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

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Abbreviated title:

Cold temperature metabolic phenotypes

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4 **Abstract**
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6 Atmospheric temperature is a key factor in determining the distribution of a plant
7 species. Alongside this, plant populations growing at the margin of their range may
8 exhibit traits that indicate genetic differentiation and adaptation to their local abiotic
9 environment. We investigated whether geographically separated marginal
10 populations of *Arabidopsis lyrata* ssp. *petraea* have distinct metabolic phenotypes
11 associated with exposure to cold temperatures. Seeds of *A. petraea* were obtained
12 from populations along a latitudinal gradient, namely Wales, Sweden and Iceland and
13 grown in a controlled cabinet environment. Mannose, glucose, fructose, sucrose and
14 raffinose concentrations were different between cold treatments and populations,
15 especially in the Welsh population, but polyhydric alcohol concentrations were not.
16 The free amino acid compositions were population specific, with fold differences in
17 most amino acids, especially in the Icelandic populations, with gross changes in
18 amino acids, particularly those associated with glutamine metabolism. Metabolic
19 fingerprints and profiles were obtained. Principal component analysis (PCA) of
20 metabolite fingerprints revealed metabolic characteristic phenotypes for each
21 population and temperature. It is suggested that amino acids and carbohydrates were
22 responsible for discriminating populations within the PCA. Metabolite fingerprinting
23 and profiling has proved to be sufficiently sensitive to identify metabolic differences
24 between plant populations at different atmospheric temperatures. These findings show
25 that there is significant natural variation in cold metabolism among populations of *A. l.*
26 *petraea* which may signify plant adaptation to local climates.
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53 **Key words:**
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55 *Arabidopsis lyrata* ssp. *petraea*; Cold; Direct Injection Mass Spectrometry;
56 Environmental Metabolomics; Metabolic Phenotypes; Metabolite Fingerprinting.
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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>).

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4 Characterising phenotypes of such marginal populations where temperature regimes
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6 differ is of interest as they pose pertinent questions about evolutionary adaptations,
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8 along with the limits of such adaptations (Vergeer et al., 2008). Alongside the genetic
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10 differences between populations, we have already discovered that populations of this
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12 species can be distinguished by their metabolic phenotypes when grown under the
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14 same controlled conditions (Davey et al., 2008). Therefore, we hypothesised that
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16 populations growing at more northern latitudes where the minimum temperatures are
17
18 different will exhibit metabolic phenotypes that are site and temperature specific.
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22 Assessing such spatial variation in metabolic responses to cold will provide
23
24 information on cold acclimation and possible adaptive processes. To this end, we
25
26 examined in detail the global metabolic phenotypes of plant foliage from three
27
28 populations at control and cold temperatures. Metabolite fingerprints were obtained
29
30 by direct injection mass spectrometry and metabolite profiling was targeted on the
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32 soluble, free carbohydrates, polyhydric alcohols and amino acids because of their
33
34 known association with plant abiotic responses (Hannah et al., 2006; Smirnov, 1998;
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36 Stitt and Hurry, 2002, Usadel et al., 2008).
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42 **2 Methods**

43 44 45 46 47 *2.1 Growth*

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52 Seeds of *Arabidopsis lyrata* (L.) ssp. *petraea* were collected from populations in
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54 Iceland, Sweden and Wales as described in Davey et al. (2008). Approximately 30
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56 seeds were sown in Levington M3 compost within individual seed trays covered with
57
58 an incubator lid (16.5 x 9.5 x 4.5 cm). Trays were placed inside one of two
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60 controlled-environment growth cabinets (Conviron Controlled Environments Limited,
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4 Canada). Cabinet conditions within a 1.5 m² growth area were 16/8 hours day/night;
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6 20 °C day/night; 60% humidity; atmospheric CO₂ ca. 400 ppm CO₂; light 250 μmol
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8 m⁻² sec⁻¹. Growth rates were similar and so after 7 days when the seeds had fully
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10 expanded cotyledons, up to 20 seedlings from each population were individually
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12 transferred to larger plant pots (7 x 7 x 8 cm) containing Levington M3 compost.
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14 Each growth cabinet had an equal number of plants per population. Plants were
15
16 watered from the base of the pot so the soil was moist, rather than sodden, when
17
18 required with reverse osmosis (RO) water. No additional nutrients were added to the
19
20 soil or water. After a further three weeks, ten of each *A. petraea* populations were
21
22 randomly transferred in equal numbers to one of two growth cabinets set at a
23
24 day/night temperature of 5 °C (all other conditions were as above, apart from relative
25
26 humidity which increased to 80-90 %). There were slight visual differences in leaf
27
28 morphology between populations (Data not shown) (Davey et al., 2008, Vergeer et al.,
29
30 2008). After another 7 days and after six hours into the daylight period the foliage
31
32 was excised at soil level with a razor blade. Leaf tissue was chosen for this study, as
33
34 this organ is regularly exposed to changes in air temperature in the wild. The foliage
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36 was immediately immersed in liquid nitrogen and stored at -80 °C.
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45 *2.2 Metabolite extraction and analyses*

