Intraspecfic variation in cold-temperature metabolic phenotypes of *Arabidopsis lyrata* ssp. *petraea*

Authors:

Matthew P. Davey

F. Ian Woodward

W. Paul Quick

Affiliation:

Animal and Plant Sciences, Western Bank, University of Sheffield, Sheffield, UK

Corresponding author:

Matthew P. Davey

Animal and Plant Sciences, Western Bank, University of Sheffield, Sheffield, UK

email m.davey@sheffield.ac.uk

tel. 44 (0) 114 222 4649

fax 44 (0) 114 222 0002

Abbreviated title:

Cold temperature metabolic phenotypes

Abstract

Atmospheric temperature is a key factor in determining the distribution of a plant species. Alongside this, plant populations growing at the margin of their range may exhibit traits that indicate genetic differentiation and adaptation to their local abiotic environment. We investigated whether geographically separated marginal populations of *Arabidopsis lyrata* ssp. *petraea* have distinct metabolic phenotypes associated with exposure to cold temperatures. Seeds of *A. petraea* were obtained from populations along a latitudinal gradient, namely Wales, Sweden and Iceland and grown in a controlled cabinet environment. Mannose, glucose, fructose, sucrose and raffinose concentrations were different between cold treatments and populations, especially in the Welsh population, but polyhydric alcohol concentrations were not. The free amino acid compositions were population specific, with fold differences in most amino acids, especially in the Icelandic populations, with gross changes in amino acids, particularly those associated with glutamine metabolism. Metabolic fingerprints and profiles were obtained. Principal component analysis (PCA) of metabolite fingerprints revealed metabolic characteristic phenotypes for each population and temperature. It is suggested that amino acids and carbohydrates were responsible for discriminating populations within the PCA. Metabolite fingerprinting and profiling has proved to be sufficiently sensitive to identify metabolic differences between plant populations at different atmospheric temperatures. These findings show that there is significant natural variation in cold metabolism among populations of *A. l. petraea* which may signify plant adaptation to local climates.

Key words:

Arabidopsis lyrata spp. *petraea*; Cold; Direct Injection Mass Spectrometry; Environmental Metabolomics; Metabolic Phenotypes; Metabolite Fingerprinting.

1 Introduction

Temperature is a paramount factor in controlling ecosystems with regards to plant productivity, reproduction and ultimately distribution (Thomas et al., 2004; Walther et al., 2002, 2005). Plant growth has to be constantly controlled by a variety of molecular and metabolic networks allowing the protection and repair of plant cells in order to provide an appropriate response to ever changing environmental and resource conditions (Meyer et al., 2007; Vinocur and Altman, 2005). Such gross changes in growth will be preceded by alterations in the plant's metabolism. Generally, changes in the metabolite content of a plant during cold temperatures may play an advantageous role in cell cryoprotection prior to freezing temperatures. This process is known as cold acclimation (Thomashow, 1999). This metabolic phenotype is likely to differ according to a plant species' inherent ability to adapt or acclimate to cold temperatures. Intraspecific variation in cold and freezing tolerances have been found in plant species with a broad geographical distribution and such variation has been attributed to specific environmental parameters, such as temperature, at each location (Alonso-Blanco et al., 2005; Hannah et al., 2006; Sackville Hamilton et al., 2002; Skøt et al., 2002; Zhen and Ungerer, 2008). Cook et al. (2004) reported significant natural variation for freezing tolerances and the preceding acclamatory processes within the metabolome of two contrasting ecotypes in *Arabidopsis thaliana*. Intraspecific genetic variation has also been found between populations of the arctic-alpine species *Arabidopsis lyrata* ssp. *petraea* (hereafter *A. petraea*). This species occurs in small geographically isolated populations across latitudinal and temperature gradients in Wales, Scotland, Germany, Norway, Sweden and Iceland, usually growing on rocky or stony cliffs and shores (Clauss and Koch, 2006; Clauss and Mitchell-Olds, 2006; Jonsell et al., 1995; http://www.petraea.shef.ac.uk).

Characterising phenotypes of such marginal populations where temperature regimes differ is of interest as they pose pertinent questions about evolutionary adaptations, along with the limits of such adaptations (Vergeer et al., 2008). Alongside the genetic differences between populations, we have already discovered that populations of this species can be distinguished by their metabolic phenotypes when grown under the same controlled conditions (Davey et al., 2008). Therefore, we hypothesised that populations growing at more northern latitudes where the minimum temperatures are different will exhibit metabolic phenotypes that are site and temperature specific. Assessing such spatial variation in metabolic responses to cold will provide information on cold acclimation and possible adaptive processes. To this end, we examined in detail the global metabolic phenotypes of plant foliage from three populations at control and cold temperatures. Metabolite fingerprints were obtained by direct injection mass spectrometry and metabolite profiling was targeted on the soluble, free carbohydrates, polyhydric alcohols and amino acids because of their known association with plant abiotic responses (Hannah et al., 2006; Smirnoff, 1998; Stitt and Hurry, 2002, Usadel et al., 2008).

2 Methods

2.1 Growth

Seeds of *Arabidopsis lyrata* (L.) ssp. *petraea* were collected from populations in Iceland, Sweden and Wales as described in Davey et al. (2008). Approximately 30 seeds were sown in Levington M3 compost within individual seed trays covered with an incubator lid (16.5 x 9.5 x 4.5 cm). Trays were placed inside one of two controlled-environment growth cabinets (Conviron Controlled Environments Limited,

Canada). Cabinet conditions within a 1.5 m^2 growth area were 16/8 hours day/night; 20 °C day/night; 60% humidity; atmospheric CO_2 ca. 400 ppm CO_2; light 250 µmol m^{-2} sec^{-1}. Growth rates were similar and so after 7 days when the seeds had fully expanded cotyledons, up to 20 seedlings from each population were individually transferred to larger plant pots (7 x 7 x 8 cm) containing Levington M3 compost. Each growth cabinet had an equal number of plants per population. Plants were watered from the base of the pot so the soil was moist, rather than sodden, when required with reverse osmosis (RO) water. No additional nutrients were added to the soil or water. After a further three weeks, ten of each *A. petraea* populations were randomly transferred in equal numbers to one of two growth cabinets set at a day/night temperature of 5 °C (all other conditions were as above, apart from relative humidity which increased to 80-90 %). There were slight visual differences in leaf morphology between populations (Data not shown) (Davey et al., 2008, Vergeer et al., 2008). After another 7 days and after six hours into the daylight period the foliage was excised at soil level with a razor blade. Leaf tissue was chosen for this study, as this organ is regularly exposed to changes in air temperature in the wild. The foliage was immediately immersed in liquid nitrogen and stored at -80 ºC.

