. Single detached leaves of three genotypes (AAS: Ås; TGV: Tingvoll; ALT: Alta) were exposed to different freezing temperatures after cold-acclimation (240 h). The upper graph (A) shows tissue damage based on visual scores (1: <10% damaged; 2: 10–25% damaged; 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 ($p \leqslant 0.05$).

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).

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≥50%

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 Fig. 2 (Supplementary Table 1). 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 five 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 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 Fig. 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) resembled those seen in hierarchical cluster (HCL) analysis of the total set of compounds (Fig. 5). Metabolite pools established two 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

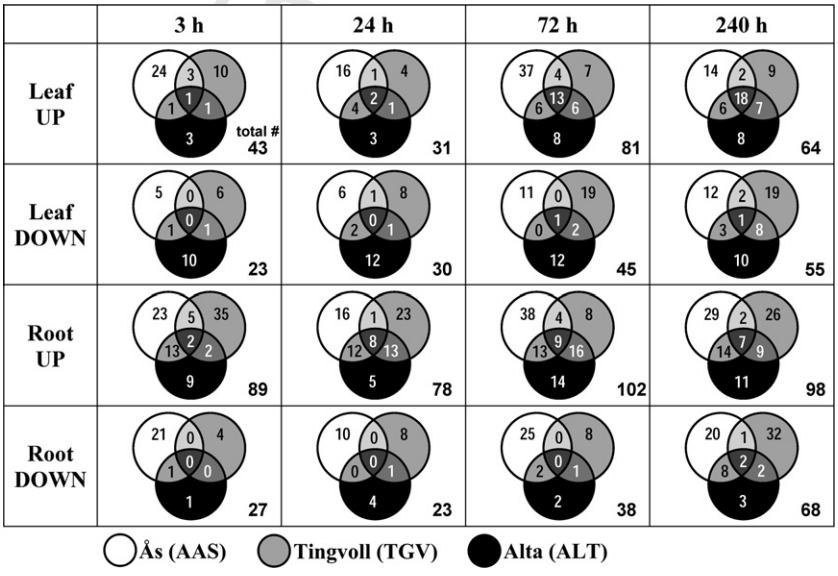

Fig. 2. Venn diagrams showing the co-ordinate up and down-regulation of metabolism. Diagrams are based on a set of 160 identified metabolites and non-identified mass spectral tags of cold-acclimation time points 3, 24, 72, and 240 h. Similarities in number of increased (UP) and decreased metabolites (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.

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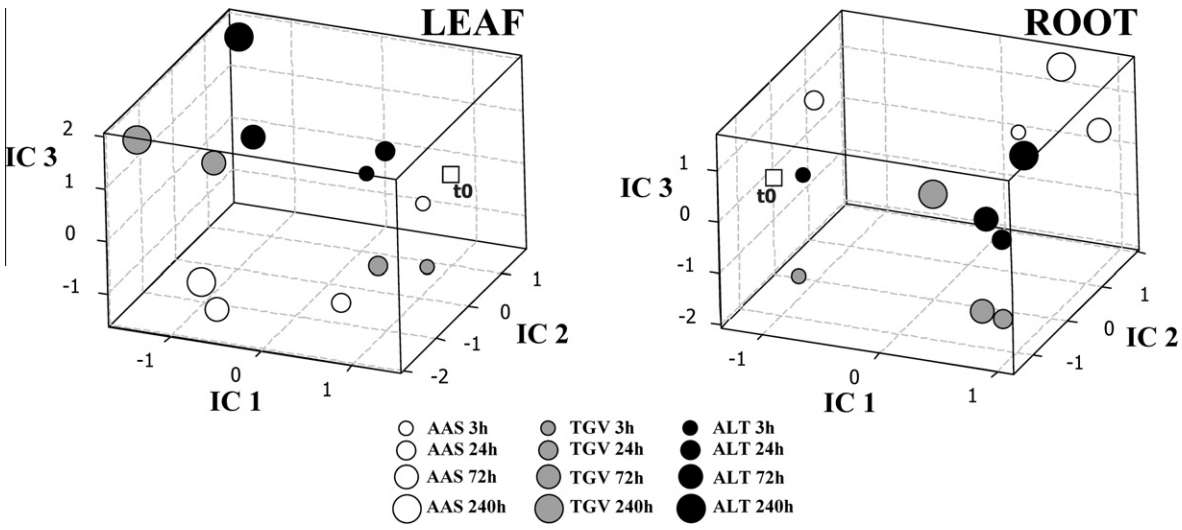