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50 Metabolites were extracted and analysed as described in Davey et al. (2008). Briefly,
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52 approximately 100 mg leaf tissue per plant was extracted using 2 ml
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54 MeOH/CHCl₃/H₂O (2.5:1:1) followed by 1 ml MeOH/CHCl₃ (1:1). The organic
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56 CHCl₃ phase was separated from the aqueous MeOH:H₂O phase by adding 500 μl
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58 H₂O. The aqueous phase was analysed for free amino acids by HPLC and free
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60 carbohydrates and polyhydric alcohols by Gas Chromatography (GC). Aqueous and
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4 organic phases were directly injected into a LCT mass spectrometer (Waters Ltd.
5
6 Manchester, UK) using a MassLynx V.4.0 data system in negative and positive
7
8 ionisation modes (50-800 m/z).
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10 11 12 13 *2.3 Metabolite fingerprinting – chemometrics* 14

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17 Raw centroid mass/charge (m/z) ratios from triplicate analytical runs were combined
18
19 into 0.2 Da mass unit ‘bins’ for noise reduction and data alignment using in-house
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21 software. m/z peaks were assigned to bins only if an ion count of similar intensity and
22
23 m/z range is detected for a peak in each of the three analytical runs for each biological
24
25 sample. Binned m/z and percent total ion count (%TIC) values from the aqueous and
26
27 organic phases, analysed in both negative and positive ion mode on the mass
28
29 spectrometer, were explored by Principal Component Analysis (PCA) using Simca-P
30
31 V.11.5 (Umetrics, Sweden) as described in Davey et al. (2008). The statistical
32
33 differences between the %TIC of each 0.2Da bin was carried out using ANOVA
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35 followed by Post-Hoc tests for multiple comparisons. Significant differences are given
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37 at $P \leq 0.05$ and at a Bonferroni P value of $0.05/n$ where n = number of bins tested.
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42 Metabolites for each bin were putatively identified using KNApSAcK v1.2.
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44 <http://kanaya.naist.jp/KNApSAcK/> using a search resolution of ± 0.1 Da. Only
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46 metabolites that have been reported in *Arabidopsis thaliana* were selected in
47
48 KNApSAcK. A Multivariate General Linear Model analysis of variance (ANOVA)
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50 followed by a Tukeys Post-Hoc test for multiple comparisons was used to test for
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52 significant differences in amino acid and carbohydrate composition between
53
54 populations and temperatures using SPSS v12.0.1 (Chicago, Illinois, USA).
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61 **3 Results** 62 63 64 65

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6 3.1 Targeted analysis
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10 3.1.1 Free soluble carbohydrates
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15 The concentration of soluble carbohydrates and polyhydric alcohols varied between
16 populations and treatments (Fig. 1). The concentration of the majority of
17 carbohydrates was increased in all populations after the cold treatment. The Welsh
18 population had the largest number of mono-, di- and trisaccharides that significantly
19 increased with the cold temperature (mannose, glucose, fructose, sucrose, raffinose).
20 There were two carbohydrates that increased with cold in the Swedish population
21 (sucrose, raffinose) and no carbohydrates were significantly affected in the Icelandic
22 population. The largest increase in response to cold was the mannose concentration in
23 the Welsh population. Polyhydric alcohols were not significantly increased by cold
24 treatment.
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38 There were significant temperature increases in the total monosaccharide pool after
39 exposure to cold in the Welsh population ($P \geq 0.001$) and in the total di- and
40 trisaccharide pools in the Swedish population ($P \geq 0.001$). There were no significant
41 differences in the total polyol or total carbohydrate or polyol pool for any population.
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60 3.1.2 Free amino acids
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4 The concentration of soluble amino acids varied between populations and treatments,
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6 with the majority of amino acids increasing in concentration after the cold treatment
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8 (Fig. 2). The Welsh population had the lowest number of amino acids that
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10 significantly increased with the cold temperature (serine, glycine, alanine, glutamine).
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12 There were five amino acids that increased with cold in the Swedish population
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14 (phenylalanine, alanine, glutamic acid, GABA, aspartic acid) and there were ten
15
16 amino acids that were significantly affected in the Icelandic population (glycine,
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18 histidine, alanine, glutamic acid, glutamine, arginine, GABA, aspartic acid,
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20 asparagine, isoleucine). In particular, amino acids derived from the citric acid cycle
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22 all were found at higher concentrations in cold-treated Icelandic plants. The total
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24 amino acid pools for the Swedish and Icelandic populations were significantly
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26 increased by cold treatment ($P \geq 0.01$ total data not shown). There were significant
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28 site differences with the Welsh population having higher concentrations of histidine
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30 and threonine than the Swedish population (Table 1). There were also significant
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32 site*temperature interactions for histidine, arginine, aspartic acid and isoleucine as the
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34 Icelandic populations had a different response to cold treatment than the other
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36 populations, whereas serine had a significantly different response to cold in the
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38 Swedish population than the Welsh population.
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45 46 47 *3.2 Metabolite fingerprinting* 48 49