2.2 Metabolite extraction and analyses

Metabolites were extracted and analysed as described in Davey et al. (2008). Briefly, approximately 100 mg leaf tissue per plant was extracted using 2 ml $MeOH/CHCL_3/H_2O$ (2.5:1:1) followed by 1 ml $MeOH/CHCL_3$ (1:1). The organic $CHCl_3$ phase was separated from the aqueous $MeOH:H_2O$ phase by adding 500 µl H_2O. The aqueous phase was analysed for free amino acids by HPLC and free carbohydrates and polyhydric alcohols by Gas Chromatography (GC). Aqueous and

organic phases were directly injected into a LCT mass spectrometer (Waters Ltd. Manchester, UK) using a MassLynx V.4.0 data system in negative and positive ionisation modes (50-800 *m/z*).

2.3 Metabolite fingerprinting – chemometrics

Raw centroid mass/charge (*m/z*) ratios from triplicate analytical runs were combined into 0.2 Da mass unit 'bins' for noise reduction and data alignment using in-house software. *m/z* peaks were assigned to bins only if an ion count of similar intensity and *m/z* range is detected for a peak in each of the three analytical runs for each biological sample. Binned *m/z* and percent total ion count (%TIC) values from the aqueous and organic phases, analysed in both negative and positive ion mode on the mass spectrometer, were explored by Principal Component Analysis (PCA) using Simca-P V.11.5 (Umetrics, Sweden) as described in Davey et al. (2008). The statistical differences between the %TIC of each 0.2Da bin was carried out using ANOVA followed by Post-Hoc tests for multiple comparisons. Significant differences are given at $P \leq 0.05$ and at a Bonferroni P value of 0.05/n where n = number of bins tested. Metabolites for each bin were putatively identified using KNApSAcK v1.2. http://kanaya.naist.jp/KNApSAcK/ using a search resolution of ±0.1Da. Only metabolites that have been reported in *Arabidopsis thaliana* were selected in KNApSAcK. A Multivariate General Linear Model analysis of variance (ANOVA) followed by a Tukeys Post-Hoc test for multiple comparisons was used to test for significant differences in amino acid and carbohydrate composition between populations and temperatures using SPSS v12.0.1 (Chicago, Illinois, USA).

3 Results

3.1 Targeted analysis

3.1.1 Free soluble carbohydrates

The concentration of soluble carbohydrates and polyhydric alcohols varied between populations and treatments (Fig. 1). The concentration of the majority of carbohydrates was increased in all populations after the cold treatment. The Welsh population had the largest number of mono-, di- and trisaccharides that significantly increased with the cold temperature (mannose, glucose, fructose, sucrose, raffinose). There were two carbohydrates that increased with cold in the Swedish population (sucrose, raffinose) and no carbohydrates were significantly affected in the Icelandic population. The largest increase in response to cold was the mannose concentration in the Welsh population. Polyhydric alcohols were not significantly increased by cold treatment.

There were significant temperature increases in the total monosaccharide pool after exposure to cold in the Welsh population ($P \geq 0.001$) and in the total di- and trisaccharide pools in the Swedish population ($P \geq 0.001$). There were no significant differences in the total polyol or total carbohydrate or polyol pool for any population. There were significant site differences with the Swedish population having higher concentrations of monosaccharides, inositol and the total carbohydrate and polyol pool (Table 1) and the Welsh population having higher concentrations of maltose and mannitol. There were no significant site*temperature interactions.

3.1.2 Free amino acids

The concentration of soluble amino acids varied between populations and treatments, with the majority of amino acids increasing in concentration after the cold treatment (Fig. 2). The Welsh population had the lowest number of amino acids that significantly increased with the cold temperature (serine, glycine, alanine, glutamine). There were five amino acids that increased with cold in the Swedish population (phenylalanine, alanine, glutamic acid, GABA, aspartic acid) and there were ten amino acids that were significantly affected in the Icelandic population (glycine, histidine, alanine, glutamic acid, glutamine, arginine, GABA, aspartic acid, asparagine, isoleucine). In particular, amino acids derived from the citric acid cycle all were found at higher concentrations in cold-treated Icelandic plants. The total amino acid pools for the Swedish and Icelandic populations were significantly increased by cold treatment ($P \geq 0.01$ total data not shown). There were significant site differences with the Welsh population having higher concentrations of histidine and threonine than the Swedish population (Table 1). There were also significant site*temperature interactions for histidine, arginine, aspartic acid and isoleucine as the Icelandic populations had a different response to cold treatment than the other populations, whereas serine had a significantly different response to cold in the Swedish population than the Welsh population.

3.2 Metabolite fingerprinting

To check the overall variation between populations and cold treatment in other metabolites alongside those detected in the targeted analysis, a DIMS analysis was carried out. There were between 155 and 240 bins (0.2 Da range Bins) that had a significantly different %TIC between control and cold-treated plants among all populations with a *P* value below 0.05 (Table 2). To reduce the risk of false positive

discoveries, a Bonferroni P value was calculated for each extraction phase and ionisation mode. In terms of percent change, the metabolites that were detectable in the aqueous phase by negative ionisation showed the largest response to cold (Fig. 3). Across all extract phases and ionisation modes, the Icelandic population had the most significantly changing bins in response to cold. The Icelandic and Welsh populations shared the most significantly different bins from the aqueous phase between temperatures.