Fig. 3. Independent component analysis (ICA). 3D ICA based on components IC1, IC2, and IC3 from metabolite profiles (160 identified metabolites and non-identified mass spectral tags). Segregation patterns of leaf and root samples of *F. vesca* (AAS: Ås; TGV: Tingvoll; ALT: Alta) harvested at different 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 Fig. 1.

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. 6–8). Fig. 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 slightly 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 which 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 (*F. vesca*) has been adopted as one of the most important Rosaceae model species within *Fragaria* and closely related genera. 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 are 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 also 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, detection of initial metabolic responses in roots by 3 h of cold acclimation as displayed in Figs. 6–8, counters this assumption.

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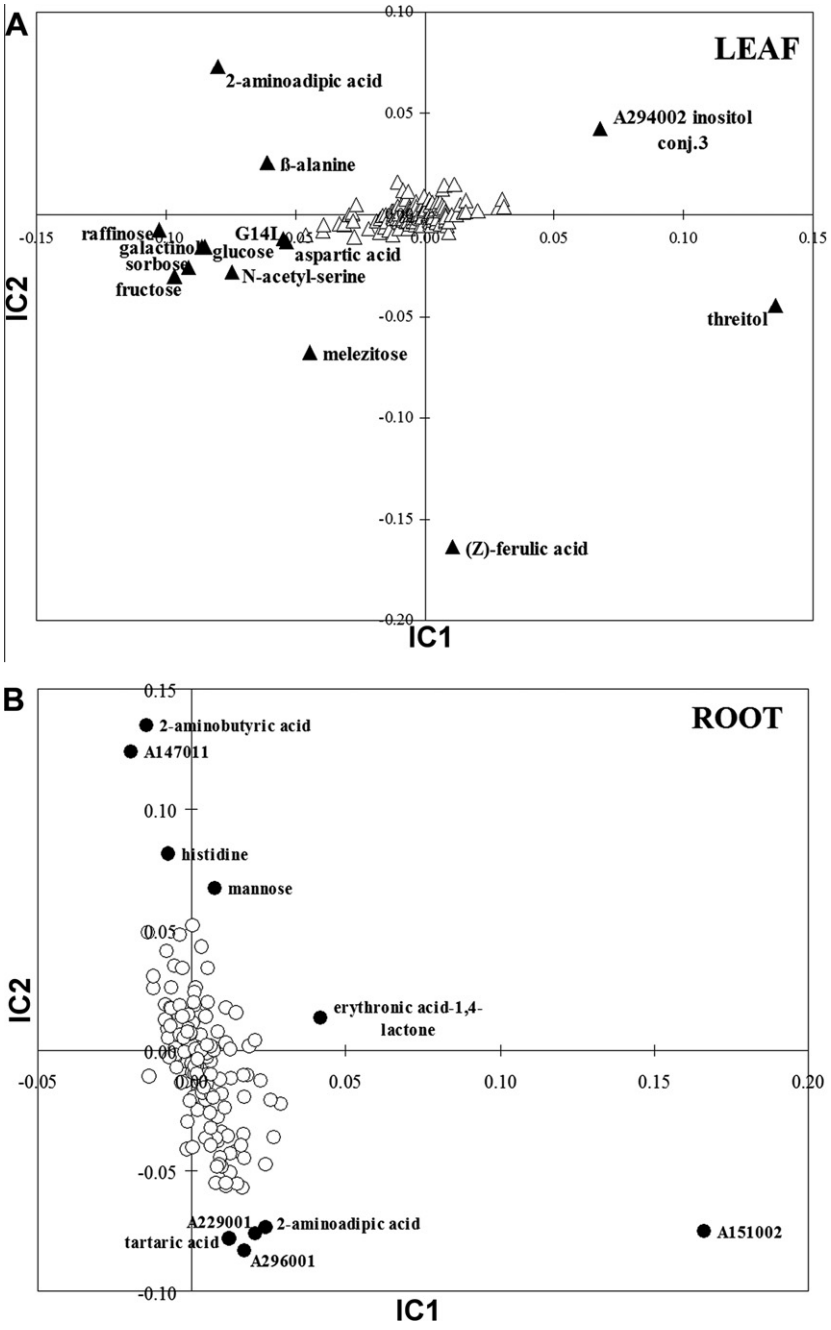