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52 To check the overall variation between populations and cold treatment in other
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54 metabolites alongside those detected in the targeted analysis, a DIMS analysis was
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56 carried out. There were between 155 and 240 bins (0.2 Da range Bins) that had a
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58 significantly different %TIC between control and cold-treated plants among all
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60 populations with a P value below 0.05 (Table 2). To reduce the risk of false positive
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4 discoveries, a Bonferroni P value was calculated for each extraction phase and
5
6 ionisation mode. In terms of percent change, the metabolites that were detectable in
7
8 the aqueous phase by negative ionisation showed the largest response to cold (Fig. 3).
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10 Across all extract phases and ionisation modes, the Icelandic population had the most
11
12 significantly changing bins in response to cold. The Icelandic and Welsh populations
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14 shared the most significantly different bins from the aqueous phase between
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16 temperatures.
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22 *3.3 Principal component analysis (PCA)*

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27 Masses detected within the aqueous phase analysed in the negative and positive
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29 ionisation mode showed separate clusters of Welsh, Swedish and Icelandic samples
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31 along principal component (PC) 2 (Figs. 4a and 4b). Samples that were exposed to
32
33 cold were clustered separately from the control samples along PC1. In particular, the
34
35 Welsh samples had distinct clusters away from Swedish and Icelandic plants.
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37 There was no distinct separation of populations and treatments of m/z values acquired
38
39 in the organic fraction when analysed in the negative ion mode. There was only some
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41 separation of cold treatment along PC1 (Fig. 4c). However, when analysed in the
42
43 positive mode there were separate clusters of Swedish, Icelandic and in particular
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45 Welsh samples along principal component 1 (Fig. 4d). Samples that were exposed to
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47 cold were very weakly separated from the control samples along PC3.
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54 The scores contribution plots (Figs. 4e - h) indicate which bins differ the most
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56 between control (more positive) and cold-treated (more negative) plants along the
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58 selected principal components for each phase and ionisation mode. There were few
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60 bins that differed in intensity in the aqueous phase analysed in the negative ionisation
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4 mode. However, the score contribution values were very high for these bins,
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6 indicating a strong influence on separating control and cold-treated plants. There was
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8 a stronger and a more even distribution of bins that differed between control and
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10 treatment from the aqueous phase analysed in the positive mode (Fig. 4f). The
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12 organic phase in negative ionisation mode showed a different distribution of bins that
13
14 differed between control and cold samples as more low mass bins were related to
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16 control conditions but more mid-range (>140 m/z) and high-range (>500 m/z) mass
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18 bins were related to cold conditions (Fig. 4g). However, the organic phase analysed in
19
20 the positive mode showed that most bins were related to control conditions with only
21
22 a few bins in the low mass range (<200 m/z) that were increased under cold
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24 conditions (Fig. 4h).
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29 The putative metabolite identification for the top four most positive (control) and
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31 negative (cold) PCA contribution scores (Fig. 4e – h) for each extraction and
32
33 ionisation mode are presented in table 3. As expected from the solvent extraction
34
35 procedure used, the majority of the top bins that were responding to cold were
36
37 assigned to low molecular weight amino acids, carbohydrates, organic acids,
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39 phenylpropanoids and phenolics. Most of the bins detected in the aqueous phase that
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41 were identified as significantly different in the ANOVA within the Bonferroni *P*-
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43 value limit (Table 2) were also detected as major contributing bins in the PCA (Table
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45 3) implying that the correct principal components were used. The complete lists of
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47 bins and respective putative compound identifications that were separating out
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49 populations and cold treatment of each such mass for each ion mode and fraction are
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51 presented as supplementary data.
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59 **4 Discussion**

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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