3.3 Principal component analysis (PCA)

Masses detected within the aqueous phase analysed in the negative and positive ionisation mode showed separate clusters of Welsh, Swedish and Icelandic samples along principal component (PC) 2 (Figs. 4a and 4b). Samples that were exposed to cold were clustered separately from the control samples along PC1. In particular, the Welsh samples had distinct clusters away from Swedish and Icelandic plants. There was no distinct separation of populations and treatments of *m/z* values acquired in the organic fraction when analysed in the negative ion mode. There was only some separation of cold treatment along PC1 (Fig. 4c). However, when analysed in the positive mode there were separate clusters of Swedish, Icelandic and in particular Welsh samples along principal component 1 (Fig. 4d). Samples that were exposed to cold were very weakly separated from the control samples along PC3.

The scores contribution plots (Figs. 4e - h) indicate which bins differ the most between control (more positive) and cold-treated (more negative) plants along the selected principal components for each phase and ionisation mode. There were few bins that differed in intensity in the aqueous phase analysed in the negative ionisation

mode. However, the score contribution values were very high for these bins, indicating a strong influence on separating control and cold-treated plants. There was a stronger and a more even distribution of bins that differed between control and treatment from the aqueous phase analysed in the positive mode (Fig. 4f). The organic phase in negative ionisation mode showed a different distribution of bins that differed between control and cold samples as more low mass bins were related to control conditions but more mid-range (>140 m/z) and high-range (>500 m/z) mass bins were related to cold conditions (Fig. 4g). However, the organic phase analysed in the positive mode showed that most bins were related to control conditions with only a few bins in the low mass range (<200 m/z) that were increased under cold conditions (Fig. 4h).

The putative metabolite identification for the top four most positive (control) and negative (cold) PCA contribution scores (Fig. 4e – h) for each extraction and ionisation mode are presented in table 3. As expected from the solvent extraction procedure used, the majority of the top bins that were responding to cold were assigned to low molecular weight amino acids, carbohydrates, organic acids, phenylpropanoids and phenolics. Most of the bins detected in the aqueous phase that were identified as significantly different in the ANOVA within the Bonferroni *P*-value limit (Table 2) were also detected as major contributing bins in the PCA (Table 3) implying that the correct principal components were used. The complete lists of bins and respective putative compound identifications that were separating out populations and cold treatment of each such mass for each ion mode and fraction are presented as supplementary data.

4 Discussion

4.1 Targeted metabolite profiling

Although only a snapshot of the metabolism was measured at one point in time the concentrations of carbohydrates and amino acids that were altered in cold treated plants were shown to be population specific. The response to cold in carbohydrate metabolism, in the number of metabolites that were accumulated during the cold treatment, was population specific in that Wales > Sweden > Iceland. However, this trend was reversed in the free amino acid pool where the response was Iceland > Sweden > Wales. There were no significant site*temperature interactions for carbohydrates but there were five amino acids that had a statistically significant site*temperature interaction.

Carbohydrates and amino acids have been commonly reported to increase in concentration after cold treatments. Recent results by Usadel et al. (2008) show that sucrose, glucose and fructose concentrations increase with cold in *A. thaliana*. They also found that initially (6h) some organic and amino acids decreased in concentration with cold while others such as glutamine increased with cold. After 78 hours cold in their study, most amino and organic acids and carbohydrates had increased in concentration. In particular, Usadel et al. (2008) also reported increases in raffinose and proline, which we detected via HPLC or DIMS.

There were significant increases in carbohydrates in response to cold in this study, namely glucose, fructose, sucrose, raffinose and mannose. This is in agreement with Klotke et al. (2004) who also reported increases in glucose, sucrose, raffinose and fructose concentrations, together with an increase in freezing tolerance, in *A. thaliana*. This increase in carbohydrates may be due to increased synthesis, or to reduced usage as growth and phloem transport is decreased. Sucrose in particular is increased (Fig. 1) probably because it can be quickly mobilised and stored throughout

the plant and used for respiratory needs as more gross morphological and biochemical changes occurs during acclimation (Atkin et al., 2005; Guy et al., 1992; Strand et al., 1999).

Amino acids have been shown to increase in concentration in a variety of perennial plant species during colder winter months (Sagisaka and Araki, 1983). Alterations in amino acid accumulation in response to cold were also measured in *A. petraea*. Most alterations occurred in compounds derived from the citric acid cycle, especially in the Icelandic population. This response in *A. lyrata* is comparable to cold responses in other species such as *Arabidopsis thaliana* as Kaplan et al. (2004) also found that amino acids derived from the citric acid cycle increased in concentration with cold treatments. Glutamine, glutamate, aspartate and asparagine are important amino acids in nitrogen assimilation, storage and N transport in plants and all were shown in increase in concentration during cold treatment (Iceland > Sweden > Wales). By far the most significant response to cold was the accumulation of glutamine (Fig. 2). Glutamine has also been reported to increase in concentration with cold in *A. thaliana* (Klotke et al., 2004) and asparagine is known to transport N around the plant.

The enzymes glutamine synthetase (GS); glutamate synthase (GOGAT); aspartate aminotransferase (AAT) and asparagine synthetase (ASN) play an important role in accumulating these amino acids. Increased aspartic acid may be beneficial to the plant during cold as mutants of AAT, that decreased aspartic production, showed reduced growth phenotypes (Coruzzi, 2003; Lam et al., 1995). With the Icelandic and Swedish populations having more aspartic acid in cold leaves over the Welsh population, this may prove to be advantageous during cold acclimation and growth.

