

Development of a Model System to Identify Differences in Spring and Winter Oat

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Abstract

Our long-term goal is to develop a Swedish winter oat (*Avena sativa*). To identify molecular differences that correlate with winter hardiness, a winter oat model comprising of both non-hardy spring lines and winter hardy lines is needed. To achieve this, we selected 294 oat breeding lines, originating from various Russian, German, and American winter oat breeding programs and tested them in the field in south- and western Sweden. By assaying for winter survival and agricultural properties during four consecutive seasons, we identified 14 breeding lines of different origins that not only survived the winter but also were agronomically better than the rest. Laboratory tests including electrolytic leakage, controlled crown freezing assay, expression analysis of the *AsVrn1* gene and monitoring of flowering time suggested that the American lines had the highest freezing tolerance, although the German lines performed better in the field. Finally, six lines constituting the two most freezing tolerant lines, two intermediate lines and two spring cultivars were chosen to build a winter oat model system. Metabolic profiling of non-acclimated and cold acclimated leaf tissue samples isolated from the six selected lines revealed differential expression patterns of 245 metabolites including several sugars, amino acids, organic acids and 181 hitherto unknown metabolites. The expression patterns of 107 metabolites showed significant interactions with either a cultivar or a time-point. Further identification, characterisation and validation of these metabolites will lead to an increased understanding of the cold acclimation process in oats. Furthermore, by using the winter oat model system, differential sequencing of crown mRNA populations would lead to identification of various biomarkers to facilitate winter oat breeding.

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Introduction

Oat is the sixth most important cereal crop in the world [1]. During the last 10 years, an average of approx. 900,000 ton of oat was harvested per year in Sweden (www.jordbruksverket.se). The commercial value of oat is derived both from the feed and the food value of the grain, which have unique health promoting properties. In addition, oat has superior break crop benefits. Although only spring oats are grown in Sweden, several European countries grow winter oats, which allow an earlier harvest and give comparably higher yields. In addition, winter oats are more environmentally beneficial than spring oats, as during the winter, cultivated fields bind nutrients and reduce soil erosion. As of now, no winter oat cultivar exists that is hardy enough to be grown commercially in Sweden. Since winter hardiness is a quantitative trait controlled by several genes [2], the traditional plant breeding programs have so far been of limited success in improving the winter hardiness of oats.

Plants adapt to the lower temperatures by a process known as cold acclimation, leading to several physiological, biochemical,

metabolic and molecular changes that allow plants to avoid, tolerate or adapt to the changing environment. Whole genome microarray experiments in the model organism *Arabidopsis* lead to the identification of several differentially expressed genes during cold acclimation [3,4]. Amongst these are genes encoding transcription factors, signal transduction components, osmoregulatory proteins, membrane stabilisation proteins, protein folding regulatory factors, ice nucleation proteins and enzymes involved in the biosynthesis of various kinds of small molecules like polyhydroxylated sugar alcohols, amino acids and derivatives, tertiary sulphonium compounds and quaternary ammonium compounds [2,3,5,6,7]. Putative gene regulatory networks demonstrating the complexities of gene interactions in cold stress in *Arabidopsis* have also been presented [8,9,10]. Collectively, these studies have shown that the most important cold acclimation regulon is encoded by the CBF (C-Repeat Binding Protein) gene family [11].

It is a well known phenomenon that winter cereal cultivars have a vernalisation requirement. They display delayed flowering or no

flowering at all if not cold-treated for prolonged times, while spring cultivars flower without cold treatment [12,13,14]. This is also reflected at the molecular level, since it has been shown that the Vernalization1 (VRN1) gene is regulated by low temperature only in winter cultivars [13,15,16] and thus can be used as a molecular marker in breeding for winter hardiness [17].