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4 the plant and used for respiratory needs as more gross morphological and biochemical
5 changes occurs during acclimation (Atkin et al., 2005; Guy et al., 1992; Strand et al.,
6 1999).
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10 Amino acids have been shown to increase in concentration in a variety of
11 perennial plant species during colder winter months (Sagisaka and Araki, 1983).
12 Alterations in amino acid accumulation in response to cold were also measured in *A.*
13 *petraea*. Most alterations occurred in compounds derived from the citric acid cycle,
14 especially in the Icelandic population. This response in *A. lyrata* is comparable to
15 cold responses in other species such as *Arabidopsis thaliana* as Kaplan et al. (2004)
16 also found that amino acids derived from the citric acid cycle increased in
17 concentration with cold treatments. Glutamine, glutamate, aspartate and asparagine
18 are important amino acids in nitrogen assimilation, storage and N transport in plants
19 and all were shown in increase in concentration during cold treatment (Iceland >
20 Sweden > Wales). By far the most significant response to cold was the accumulation
21 of glutamine (Fig. 2). Glutamine has also been reported to increase in concentration
22 with cold in *A. thaliana* (Klotke et al., 2004) and asparagine is known to transport N
23 around the plant.
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43 The enzymes glutamine synthetase (GS); glutamate synthase (GOGAT);
44 aspartate aminotransferase (AAT) and asparagine synthetase (ASN) play an important
45 role in accumulating these amino acids. Increased aspartic acid may be beneficial to
46 the plant during cold as mutants of AAT, that decreased aspartic production, showed
47 reduced growth phenotypes (Coruzzi, 2003; Lam et al., 1995). With the Icelandic and
48 Swedish populations having more aspartic acid in cold leaves over the Welsh
49 population, this may prove to be advantageous during cold acclimation and growth.
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59 Glutamine synthetase is encoded by multiple genes (*GLN*) (Peterman and
60 Goodman, 1991) with the expression being increased by exposing plants to light and
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4 interestingly, by carbohydrate accumulation such as sucrose (Oliveira and Coruzzi,
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6 1999; Suzuki and Knaff, 2005). An increased expression of *GLN1.1* to form
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8 glutamine would increase the N available for assimilation into maintained growth or
9
10 increased translocation from senescing or damaged leaves to form newly acclimated
11
12 leaves (Bleeckerl and Patterson, 1997; Lam et al., 1995; Li et al., 2006). However,
13
14 the gene family encoding ASN (*ASN1-3*) is mainly expressed, and asparagine
15
16 accumulated, at night (Lam et al., 1998; Miesak and Coruzzi, 2002) as the daytime
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18 repression of *ASN* expression is also linked to light and carbohydrate accumulation
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20 (Coruzzi, 2003; Lam et al., 1994, 1995). Therefore, if carbohydrate concentrations
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22 are increased during cold, then the gene expression for GS and ASN should be further
23
24 increased and decreased respectively. We found this to be true in other populations of
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26 *A. petraea* (Norway and Ireland) that were cold shocked for 8 hours as *GLN1.1*
27
28 increased and *ASN3* decreased its expression (Dr Catherine Lilley – paper in
29
30 preparation). Kilian et al. (2007) also reported a 45% decrease in *ASN3* and a 2000%
31
32 increase in *GLN1.1* in expression in 24h cold-treated *A. thaliana* (eFP-brower
33
34 <http://bar.utoronto.ca/efp/cgi-bin/efpweb.cgi>). Alongside this, there was a reported
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36 increase of more than 3000% in sucrose synthetase (*SUS1*) (Kilian et al., 2007) in
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38 response to cold, which was also shown to increase in the other two populations of *A.*
39
40 *petraea* (unpublished data). The accumulation of glutamine may relieve some of the
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42 repression on *ASN3* as studies have shown that addition of glutamine alleviates the
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44 repression made by sucrose abundance on *ASN* expression and may explain the
45
46 increase in asparagine in response to cold in our study (Lam et al., 1994, 1995). It is
47
48 still unclear whether this activation and repression of such gene expressions is
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50 continued into the night in cold treated plants that accumulate carbohydrates and
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52 whether an increase in carbohydrates is more important to the plant than an increase
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54 or mobilisation of N.
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4 Although the variation in relationships between gene expression and metabolite
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6 concentrations can be high in cold acclimating plants (Kaplan et al., 2007) an increase
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8 in *SUS1* and *GLN1.1* and a decrease in *ASN3* gene expression infer tight signalling
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10 processes involved in the metabolic control of cold exposure and cold acclimation.
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12 This may explain the inverse relationship between the number of significantly higher
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14 concentrations of amino acids and carbohydrates in cold-treated Icelandic (10 amino
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16 acids to 0 CHO) when compared to Swedish (5 to 2) and Welsh (4 to 5) populations.
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22 *4.2 Metabolite fingerprinting*