Glutamine synthetase is encoded by multiple genes (*GLN*) (Peterman and Goodman, 1991) with the expression being increased by exposing plants to light and

interestingly, by carbohydrate accumulation such as sucrose (Oliveira and Coruzzi, 1999; Suzuki and Knaff, 2005). An increased expression of *GLN1.1* to form glutamine would increase the N available for assimilation into maintained growth or increased translocation from senescing or damaged leaves to form newly acclimated leaves (Bleeckerl and Patterson, 1997; Lam et al., 1995; Li et al., 2006). However, the gene family encoding ASN (*ASN1-3*) is mainly expressed, and asparagine accumulated, at night (Lam et al., 1998; Miesak and Coruzzi, 2002) as the daytime repression of *ASN* expression is also linked to light and carbohydrate accumulation (Coruzzi, 2003; Lam et al., 1994, 1995). Therefore, if carbohydrate concentrations are increased during cold, then the gene expression for GS and ASN should be further increased and decreased respectively. We found this to be true in other populations of *A. petraea* (Norway and Ireland) that were cold shocked for 8 hours as *GLN1.1* increased and *ASN3* decreased its expression (Dr Catherine Lilley – paper in preparation). Kilian et al. (2007) also reported a 45% decrease in *ASN3* and a 2000% increase in *GLN1.1* in expression in 24h cold-treated *A. thaliana* (eFP-brower http://bar.utoronto.ca/efp/cgi-bin/efpweb.cgi). Alongside this, there was a reported increase of more than 3000% in sucrose synthetase (*SUS1*) (Kilian et al., 2007) in response to cold, which was also shown to increase in the other two populations of *A. petraea* (unpublished data). The accumulation of glutamine may relieve some of the repression on *ASN3* as studies have shown that addition of glutamine alleviates the repression made by sucrose abundance on *ASN* expression and may explain the increase in asparagine in response to cold in our study (Lam et al., 1994, 1995). It is still unclear whether this activation and repression of such gene expressions is continued into the night in cold treated plants that accumulate carbohydrates and whether an increase in carbohydrates is more important to the plant than an increase or mobilisation of N.

Although the variation in relationships between gene expression and metabolite concentrations can be high in cold acclimating plants (Kaplan et al., 2007) an increase in *SUS1* and *GLN1.1* and a decrease in *ASN3* gene expression infer tight signalling processes involved in the metabolic control of cold exposure and cold acclimation. This may explain the inverse relationship between the number of significantly higher concentrations of amino acids and carbohydrates in cold-treated Icelandic (10 amino acids to 0 CHO) when compared to Swedish (5 to 2) and Welsh (4 to 5) populations.

4.2 Metabolite fingerprinting

This study has shown that there is intraspecific variation in the metabolic phenotype that plants have in response to cold temperature. The metabolite fingerprinting approach shows that shifts in the metabolome of *A. petraea* that were exposed to cold is detectable, with Icelandic and Welsh populations sharing the most significantly different changing bins. The control population phenotypes were similar to those reported in Davey et al. (2008).

The initial screen of the 0.2Da bins showed significant differences between control and cold-treated plants for each population. We detected approximately 3000 masses (0.2 Da bins) across all extraction phases and ionisation modes. Gray and Heath (2005) found 1187 masses (DIMS-Fourier Transform-Ion Cyclotron Resonance) when masses were pooled and compared from all ion modes and extraction phases, therefore as one metabolite may produce more than one mass peak and may be detectable in more than one ionisation mode or extraction phase the number of masses detected are comparable with their findings. About 8% of the masses found by Gray and Heath (2005) significantly increased or decreased in intensity after seven days cold treatment. This is in agreement with our findings that

on average across ion modes, extraction phases and populations 7.2% of the bins detected had significantly altered ion intensities with cold treatment (Fig. 3). There were more bins detected in extracts that were analysed in the positive ionisation mode, which could either be the result of more metabolites being able to ionise in positive mode than negative, or more adducts being formed. However, none of the most significant bins were found to match possible K or Na adducts of metabolites.

Metabolites that were associated with cold treatment were detected using both Bonferroni statistics and PCA loading scores, the majority of which were assigned to amino acids, carbohydrates, organic acids, phenylpropanoids and phenolics. Many of the highly statistically significant metabolites putatively identified by DIMS (Table 3 and supplementary data) are associated with the citric acid cycle and similar to the targeted analysis were also up regulated more so in the Icelandic population. From the DIMS we also found that masses for malate and glutamine increased and fumarate and succinate decreased. It is likely that the metabolite found at bin 145 (negative ion) and 147 (positive ion) is glutamine (Table 3). The raw unbinned mass spectrum for this bin was 145.099 m/z^- and 147.029 m/z^+. The difference between the monoisotopic mass of glutamine (146.069) and the unknown mass is 0.038Da in the negative ion and 0.047Da in the positive ion. The masses of α-Ketoglutaric acid and Ketopantoic acid were between 0.049 and 0.095Da away from the unknown peak. This confirms the result in the targeted analysis where glutamine concentrations increased in concentration with cold treatment.

The mass of metabolite 3-Hydroxypropyl glucosinolate (Bin 376 negative ion) was found to discriminate Welsh plants grown at 20ºC from all other populations at both control and cold temperatures. This mass had a highly positive PCA loading score (see supplementary data) and was significantly different to the other populations and treatments above the Bonferroni *P* value. This mass was also found to

discriminate Wales from other populations in our previous study (Davey et al., 2008). Therefore, although this metabolite can distinguish populations it may not be involved in cold tolerance mechanisms as it was not accumulated during the cold treatment in the Welsh, or any other population.