Apart from vernalisation-associated genes, several other genes are differentially expressed in the leaves and crowns of both winter hardy and spring cultivars during cold acclimation [18]. Among these are genes that regulate carbohydrate synthesis and metabolism, which leads to the accumulation of various carbohydrates including glucose, sucrose, fructose, maltose, raffinose and fructans during low temperature stress [19,20,21,22,23,24]. Interestingly, this accumulation occurs at different levels in spring and winter wheat cultivars [25]. Although polysaccharides are primarily used as an energy source, they can also act as osmolytes and provide stability to the plasma membrane during the phase transition [26]. Thus, carbohydrate concentrations can be excellent markers for analysing the freezing tolerance of a cultivar.

Cook et al. [19] performed metabolomic analysis of cold acclimated Arabidopsis leaf tissue and monitored expression levels of 434 metabolites using Gas Chromatography Time of Flight Mass Spectrometry (GC-TOF-MS). They found that 26 metabolites increased by more than 25-fold in the cold acclimated plants compared to the non-acclimated controls whereas 88 metabolites increased between 5- and 25-fold. Kaplan et al. [21] monitored expression levels of 497 metabolites and found 311 metabolites with altered expression under cold acclimation. Maruyama et al. [27] showed that DREB1A overexpressing transgenic Arabidopsis plants had increased freezing tolerance and increased levels of several metabolites while DREB2A overexpressors lacked the increased freezing tolerance and the corresponding metabolites, suggesting that, in DREB1A overexpressor, altered metabolite profile levels contribute to the increased freezing tolerance of the plants. Using a statistical model, Korn et al. [23] showed that metabolites such as fumaric acid, succinic acid, fructose, glucose, raffinose, galactinol, glycine and proline contribute to optimal prediction for freezing tolerance. Thus, previous studies suggest that a significant alternation in the metabolome occurs under cold acclimation in plants and is critical for an increased freezing tolerance. In oats, although there are reports on measurements of a few metabolites in crowns under low temperature stress [25,28,29], large scale metabolomics has not yet been reported to our knowledge.

Under freezing temperatures, most of the aerial plant tissue in wheat, barley, and oat is destroyed and recovery from freezing is mainly dependent on surviving cells from the crown [28,30]. Previous studies have shown that the apical meristem in the crown is the most freezing tolerant part of the plant [28]. Controlled crown freezing tests on successive generations have also been successfully used as a selection method in a progeny generated from a cross of oat cultivars Wintok and Norline, from which the cold hardy Win/Nor-1 and Win/Nor-10 oat lines were obtained [31]. Amongst cereal crops, rye is most freezing tolerant, followed by wheat, barley and oat [32]. In places with harsh winters, rye, barley, and wheat are, therefore, the winter crops of choice. Breeding for winter oats, on the other hand, has been of limited success. Winter oat Cv. Wintok released in USA in 1940 and Cv. Norline released by Purdue University in USA, in 1960, are amongst the most winter hardy oat cultivars [31,33]. In UK, winter oat Cv. Gerald released in 1993, by the Institute of Biological, Environmental, and Rural sciences (IBERS), is widely used as a commercial winter oat cultivar in South England and Wales.

Results

Oat field experiments

Before this work started, breeders at Lantmännen Seed AB, had already tested several English winter oat and Nordic spring oat cultivars for winter survival in South and Western Sweden. However, few of those cultivars survived the winter, and none grew well in the successive trials (R. Jonsson, personal communication). For the field trials, we therefore collected various accessions from Germany, Russia and the USA that had been specifically bred for winter hardiness. During four consecutive winters (2003/2004, 2004/2005, 2005/2006 and 2006/2007), these winter oat-breeding lines were tested in winter field trials at two different locations in south- and western Sweden. All lines were scored for field survival, vigour and predicted agronomic value. The field experiments were divided into two phases. Phase-Ia trials were started in 2003 with 294 lines. Winter survival and agronomic characteristics such as yield, panicle density per square meter, time of maturity, and lodging resistance of surviving lines were evaluated in the 2003/2004 growing season. Based on these investigations, 14 lines were chosen for further trials in 2004/2005 and the remaining 280 lines were discontinued from trials. In the spring of 2005, 6 lines that survived winter to at least 60% and showed agronomic value of 6 or above on a scale of 1–9, were selected for further trials. These were planted in the field in the autumn 2005 for phase-II trials along with 7 additional newly introduced lines for phase-Ib trials. A schematic representation of the conducted experiments is shown in Figure 1.