Fig. 4. Loading plots of independent components. ICA loading plots of leaf (A) and root variables (B) are based on the two independent components IC1 and IC2. Metabolites showing highest discrimination are tagged in the graphs (black-filled symbols).

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 profiling approach adopted in this study, metabolic changes are discussed with regard to primary metabolism. Obvious alterations (>2.5-fold) in sugars found in our study 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). 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.

In *Arabidopsis*, sucrose has been shown to be transiently up-regulated after 48 h (Kaplan et al., 2007), and is involved in the regulation of cold acclimation during diurnal dark periods

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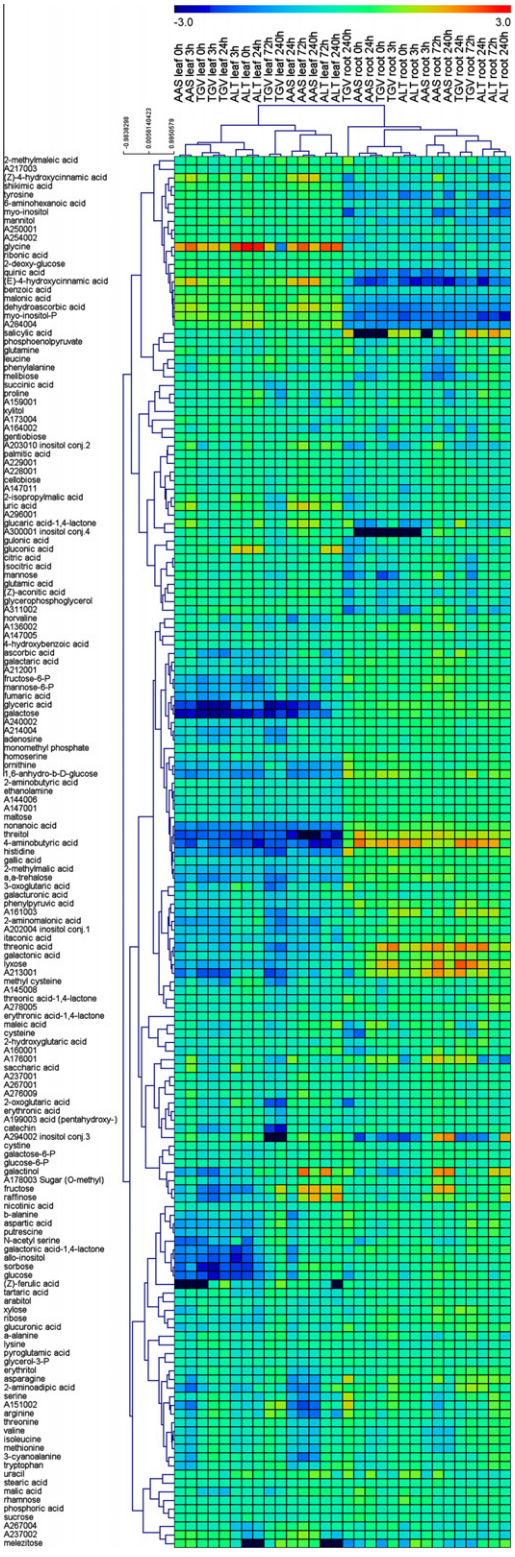