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27 This study has shown that there is intraspecific variation in the metabolic phenotype
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29 that plants have in response to cold temperature. The metabolite fingerprinting
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31 approach shows that shifts in the metabolome of *A. petraea* that were exposed to cold
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33 is detectable, with Icelandic and Welsh populations sharing the most significantly
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35 different changing bins. The control population phenotypes were similar to those
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37 reported in Davey et al. (2008).
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40 The initial screen of the 0.2Da bins showed significant differences between
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42 control and cold-treated plants for each population. We detected approximately 3000
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44 masses (0.2 Da bins) across all extraction phases and ionisation modes. Gray and
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46 Heath (2005) found 1187 masses (DIMS-Fourier Transform-Ion Cyclotron
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48 Resonance) when masses were pooled and compared from all ion modes and
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50 extraction phases, therefore as one metabolite may produce more than one mass peak
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52 and may be detectable in more than one ionisation mode or extraction phase the
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54 number of masses detected are comparable with their findings. About 8% of the
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56 masses found by Gray and Heath (2005) significantly increased or decreased in
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58 intensity after seven days cold treatment. This is in agreement with our findings that
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4 on average across ion modes, extraction phases and populations 7.2% of the bins
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6 detected had significantly altered ion intensities with cold treatment (Fig. 3). There
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8 were more bins detected in extracts that were analysed in the positive ionisation mode,
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10 which could either be the result of more metabolites being able to ionise in positive
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12 mode than negative, or more adducts being formed. However, none of the most
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14 significant bins were found to match possible K or Na adducts of metabolites.
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18 Metabolites that were associated with cold treatment were detected using both
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20 Bonferroni statistics and PCA loading scores, the majority of which were assigned to
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22 amino acids, carbohydrates, organic acids, phenylpropanoids and phenolics. Many of
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24 the highly statistically significant metabolites putatively identified by DIMS (Table 3
25
26 and supplementary data) are associated with the citric acid cycle and similar to the
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28 targeted analysis were also up regulated more so in the Icelandic population. From
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30 the DIMS we also found that masses for malate and glutamine increased and fumarate
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32 and succinate decreased. It is likely that the metabolite found at bin 145 (negative
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34 ion) and 147 (positive ion) is glutamine (Table 3). The raw unbinned mass spectrum
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36 for this bin was $145.099\ m/z^-$ and $147.029\ m/z^+$. The difference between the
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38 monoisotopic mass of glutamine (146.069) and the unknown mass is 0.038Da in the
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40 negative ion and 0.047Da in the positive ion. The masses of α -Ketoglutaric acid and
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42 Ketopantoic acid were between 0.049 and 0.095Da away from the unknown peak.
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44 This confirms the result in the targeted analysis where glutamine concentrations
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46 increased in concentration with cold treatment.
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52 The mass of metabolite 3-Hydroxypropyl glucosinolate (Bin 376 negative ion)
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54 was found to discriminate Welsh plants grown at 20°C from all other populations at
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56 both control and cold temperatures. This mass had a highly positive PCA loading
57
58 score (see supplementary data) and was significantly different to the other populations
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60 and treatments above the Bonferroni *P* value. This mass was also found to
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4 discriminate Wales from other populations in our previous study (Davey et al., 2008).
5
6 Therefore, although this metabolite can distinguish populations it may not be involved
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8 in cold tolerance mechanisms as it was not accumulated during the cold treatment in
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10 the Welsh, or any other population.
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15 **5 Conclusion**

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20 Metabolite fingerprinting and profiling has proved to be sufficiently sensitive to detect
21
22 unique cold-induced metabolic phenotypes between populations of *A. petraea* across a
23
24 broad geographical and climate distribution. A mixture of unique metabolic changes
25
26 to cold and more general responses as described in other species was measured.
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29 Principal component analysis (PCA) of metabolite fingerprints revealed metabolic
30
31 phenotypes for each population and temperature. Mannose, glucose, fructose, sucrose
32
33 and raffinose concentrations were different between cold treatments and populations,
34
35 especially in the Welsh population but polyhydric alcohol concentrations were not.
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38 The free amino acid compositions were population specific, with fold differences in
39
40 most amino acids, especially in the Icelandic populations with gross changes in amino
41
42 acids associated with the citric acid cycle, in particular glutamine metabolism. Such
43
44 intraspecific variation in the metabolome of cold-treated plants may ultimately affect
45
46 the plants ability to acclimate and adapt to new environments. This will be
47
48 particularly important for plant populations living at the margin of their range and
49
50 threatened by rapid climate change.
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56 **Acknowledgements**

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14 expression data.
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Figure1

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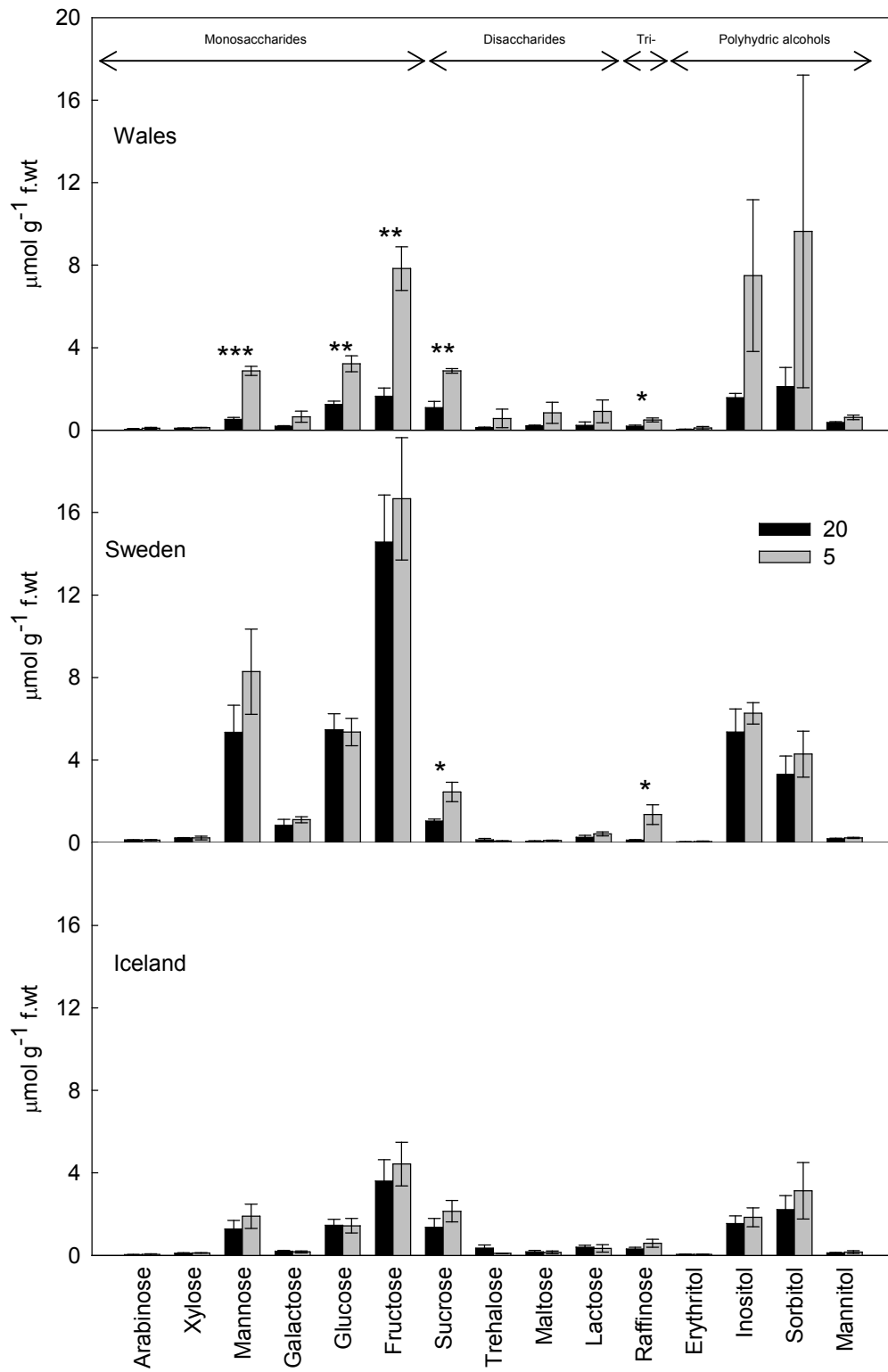