While the survival rate of the commercial oat spring cultivars used as control was 0% in all trials, large differences in survival rates amongst the selected winter breeding lines were noticed. The survival rate in the hardest lines varied from 20%–70% in 2004 and 2005 to 90% in 2007. This variation can be related to variation in winter temperatures (Figure S1). The results of the winter trials are summarised in Table 1. From these trials, we finally identified 14 superior lines that survived not only two or more winters but also scored above average on agronomic characteristics.

Follow up laboratory experiments

Electrolytic leakage. To quantify freezing tolerance in a controlled environment, electrolytic leakage (EL) was estimated in the 14 lines that ranked the best in the field trials. In addition, one spring cultivar, Stork, one English winter hardy cultivar Gerald and one barley winter hardy cultivar, Hampus, were included for comparison. In these experiments, both non-acclimated plants and plants acclimated at +4°C for 48 hours were used. To titrate in appropriate acclimation times and freezing temperatures, the LPWH992209 line, which was one of the best performing lines in the field, was used. At the conditions finally used, LPWH992209 showed a 50% EL at –8°C after acclimation. The same protocol was then used for all lines. As expected, in general the leakage was higher for the non-acclimated plants compared to the acclimated ones. Win/Nor-1 displayed the lowest leakage of all the oat lines even when not cold acclimated. Significant differences were found between the spring and the winter lines. On the other hand, no significant differences between the EL of acclimated and non-acclimated spring oat Stork was found, demonstrating the inability of spring cultivars to adjust to and tolerate extreme freezing temperatures. Gerald, from which we earlier characterised ESTs (Expressed Sequence Tags) expressed under cold stress [34], leaked ca 50% upon cold acclimation indicating that it was only slightly better than Stork. Hampus, the winter hardy barley,

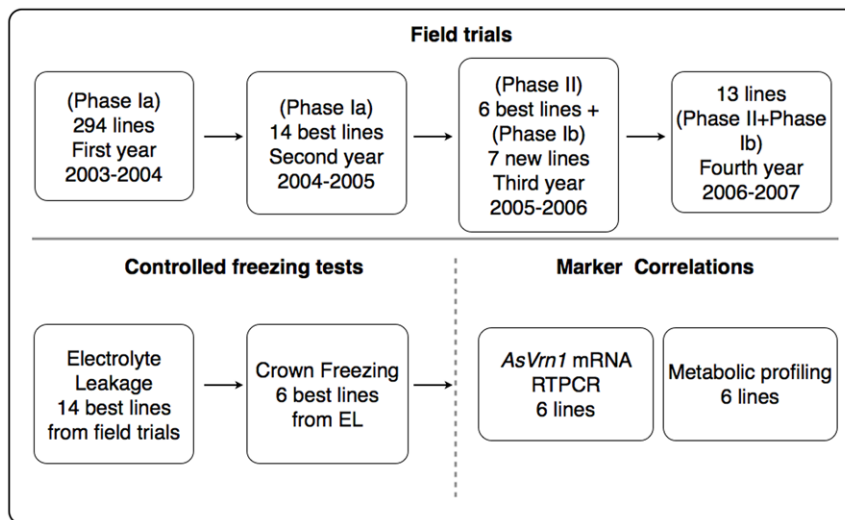


Figure 1. Schematics of experiments. Figure shows the workflow and the approach used for identifying the cultivars in the winter oat model system. Four years of field trials were conducted to identify cultivars that are most tolerant to Swedish winters. The best performing cultivars were then tested by EL, crown freezing assay, molecular and metabolomic analysis. doi:10.1371/journal.pone.0029792.g001

performed as expected the best but only marginally better than the best oat lines (Figure 2).