Fig. 5. Hierarchical clustering (HCL) of metabolite pools. Hierarchical trees (Pearson correlation) were drawn, based on 160 identified metabolites and non-identified mass spectral tags, from leaves and roots of *F. vesca* (AAS: Ås; TGV: Tingvoll; ALT: Alta) sampled at different time points upon cold 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 colors indicate decreased concentration levels of metabolites, yellow-reddish colors increased metabolite levels (see color scale). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Rekarte-Cowie et al., 2008). The non-CBF regulation of sucrose synthases underlies clock-gene regulation, and 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. The expected initial strong up-regulation of disaccharides such as sucrose and the osmolyte trehalose (Cook et al., 2004) was not observed in our experiments as 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. 6–8), corroborate starch breakdown patterns described by Kaplan et al. (2007), leading to rapidly increasing hexose-phosphate pools and ultimately, 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, emphasizing a coordinated starch and sucrose breakdown in *F. vesca* during cold acclimation. Seen in a different context, for 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 rather than sucrose were accumulated *in planta* in leaf and root tissues after long-term (10 days) of cold exposure.

3.2. Cold-induced metabolic shifts in roots are retained or transient compared 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 changes of other metabolites in roots occurring within 3 h after onset of cold when compared to the leaves. Such early induced metabolites included both amino acids (especially cysteine; 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 only small transient increases. The actual functional role of proline in cold stress tolerance is not clear (Korn et al., 2008). Considering the low levels in our study, a minor role of this amino acid in low-temperature acclimation in *F. vesca* is suggested. The polar amino acid glutamine, previously shown to be induced after 72 h of cold acclimation (Usadel et al., 2008), here showed enhanced levels in *Fragaria* (most in the roots).

The precursors arginine and ornithine and their product, the diamine putrescine, were differently regulated in root organs and between genotypes, with all genotypes having higher levels under control conditions in roots than leaves. However, putrescine increased strongly in leaves in response to cold with minimal changes in the roots. The latter metabolite has been reported to serve in several biological functions related to cold stress as a compatible solute (Kaplan et al., 2004), in modulation of antioxidant systems (Zhang et al., 2009), and in the signaling and control of ABA levels (Cuevas et al., 2009). Since putrescine concentrations were drastically enhanced in leaves, the transient increase in 'Alta' or even the decrease observed in 'Ås' root tissue suggests other