5 Conclusion

Metabolite fingerprinting and profiling has proved to be sufficiently sensitive to detect unique cold-induced metabolic phenotypes between populations of *A. petraea* across a broad geographical and climate distribution. A mixture of unique metabolic changes to cold and more general responses as described in other species was measured. Principal component analysis (PCA) of metabolite fingerprints revealed metabolic phenotypes for each population and temperature. Mannose, glucose, fructose, sucrose and raffinose concentrations were different between cold treatments and populations, especially in the Welsh population but polyhydric alcohol concentrations were not. The free amino acid compositions were population specific, with fold differences in most amino acids, especially in the Icelandic populations with gross changes in amino acids associated with the citric acid cycle, in particular glutamine metabolism. Such intraspecific variation in the metabolome of cold-treated plants may ultimately affect the plants ability to acclimate and adapt to new environments. This will be particularly important for plant populations living at the margin of their range and threatened by rapid climate change.

Acknowledgements

This research was funded by the Natural Environment Research Council Post-Genomics and Proteomics programme (NE/C507837/1). We thank D. Kinsman, J. Keily, J. Ramsden, H. Walker and M. Wormald for analytical help. We thank Dr. S. Ansell, Dr. W.E. Kunin, Dr. J.A. Lake, Dr. B. Lomax and Dr. P. Vergeer for seed collection and Dr. C. Lilley and Dr. N. Haigh at the University of Leeds for gene expression data.

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Figure1

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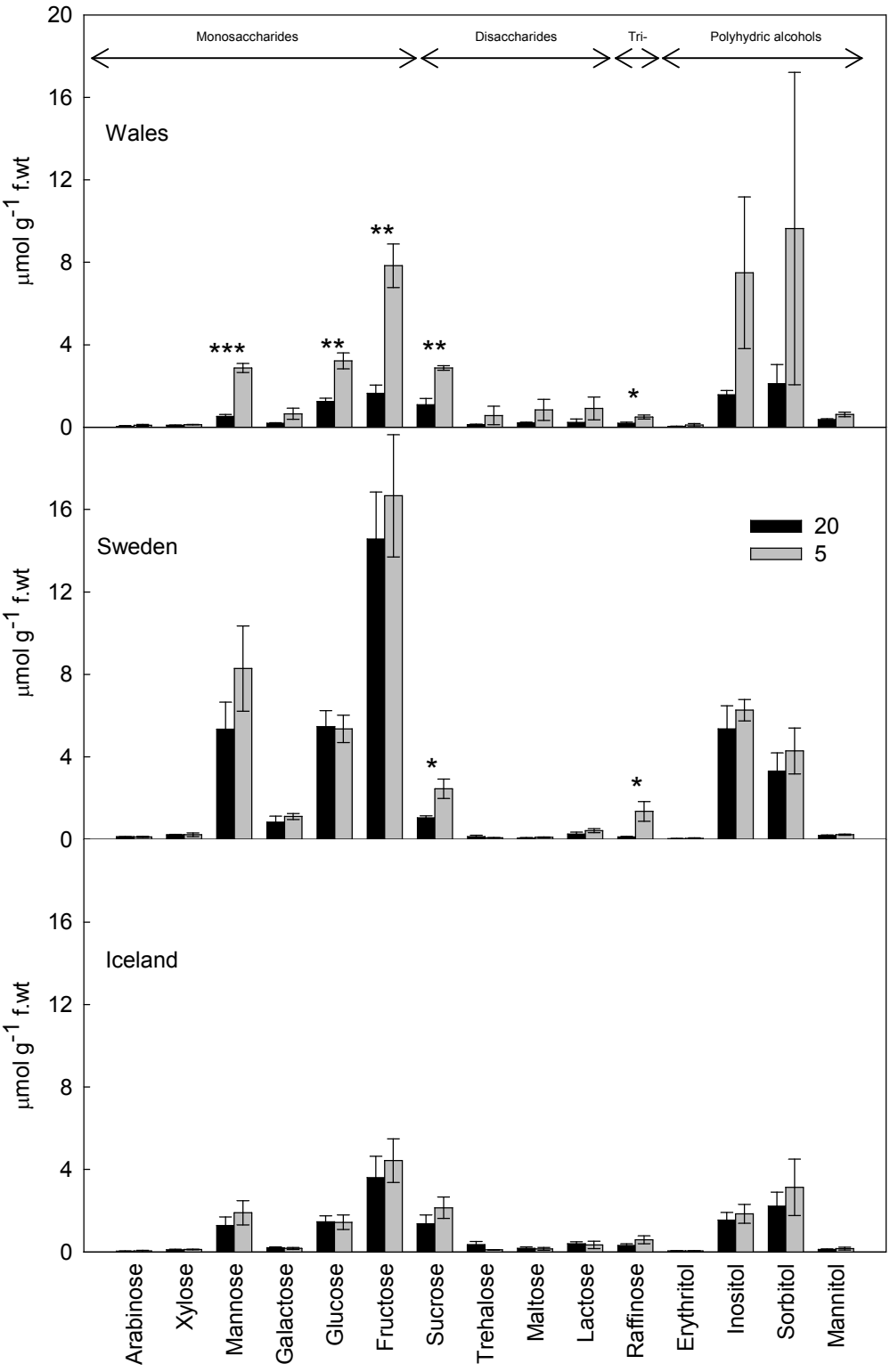

Figure 1: Concentrations of free carbohydrates and polyhydric alcohols in the leaves of *Arabidopsis lyrata* ssp. *petraea* (Wales, Sweden, Iceland) grown under control (20 ºC) or cold (5 ºC for 7 days) conditions. Significant differences between control and cold treatments within each population are given as * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$. Data are mean (± SE).