Crown freezing. Crown freezing was done on two spring cultivars Stork and Belinda, the best performing and intermediate performing lines in the field LPWH992209 and CIAV9327, the best performing line in the EL test Win/Nor-1 and the commercial English winter oat cultivar Gerald. In agreement with the EL measurements, Belinda and Stork did not survive under the conditions used, Gerald and CIAV9327 just barely survived, while LPWH992209 and Win/Nor-1 survived well and thus were the best in this test (Figure 3). However, the LPWH992209 and CIAV9327 crown freezing results contradicted with the EL test since LPWH992209 was only an average performer in the EL test but the best in the crown-freezing test. CIAV9327 was about average in the crown-freezing test and in the field, while it was amongst the better performers in the EL test.

Gene expression by RT-PCR. To determine whether known cold induced genes were differentially expressed in the six chosen lines, the vernalisation gene *AsVRN1* [13] was chosen for expression analysis. This showed that *AsVRN1* was expressed constitutively irrespective of cold treatment in the spring cultivars Belinda and Stork and the English winter oat cultivar Gerald. On the contrary, in the winter hardy lines, there was no detectable expression in the 0 hour and 2 week samples, and it was not until after 6 weeks, that expression was clearly detected in the cold acclimated samples. Expression remained high also in the 8-week sample (6 weeks of cold acclimation followed by 2 weeks of recovery at 'regular treatment condition'), in all lines and cultivars (Figure 4a).

Flowering time analysis. To study if there was a correlation between winter survival and vernalisation requirement, the time of flowering, defined as the day when the inflorescence entirely emerged out of the flag leaf, was analysed. According to this assay, the spring cultivars Belinda and Stork flowered in 59.5 and 61.7 days after germination, respectively. If vernalised for 6 weeks, flowering was slightly delayed to 60 and 70 days, respectively. On the other hand, flowering was much delayed in the two non-vernalised winter hardy lines, indicating that these lines require

vernalisation for flowering (Figure 4b). Flowering time, therefore, is a reliable indicator for winter hardiness and a possible parameter to screen for in the oat TILLING-population [35].

Metabolic profiling. GC-MS metabolic profiles were analysed from cold acclimated leaf tissue of the two spring, two intermediate and two winter hardy model lines at 4 different time-points (0 h, 2 hrs, 1day and 4days). Sample preparation, metabolite extraction, and data processing was done as described (see Methods). Overall, 64 metabolites and 181 mass spectral tags (MSTs) with unknown structure (in total 245) were profiled. From these, 235 metabolites passed the various filtering criteria used (see Methods) and were selected for further analysis. Two-way ANOVA analysis resulted in the identification of 107 metabolites that showed significant ($p \leq 0.05$) interactions with either cultivar (73) or time point (73) or both (4) (Figure 5 and Table S1). Amongst the 73 metabolites that showed significant interactions with cultivars and lines, several are well known and have been previously associated with cold stress. These include glucose, sucrose, fructose, maltose, xylose, galactinol, raffinose, glucose-6P, fructose-6P, GABA, α -tocopherol, and γ -Aminobutyrate (GABA). The 73 metabolites that showed significant interactions with time points included well-known metabolites like glucose, maltose, glucose-6P, fructose-6P, cysteine, tyrosine, galactinol, glycine, phenylalanine, proline, tryptophan, α -tocopherol and proline and 29 MSTs.

By Principal Component Analysis (PCA) on 107 metabolites and MSTs, we found that component 1 accounted for 38.2% variation, whereas component 2 accounted for 24.5% variation (Figure 6). Also, component 1 accounted for variation in the time point parameter (0 h, 2 h, 4 d) where as component 2 accounted for variation between the six lines. Thus, the PCA plot gave an overall view of the trend in the obtained data. While, at the 0 h time point, the separation of the spring and winter lines was random at best, within 2 hours of cold acclimation, spring and winter lines started to separate. At the 4 days time point, spring cultivars Belinda and Stork clustered together in one quadrant whereas the intermediate and the winter lines clustered in the other suggesting a difference in the metabolic profile between the spring and the freezing tolerant lines. Orthogonal Projections to

Table 1. Field trials for winter survival and agronomic properties of cultivars and breeding lines.