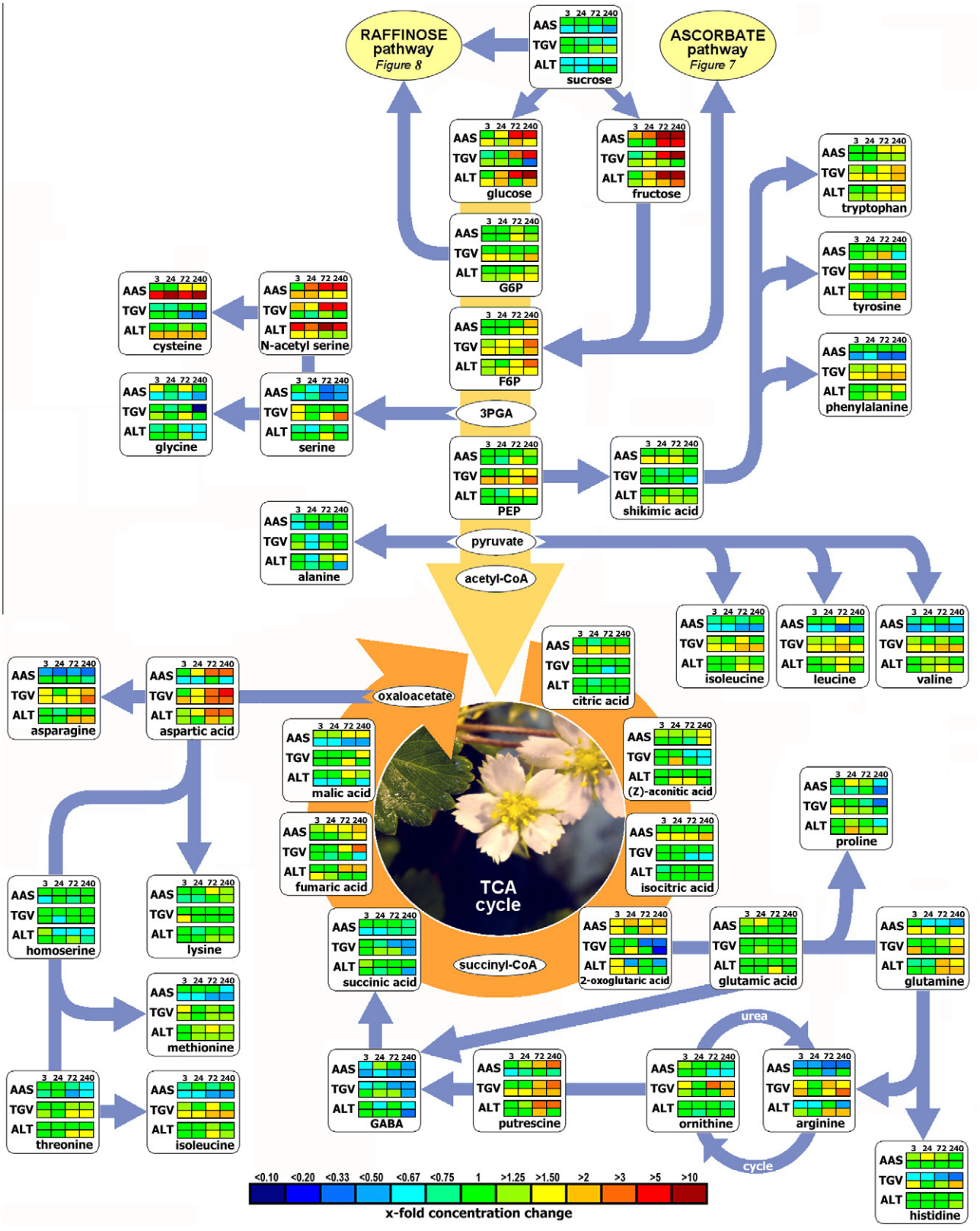

Fig. 6. Functional regulation of glycolysis, citric acid cycle (TCA), and amino acid biosynthesis are depicted as pathway maps. Leaf and root samples of *F. vesca* (AAS: Ås; TGV: Tingvoll; ALT: Alta) were harvested at different time points after cold acclimation (0, 3, 24, 72, and 240 h). Colors in metabolite 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 colors indicate decreased concentration levels of metabolites, yellow-reddish colors increased metabolite levels (see color 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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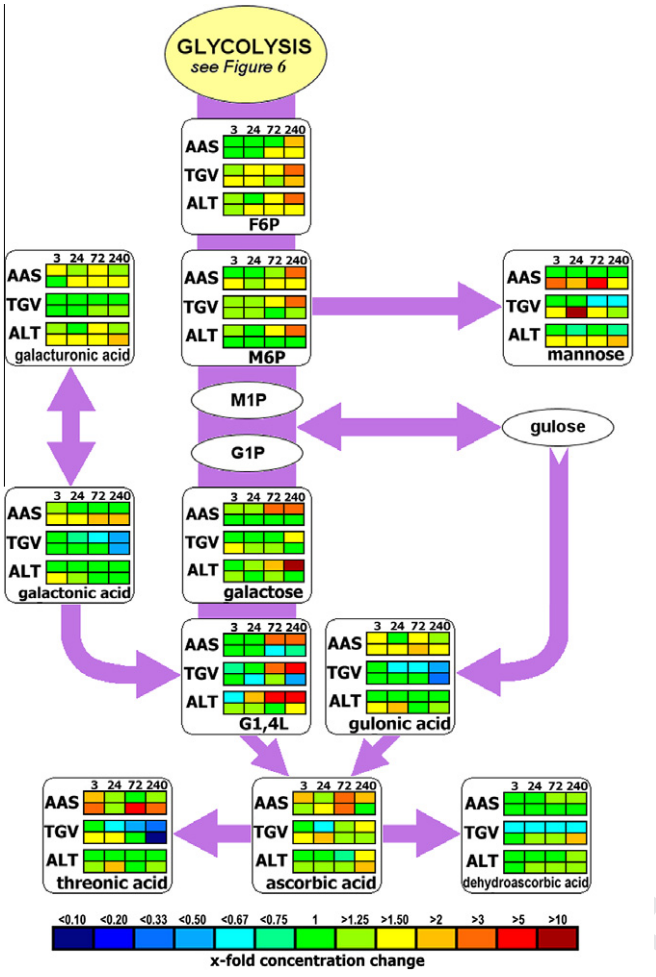