Figure2

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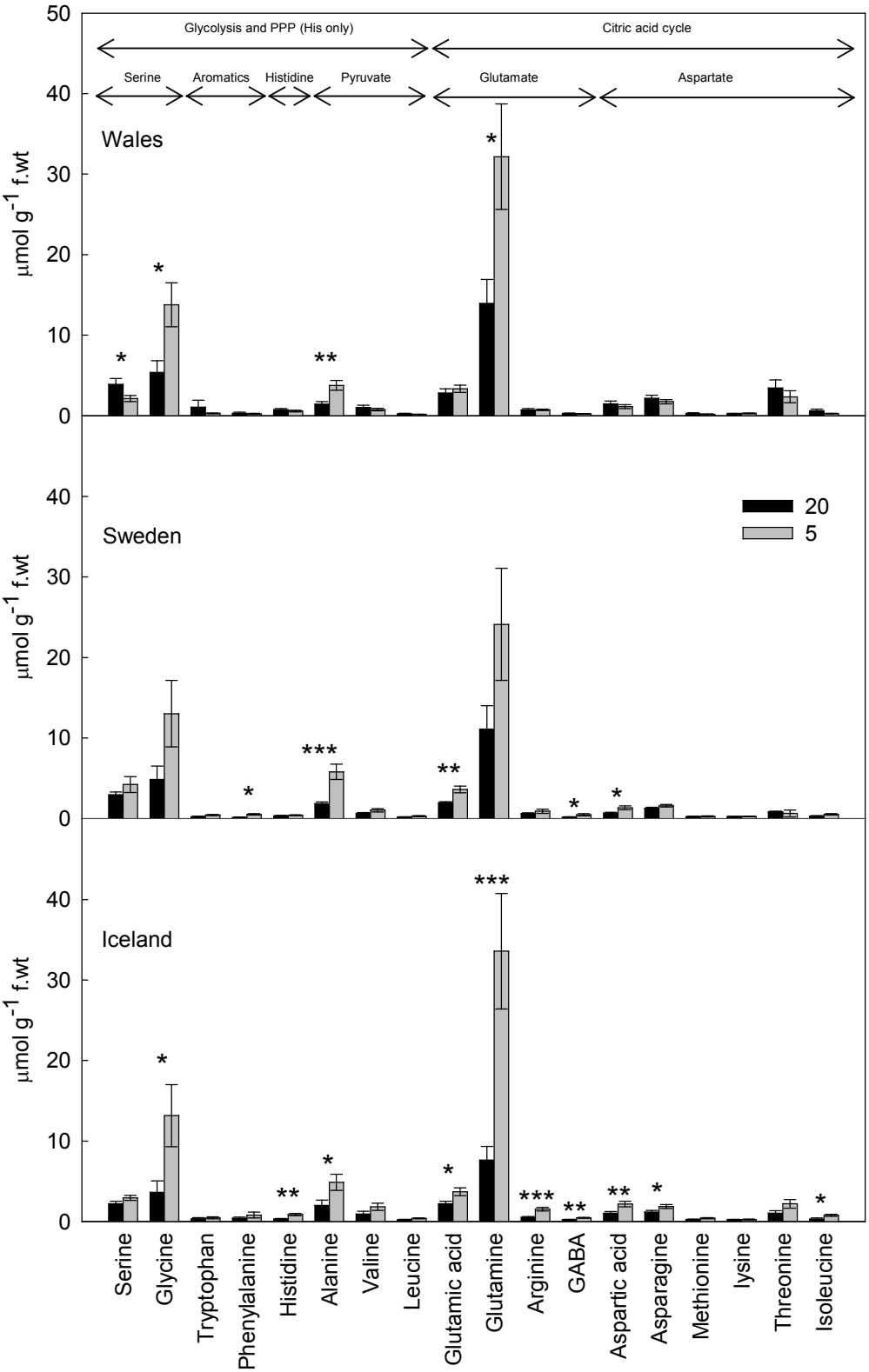

Figure 2: Concentrations of soluble free amino acids in the leaves of *Arabidopsis lyrata* ssp. *petraea* (Wales, Sweden, Iceland) grown under control (20 ºC) or cold (5 ºC for 7 days) conditions. Amino acids are listed according to their biosynthetic family and origin. Significant differences between control and cold treatments within each population are given as * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$. Data are mean (± SE).

Figure3
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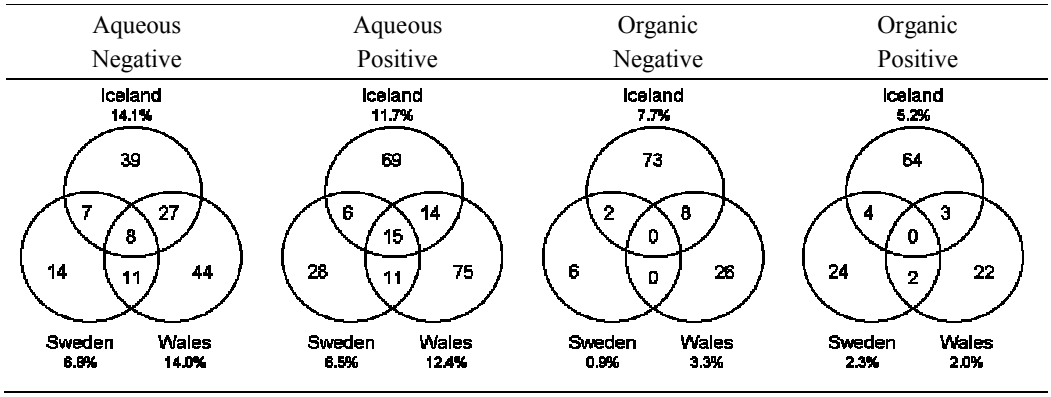

Figure 3. Venn diagram of the number of significantly different bins (P<0.05) between control and cold-treated Icelandic, Swedish and Welsh populations of *Arabidopsis lyrata* spp. *petraea* plants with overlapped regions denoting the number of shared changing bins of the selected population. The percentage of the total number of 0.2 Da Bins that had significantly different % total ion counts between control and cold-treated treatments within each country is given below the country name.