	Origin	Cultivars	Winter survival (%)				Agronomic value (1-9)			
			2004	2005	2006	2007	2004	2005	2006	2007
Control	Sweden	Stork (spring oat)	0	0	0	0				
Control	Poland	Fidelio (triticale)		90	90	100		9	9	9
Control	Sweden	Hampus (barley)	90				9			
P1a; P2	Germany	LPWH 992210	60	60	30	90	7	7	7	7
P1a; P2	Germany	LPWH 992213	20	70	40	90	3	7	5	7
P1a; P2	Germany	LPWH 002205-1	30	60	50	90	5	7	8	8
P1a; P2	Germany	LPWH 992209	60	70	40	90	7	7	7	8
P1a; P2	USA	Clav 9340	30	60	40	90	7	5	5	5
P1a; P2	USA	Clav 9349	50	70	40	90	7	7	2	3
P1a; P2	USA	Clav 9346	40	50			7	5		
P1a	USA	Clav 8313	30	60			5	3		
P1a	USA	Clav 9168	60	70			5	5		
P1a	USA	AR0213-3	30	50			5	5		
P1a	USA	Clav 9339	30	60			3	5		
P1a	USA	Clav 9327	20	60			3	5		
P1a	USA	Win/Nor-4	50	50			3	4		
P1a	USA	Win/Nor-1	50	40			3	3		
P1b	USA	Win/Nor-10b			25	90			6	4
P1b	USA	AR0213-10b			25	90			5	4
P1b	USA	AR0213-12			35	90			5	4
P1b	USA	AR0336-3			45	90			5	4
P1b	N/A	NC01-3497			25	90			5	3
P1b	N/A	NC01-3981			35	90			5	4
P1b	N/A	NC01-4365			40	90			6	5
		T_{min}	-17.15	-18.05	-17.19	-10.6				
		T_{avg}	-0.15	0.92	-1.44	0.69				
		Winter days	92	123	125	34				

The origin shows the country where the cultivar was developed. Winter survival (%) shows percent survival of the cultivar. The agronomic value is an integrated index of the agronomic properties of the surviving plants. P1a: phase Ia; P1b: phase Ib; and P2: phase II. T_{min}: minimum temperature during the winter; T_{avg}: average temperature during the winter. Phase Ia, phase Ib, and Phase II are different field trials and cultivars included in respective trials. doi:10.1371/journal.pone.0029792.t001

Latent Structures Discriminant Analysis (OPLS-DA) is a supervised multivariate projection method that aims at maximum separation between groups (here genotypes). OPLS-DA analysis of

107 metabolites showed that, spring cultivars Belinda and Stork clustered together whereas the most freezing tolerant lines Win/Nor-1 and LPWH992209 clustered together in a quadrant

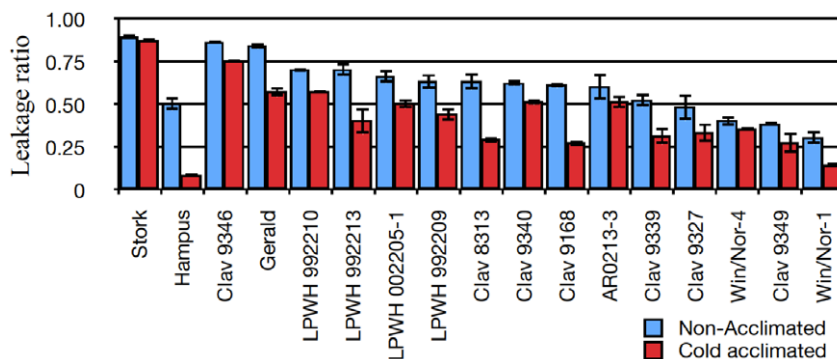


Figure 2. Electrolytic leakage for non-acclimated and cold acclimated plants. Electrolytic leakage data for non-acclimated (blue) and 4 days cold acclimated (red) plants of various cultivars. The ratio was calculated from the ratio of electrolyte leakage to 100% electrolyte leakage. Error bars are SEM. doi:10.1371/journal.pone.0029792.g002