Fig. 7. Functional regulation of ascorbate metabolism. For further details regarding pathway map, experiments and color settings, see Fig. 6. *Abbreviations:* F6P = fructose-6-phosphate; G1,4L = galactonic acid-1,4-lactone; G1P = galactose-1-phosphate; M1P = mannose-1-phosphate; M6P = mannose-6-phosphate; UDP = uridine-diphosphate. See also comprehensive metabolite information in Supplementary Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

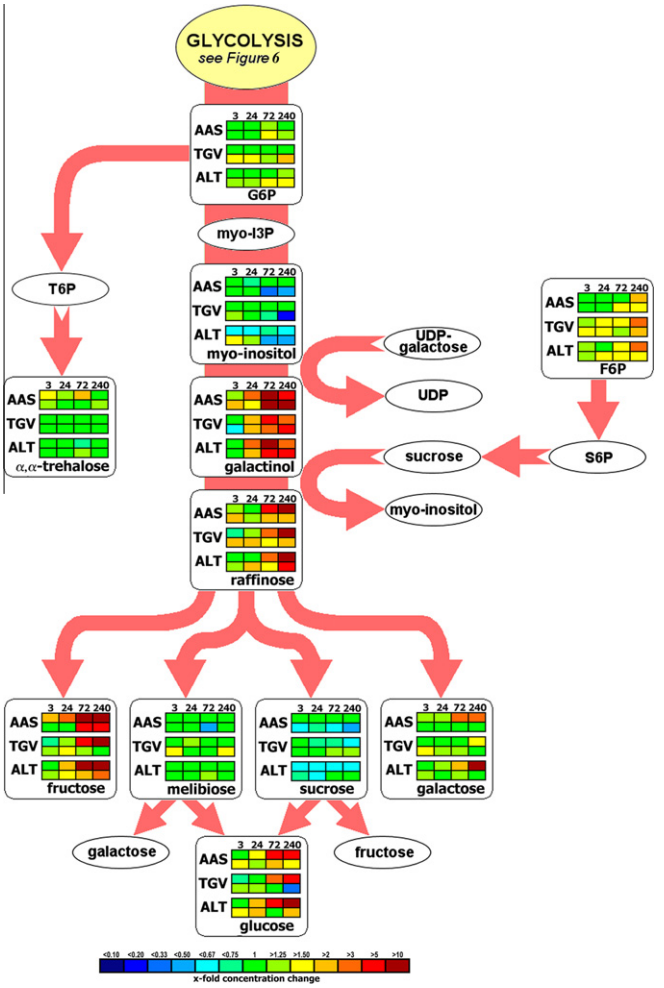

Fig. 8. Functional regulation of raffinose biosynthesis. For further details regarding pathway map, experiments and color settings, see Fig. 6. *Abbreviations:* F6P = fructose-6-phosphate; G6P = glucose-6-phosphate; myo-I3P = *myo*-inositol-3-phosphate; S6P = sucrose-6-phosphate; T6P = trehalose-6-phosphate; UDP = uridine-diphosphate. See also comprehensive metabolite information in Supplementary Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