Figure4

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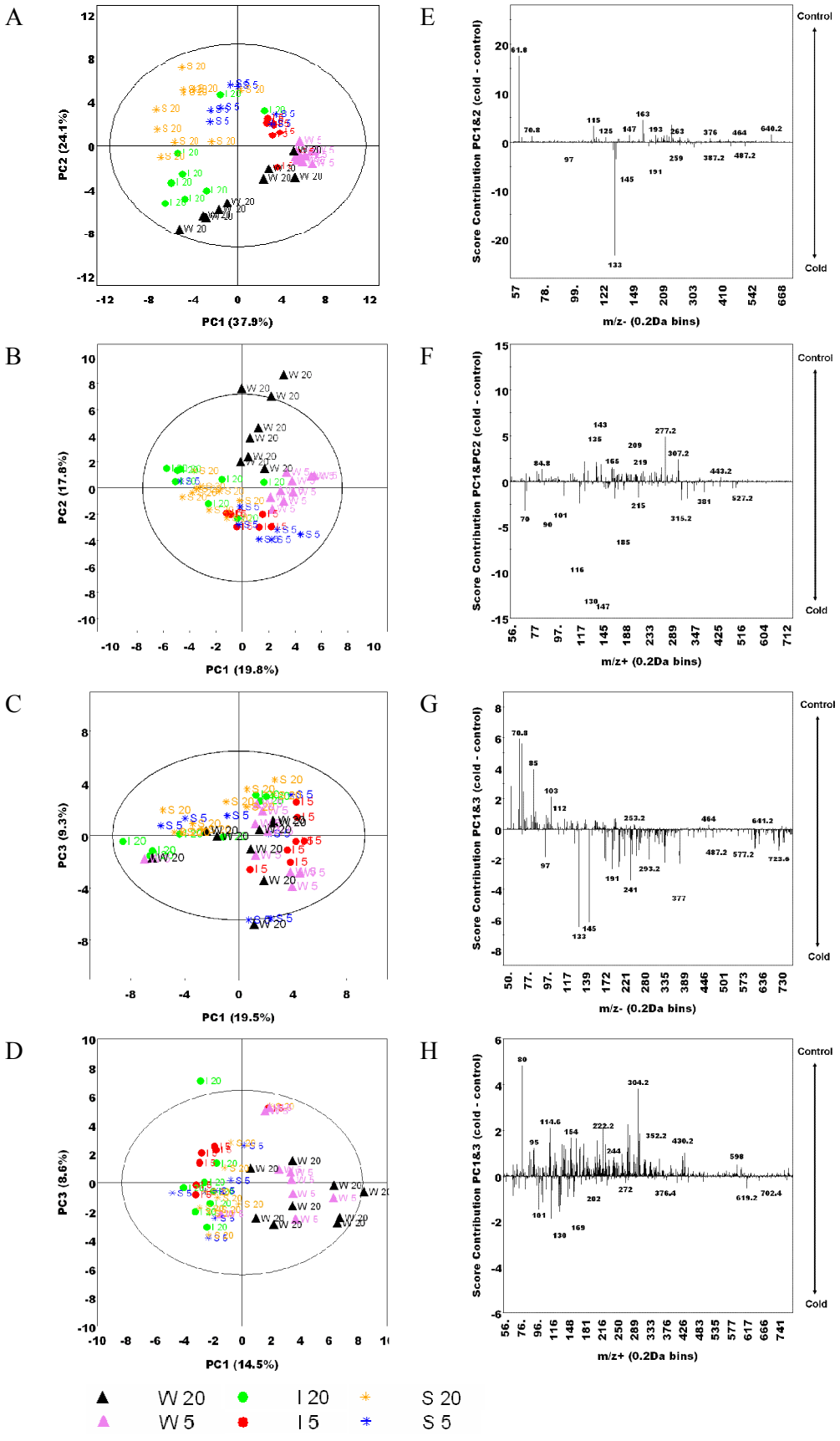

Figure 4. Score scatter plots from principal component analysis of *m/z* values (binned to 0.2Da) obtained by metabolic fingerprinting of *Arabidopsis lyrata* spp. *petraea* populations from Iceland (I), Sweden (S) and Wales (W). The percent of the variation of the data explained by each component is provided in each graph. Fingerprints were obtained from direct injection mass spectrometry of the aqueous phase in negative ionisation (1a, e); aqueous phase in positive ionisation (1b, f); organic phase in negative ionisation (1c, g); organic phase in positive ionisation (1d, h). The score contribution plots (e-h) indicate which bins differ the most between control (more positive) and cold-treated (more negative) plants along the selected principal components (note differences in x-axis scale).

Table1

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Carbohydrates and polyols			Amino acids			
	Metabolite	**All Site differences**	**Biosynthetic family (origin)**	**Metabolite**	**All site differences**	**Site*temp interactions**
Monosaccharide	Arabinose	S > I *	Glycolysis	Serine	n.s.	S > W*
	Xylose	S > I & W *		Glycine	n.s.	n.s.
	Mannose	S > I & W ***		Tryptophan	n.s.	n.s.
	Galactose	S > I & W ***		Phenylalanine	n.s.	n.s.
	Glucose	S > I & W ***	Pentose Phosphate Pathway	Histidine	W > S *	I > W&S**
	Fructose	S > I & W ***	Glycolysis	Alanine	n.s.	n.s.
Disaccharide	Sucrose	n.s.		Valine	n.s.	n.s.
	Trehalose	n.s.		Leucine	n.s.	n.s.
	Maltose	W > S *	Citric acid Cycle	Glutamic acid	n.s.	n.s.
	Lactose	n.s.		Glutamine	n.s.	n.s.
Trisaccharide	Raffinose	n.s.		Arginine	n.s.	I > W&S*
Polyhydric alcohol	Erythritol	n.s.		GABA	n.s.	n.s.
	Inositol	S > I **		Aspartic acid	n.s.	I & S > W*
	Sorbitol	n.s.		Asparagine	n.s.	n.s.
	Mannitol	W > I & S ***		Methionine	n.s.	n.s.
Total monosaccharides		S > I & W ***		Lysine	n.s.	n.s.
Total di- and saccharides		n.s.		Threonine	W > S **	n.s.
Total polyols		n.s.		Isoleucine	n.s.	I > W**
Total carbohydrates and polyols		S > I & W ***	Total amino acids		n.s.	n.s.