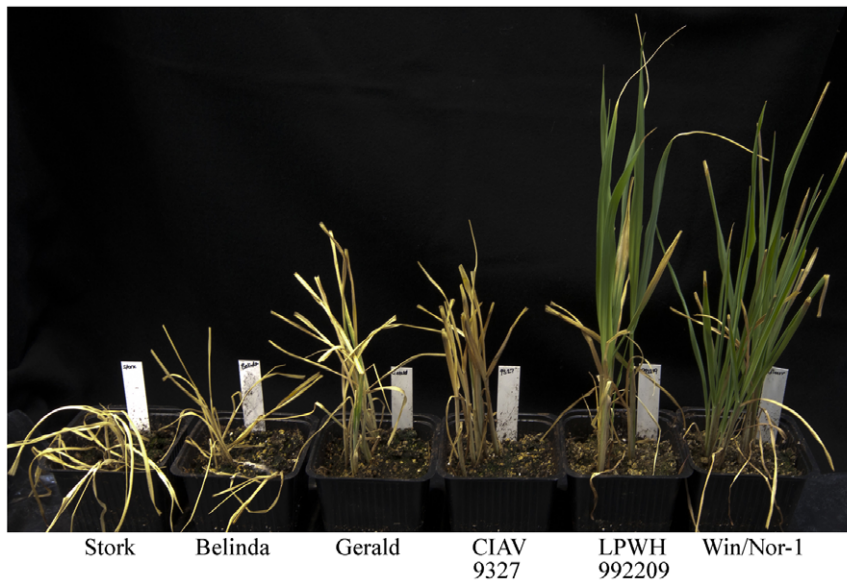


Figure 3. Crown freezing experiments. Plants cultivars as indicated below the pictures were cold acclimated at 4°C in long day light conditions for 48 hours. Crown freezing was done at -12°C for 135 minutes. Pictures were taken 20 days after re-planting of the crowns.
doi:10.1371/journal.pone.0029792.g003

opposite to that of the spring cultivars. The intermediate freezing tolerant cultivar Gerald and the CIAV 9327 genotype were located in two separate quadrants (Figure 7).

The metabolic profiling gave an insight into the dynamics of the levels of several known sugar metabolites including glucose, sucrose, fructose, maltose and raffinose (Figure 8), where maltose and raffinose were >15 fold higher in the freezing tolerant line Win/Nor-1 and >10 fold in LPWH992209. The maltose level then dropped significantly at 4 days time point in all cultivars while raffinose accumulation continued. Sucrose levels were highest after 4 days in Win/Nor-1 and lowest in Belinda, while glucose, fructose and xylose levels only changed marginally in all time points. Expression levels of several amino acids were altered including proline, glycine, tryptophan, cysteine, serine and glutamine. The most up-regulated amino acids were tryptophan, cysteine and glycine. In addition, several MSTs were differentially expressed.

To further identify differences between the lines at the pathway level, we chose to plot the metabolic data on the

sucrose metabolism pathway. Figure 9 gives an overview of the metabolic pathway leading to the biosynthesis of simple sugars. As can be seen from the figure, xylose levels were only marginally altered while the levels of galactinol and raffinose were increased in all six lines. This suggests that galactinol and raffinose accumulation is preferred over xylose under cold acclimation. Furthermore, raffinose levels were lower than the pre-cold treatment levels in Stork at time-point 1day and in CIAV9327 and LPWH992209 at time-point 2 hrs. Maltose levels were highly increased in all six lines at either or all of the three time points but also decreased in Gerald, CIAV9327 and Win/Nor-1 at 4 days, 2 hrs and 4 days, respectively. Minor but significant differences were also observed in the glucose and sucrose levels in the six lines, and was confirmed by an independent enzymatic quantification assay (Figure S2). Fructose 6-P levels were increased in all six cultivars albeit at different time-points. Levels of starch, glucose 1-P, UDP-glucose, sucrose 6-P and fructans were not identified in this work.