Figure 1: Concentrations of free carbohydrates and polyhydric alcohols in the leaves of *Arabidopsis lyrata* ssp. *petraea* (Wales, Sweden, Iceland) grown under control (20 °C) or cold (5 °C for 7 days) conditions. Significant differences between control and cold treatments within each population are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

Figure2

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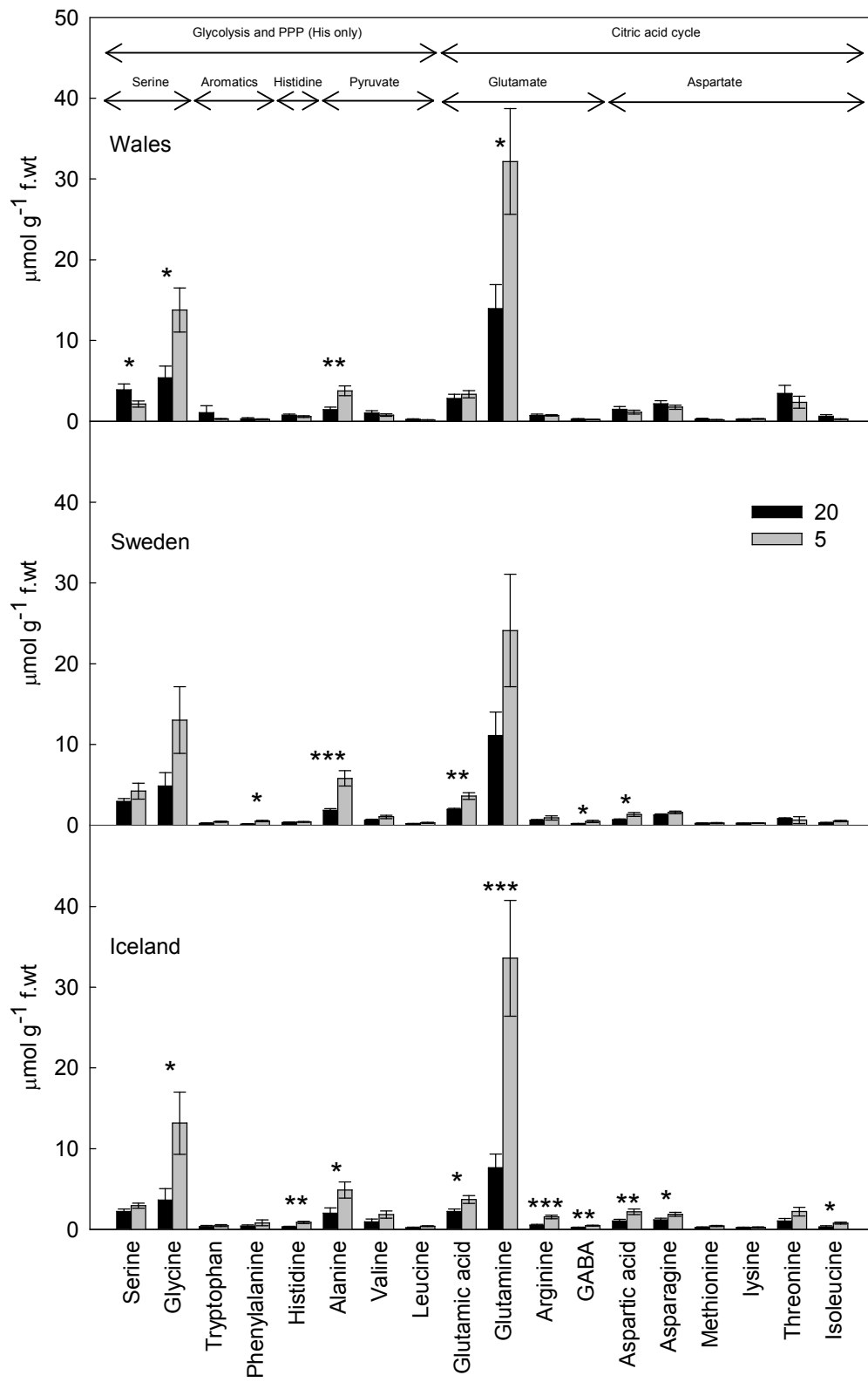