Table 1: Significant differences of carbohydrate, polyol and amino acid concentrations of *Arabidopsis lyrata* ssp. *petraea* between all sites (Wales, W; Sweden, S; Iceland, I) and site*temperature interactions from a Multivariate General Linear Model analysis of variance (ANOVA) followed by a Tukeys Post-Hoc test to identify which population has the greatest significant difference between control and treatment temperatures compared to the other populations. * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$. n.s. = not significant.

Table2

	Aqueous Negative	Aqueous Positive	Organic Negative	Organic Positive
N°. of significantly different bins between ALL treatments and populations at $P<0.05$	211	240	155	236
Percent significantly different bins between ALL treatments and populations at $P<0.05$	22	19	11	13
N°. of significantly different bins between ALL treatments and populations below the Bonferroni *P* value	68*	43†	5‡	19§
Percent significantly different bins between ALL treatments and populations below the Bonferonni *P* value	7	3	0	1

Table 2. The number and percent of significantly different bins (0.2 Da bins), based on % total ion count, obtained for each solvent extract phase and mass spectrometer ionisation mode between control (20 ºC) and cold treated (5 ºC) Icelandic, Swedish and Welsh populations of *Arabidopsis lyrata* spp. *petraea*. The number of bins below a *P* value of 0.05 and of a Bonferroni *P* value to reduce the number of false positives. Bonferonni *P* value (0.05/n°. of bins compared): * $P = (0.05/942) = 0.000053$; † $P = (0.05/1232) = 0.000041$; ‡ $P = (0.05/1454) = 0.000034$; § $P = (0.05/1788) = 0.000028$.

Table3

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Phase and ionisation mode	Comment/response	BIN [M-H] or [M+H]	PCA Score contribution	Putative metabolite identification [m/z +/- H+]				
	High I 20; low I/W 5	163*	4.49	Caffeic aldehyde	p-Coumaric acid	L-Fucose	Phenylpyruvic acid	D-Rhamnose
	High S; low W	115*	2.70	Fumaric acid	2-Ketoisovalerate	Maleate		
	High I 20; low I 5, S	193*	2.12	Ferulic acid	D-Galacturonic acid	5-Hydroxyconiferyl aldehyde		
Aqueous negative	High I 20; low S, W 5	212	1.77	L-4_Aspartyl phosphate				
	High I 5, low S 20	133*	-24.94	L-Malic acid				
	High S 5; low I	145*	-4.24	L-Glutamine	α-Ketoglutaric acid	Ketopantoic acid		
	High I 5; low S 20	191*	-4.23	Citric acid	Isocitric acid	Quinic acid		
	High I 5; low S 20 (isotope of 133?)	134*	-3.47	L-Homocysteine	Phenylacetaldoxime			
	High W 20; low S 20/5, I 20	277.2*	6.38	Saccharopine				
	High 20, low W 5	135	5.45	L-Malic acid				
	High I 20; low W 5	140*	5.03	L-Histidinal	6-Hydroxynicotinic acid			
Aqueous positive	High I 20; Low 5	209	4.11	L-Kynurenine	2-(2'-Methylthio)ethylmalic acid			
	High I 5; low I 20	147*	-15.82	L-Glutamine	α-Ketoglutaric acid	Ketopantoic acid		
	High W; low I 20	130*	-14.90	Pyroglutamic acid				
	High I/S/W 5, S 20; Low W 20	116	-12.38	L-Proline				
	High I 5; low I/W 20	175.2*	-6.71	L-Arginine				
	High I/S 20; low S 5	87	1.94	Pyruvic acid				
	High I; low W	117	1.21	Succinic acid				
	High 20 all; low 5 all	112	1.12	L-Δ1-Pyrroline-5-carboxylate				
Organic negative	High S/I 20; low S/I 5	94	1.08	2-Hydroxypyridine	4-Hydroxypyridine			
	High I/W 5; low 20, S 5	133	-4.15	L-Malic acid				
	High I 5; low all (isotope of 145?)	146	-2.64	O-Acetyl-L-serine	L-Glutamic acid			
	High I/W 5; low I/S/W 20	145	-2.44	L-Glutamine	α-ketoglutaric acid	Ketopantoic acid		
	High 5; low20 all	316.2	-2.33	4-Hydroxysphinganine				
	High W 20; low I/S	277.2*	4.84	Saccharopine				
	High I/S 20; low I/S 5, W 20/5	302.2	2.57	Sphinganine				
	High I/S 20; low W 5	135	2.51	L-Malic acid				
Organic positive	High S/I 20; low I/W 5	222.2	1.96	Dihydrozeatin				
	High 5; low20 all	130	-2.23	Pyroglutamic acid				
	High 5; low 20 all	169	-1.89	Homogentisate	Pyridoxamine	Urate	Vanillic acid	
	High 5; low20 all	116	-1.82	L-Proline				
	High W 20/5, S/I 20; low S/I 5	104	-1.74	γ-Aminobutyruc acid				

* = Bin was also significantly different between treatments at the Bonferonni *P* value level (Table 2)

Table 3. The putative metabolite identification for the top four most positive (control 20 ºC) and negative (cold 5 ºC) PCA contribution scores for each extraction and ionisation mode for *Arabidopsis lyrata* spp. *petraea*. A high positive or negative value indicates a high contribution that such a bin has on separating control (more positive) or cold treated (more negative) treatments. Metabolites for each bin [m +/- H adduct] were putatively identified using KNApSAcK v1.2. http://kanaya.naist.jp/KNApSAcK/ using a search resolution of ±0.1Da. Only metabolites found and reported in *Arabidopsis thaliana* were selected. I = Iceland; S = Sweden; W = Wales; High = high ion count; Low = low ion count; 20 = 20 ºC control; 5 = 5 ºC cold treatment.