functional 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 received little attention in terms of cold-regulated metabolism. Reasons might be their relatively low molecular weight and limited potential to function as osmolytes 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 suggest 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, the potential signaling function of leucine and its role as mediator of gene expression in plants (*Arabidopsis*) has just recently been proposed (Hannah et al., 2010), which is supported by transiently increased levels (72 h) in leaves and also roots (except 'Aas', 'Ås' of all *F. vesca* lines. Organ-dependent differences were clearly displayed in the acetyl-serine–cysteine route (Fig. 6). Large (2- to 10-fold) 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, and likewise different genotypes, upon cold treatment. To the best of our knowledge, this is the first approach addressing the simultaneous assessment of cold responses in leaf and root tissue using multiparallel metabolite profiling. Only a 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 *O. 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.

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3.3. Cold tolerance and potential metabolic phenotypes

Three genotypes, of contrasting geographical origins, were compared for their cold tolerance using two types of assays, ion leakage (indicator of cell death) and a visual tissue damage estimate. All lines were cold-tolerant, showing insignificant damage at −10 °C. However, the 'Alta' genotype was consistently and significantly more freezing-tolerant than the other two lines. For this reason we have compared trends in the most tolerant line 'Alta' to the two less tolerant 'Ås' and 'Tingvoll'. Perhaps the most striking cold response involved was the strongly induced raffinose pathway (Fig. 8 and Supplementary Table 1). The levels of galactinol and raffinose were responsive in both leaf and root tissues of all three *F. vesca lines* tested. These metabolites are associated with substantial increases in fructose, glucose, and galactose in response to cold. Of the three, 'Alta' showed the greatest increase of raffinose in the root following cold treatment. However, these responses did not seem to differentiate the most tolerant genotype from the lesser tolerant lines. 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).

Overall the main differences between 'Alta' and the less tolerant genotypes were the consistently higher levels of salicylic acid, gluconic acid, inositol conjugates and *myo*-inositol (Fig. 5) in 'Alta', and the rapid response of this accession to cold exposure (within 3 h) in regard to distinct metabolites, namely 2-oxoglutarate, proline and A159001, glycine, GABA, and gluconic acid. These responses were not all unique to 'Alta', and many appeared to be shared by two or even all three genotypes. Because the studied *F. vesca* lines unexpectedly differed little in their freezing tolerance (Fig. 1), we cannot definitively attribute these metabolic differences to directly contribute to the differential cold tolerance and thus explain the presence of distinct metabolic phenotypes.

Of the factors winter temperature and possibly summer temperature at the plant accession sites might explain some of the metabolic differences between the genotypes. In particular, the latitude implies highly varying day length conditions during the growth season with 24 h daylight during summer time for the most Northern genotype 'Alta' and thus, 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). 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 in the model *F. vesca*.

4. Conclusions

Metabolite pools in *F. vesca* were highly reprogrammed 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 regulatory roles of branched-chain amino acids (leucine, isoleucine, and valine) in cold responses. 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.* × *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 *A. 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, results may 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 likely established partially different strategies to prepare for long-term freezing temperatures. 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.

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5. Materials and methods

5.1. Plant experiment and cold acclimation

Eight weeks old runner-propagated *F. vesca* L. plants from three 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} s^{-1}) in a conditioning room prior to transfer to a cold room at 2 °C under artificial light (fluorescent tubes, ~90 µmol m^{-2} s^{-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).

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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. Three hundred and sixty microliters 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. Eighty microliters 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 and by 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 Microsoft® Word. Only those metabolites showing a ≥50% increase or ≤50% decrease in concentration, compared to the initial time point t0 of individual genotypes, were considered in order to illustrate also marked responses after short-term cold exposure (within 24 h). 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:10.1016/j.phytochem.2012.01.024.

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