Figure 2: Concentrations of soluble free amino acids in the leaves of *Arabidopsis lyrata* ssp. *petraea* (Wales, Sweden, Iceland) grown under control (20 °C) or cold (5 °C for 7 days) conditions. Amino acids are listed according to their biosynthetic family and origin. Significant differences between 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 (\pm SE).

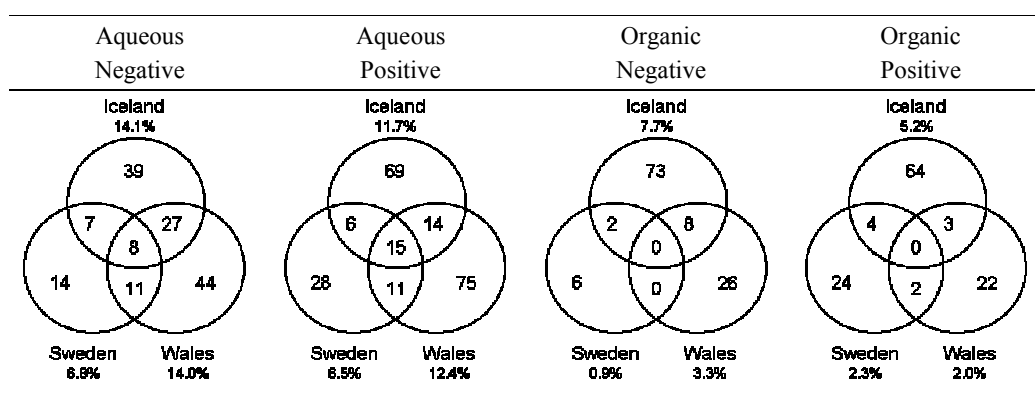
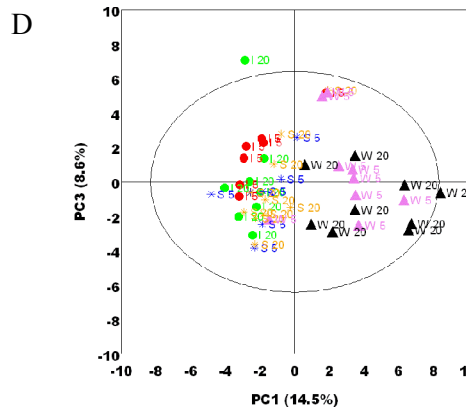
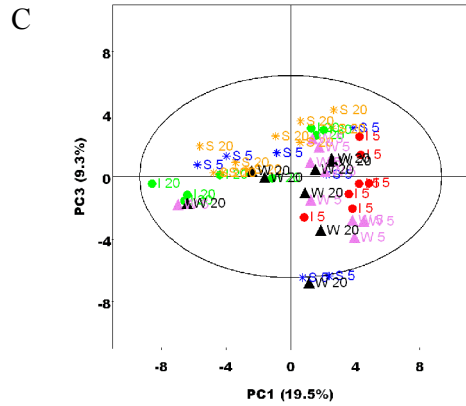
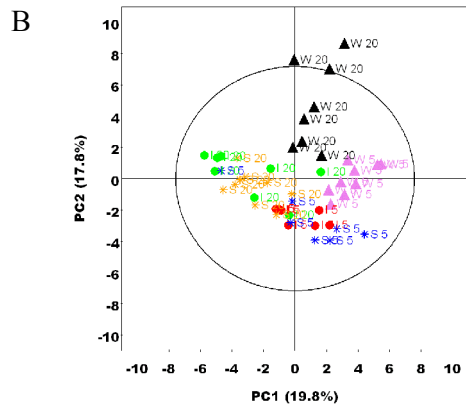
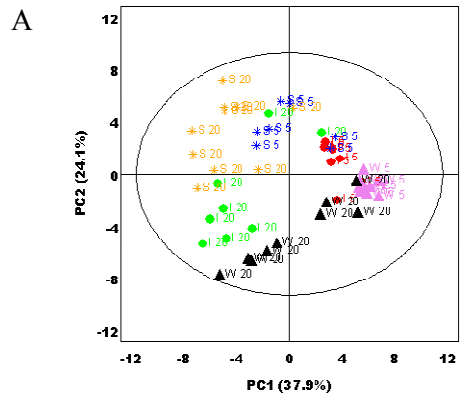