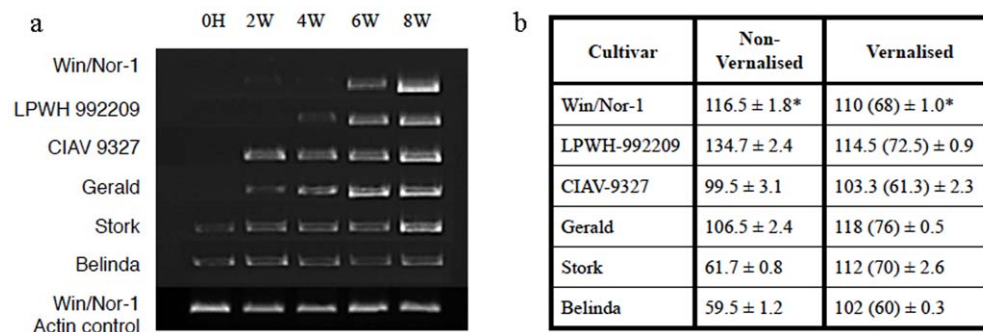


Figure 4. AsVrn1 expression by RT-PCR and flowering time. 4a: RT-PCR experiments were performed on pooled tissue samples cold acclimated for various periods from 0 hours to up-to 6 weeks. 8 weeks (8 W) is 2 weeks of recovery at regular treatment condition after 6 weeks of cold acclimation. Cultivars (cultivars and breeding lines) used are indicated to the left and time points for assay on the top of the picture; 4b: Flowering time of the cultivars with or without vernalisation. "Cultivar" indicates the different oat cultivars tested. Figure in brackets indicates number of days after subtracting the actual vernalisation time; * indicates SD.
doi:10.1371/journal.pone.0029792.g004

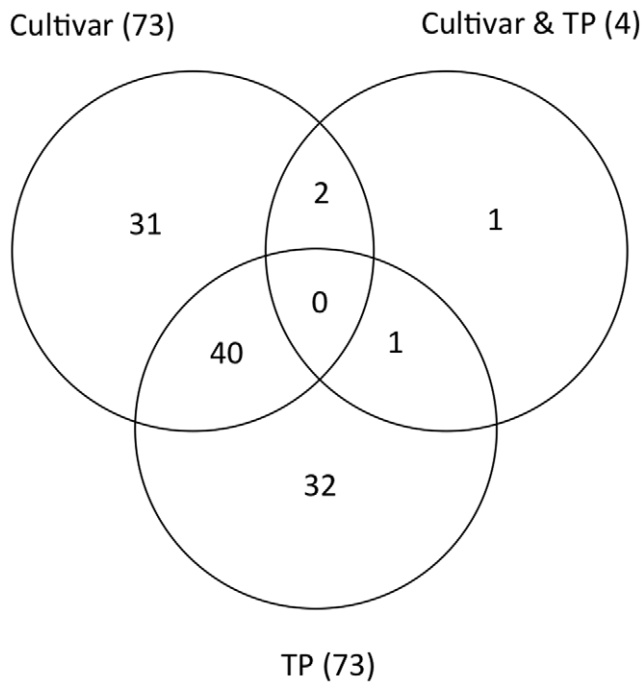


Figure 5. Two-way ANOVA analysis. Two-way ANOVA results from six cultivars (cultivars and breeding lines) and four time points. Out of 235 metabolites, 107 passed and showed significant interactions with either cultivar or time-point (TP) or both cultivar & TP ($p \leq 0.05$). 73 metabolites showed significant interactions with cultivars, 73 with TP and 4 showed interactions with both cultivar and TP. doi:10.1371/journal.pone.0029792.g005