Figure 3. Venn diagram of the number of significantly different bins ($P < 0.05$) between control and cold-treated Icelandic, Swedish and Welsh populations of *Arabidopsis lyrata* spp. *petraea* plants with overlapped regions denoting the number of shared changing bins of the selected population. The 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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▲ W 20 ● I 20 * S 20
 ▲ W 5 ● I 5 * S 5

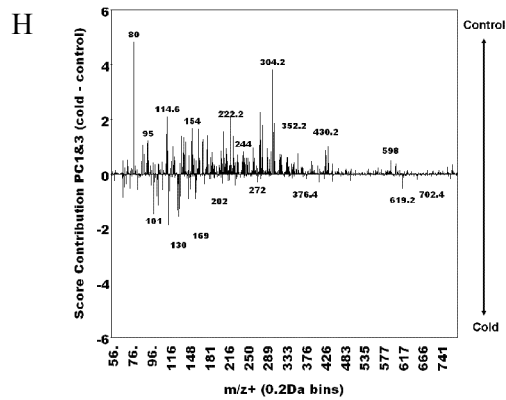
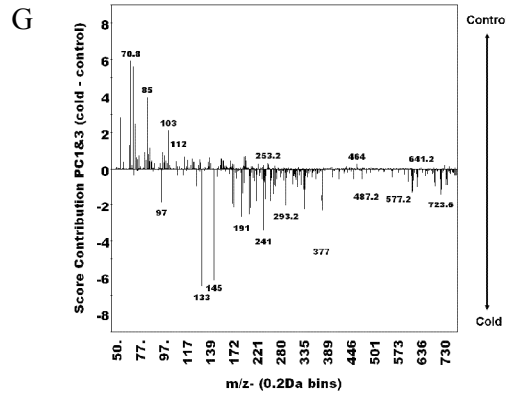
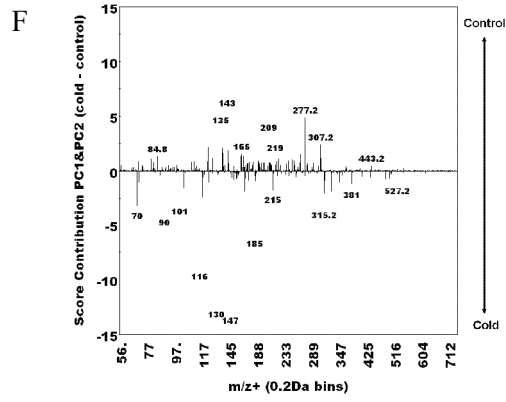
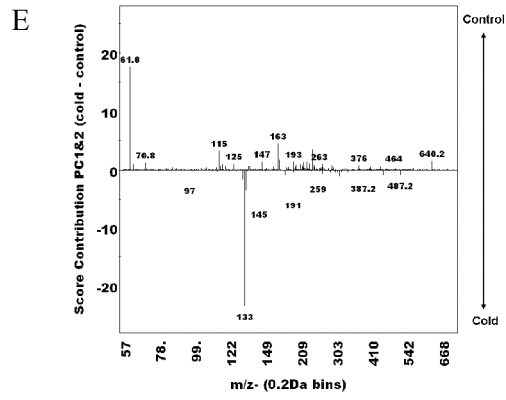


Figure 4. Score scatter plots from principal component analysis of m/z values (binned to 0.2Da) obtained by metabolic fingerprinting of *Arabidopsis lyrata* spp. *petraea* populations from Iceland (I), Sweden (S) and Wales (W). The percent of the variation of the data explained by each component is provided 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 ***	Pentose Phosphate Pathway	Phenylalanine	n.s.	n.s.
	Glucose	S > I & W ***		Histidine	W > S *	I > W&S**
	Fructose	S > I & W ***		Glycolysis	Alanine	n.s.
Disaccharide	Sucrose	n.s.	Citric acid Cycle	Valine	n.s.	n.s.
	Trehalose	n.s.		Leucine	n.s.	n.s.
	Maltose	W > S *		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

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	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 Bonferroni 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 °C) and cold treated (5 °C) 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. Bonferroni 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+]				
Aqueous negative	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		
	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				
Aqueous positive	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			
	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					
Organic negative	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				
	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; low 20 all	316.2	-2.33	4-Hydroxysphinganine					
Organic positive	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				
	High S/I 20; low I/W 5	222.2	1.96	Dihydrozeatin				
	High 5; low 20 all	130	-2.23	Pyroglutamic acid				
	High 5; low 20 all	169	-1.89	Homogentisate	Pyridoxamine	Urate	Vanillic acid	
	High 5; low 20 all	116	-1.82	L-Proline				
	High W 20/5, S/I 20; low S/I 5	104	-1.74	γ-Aminobutyric 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 °C) and negative (cold 5 °C) 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 ± 0.1 Da. 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 °C control; 5 = 5 °C cold treatment.