Discussion

Our long-term aim is to produce a Scandinavian winter oat. To succeed with this, we first need to identify a winter oat model system that can be used to identify genes and metabolites uniquely expressed during cold acclimation. Such studies, will give us an insight into the molecular mechanisms behind both winter hardiness, and other stresses related to field survival during the winter. This insight, would help in developing markers, to facilitate the selection of hardy lines in crosses between lines with strong agricultural properties and the best survivors in the field. Since previous winter tests with typical Swedish spring oat varieties showed that they did not survive the winter and no model winter oat was available at the beginning of this work, we needed to develop one. Therefore, we collected the best lines from Russian; American and German winter oat-breeding programs. After planting these in the field in South Sweden in the fall, we scored winter survival and vigour of the survivors during the following season. We also included one representative for the spring varieties, Stork, as a control. After four years of testing, it was clear that very few lines have an inherent capacity to adapt to the Swedish winter. Of the 294 cultivars tested, only 6 survived all four winters (Table 1).

The weather data was analysed as described in the experimental procedures. During the time of the experiment, the winter of 2006–2007 was the mildest lasting for 34 days with the lowest temperature (T_{\min}) of -10.6°C and an average temperature (T_{avg}) of 0.7°C . The winter T_{\min} of 2004–2005 was the lowest with -18.05°C , T_{avg} of 0.92°C and altogether 123 winter days. Although the T_{\min} of the 2005–2006 winter was -17.19°C , which was slightly higher than T_{\min} of the previous winter, the lowest plant survival was during this winter. This could be because, this winter had a much lower T_{avg} (-1.44°C) and more winter days compared to the previous winter (Figure S1).

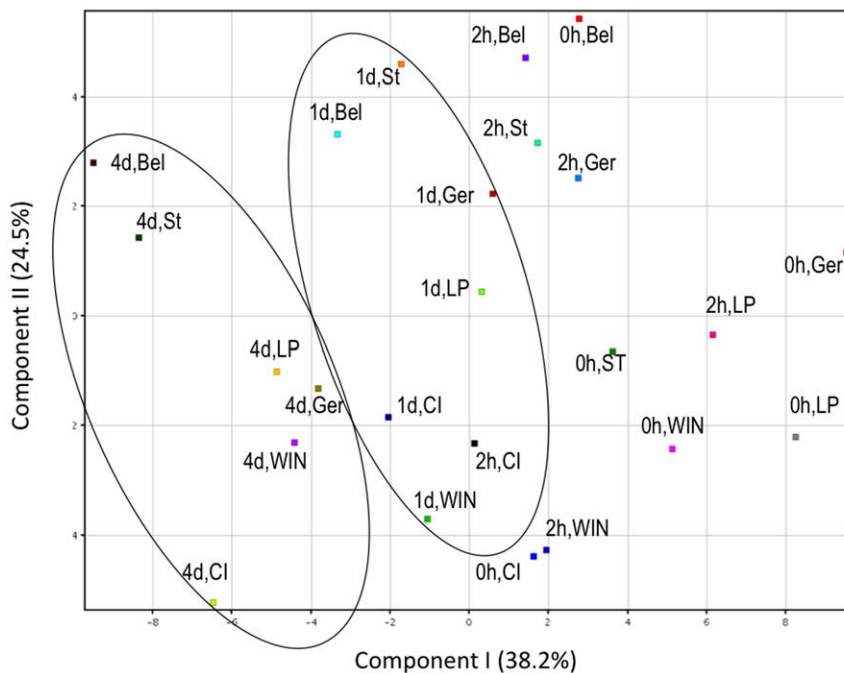


Figure 6. PCA. Principal Component Analysis (PCA). Component 1 (differential response to time points) and Component 2 (differential response to cultivars) are plotted on axes. All cultivars and time-points are represented and are indicated in the plot. doi:10.1371/journal.pone.0029792.g006

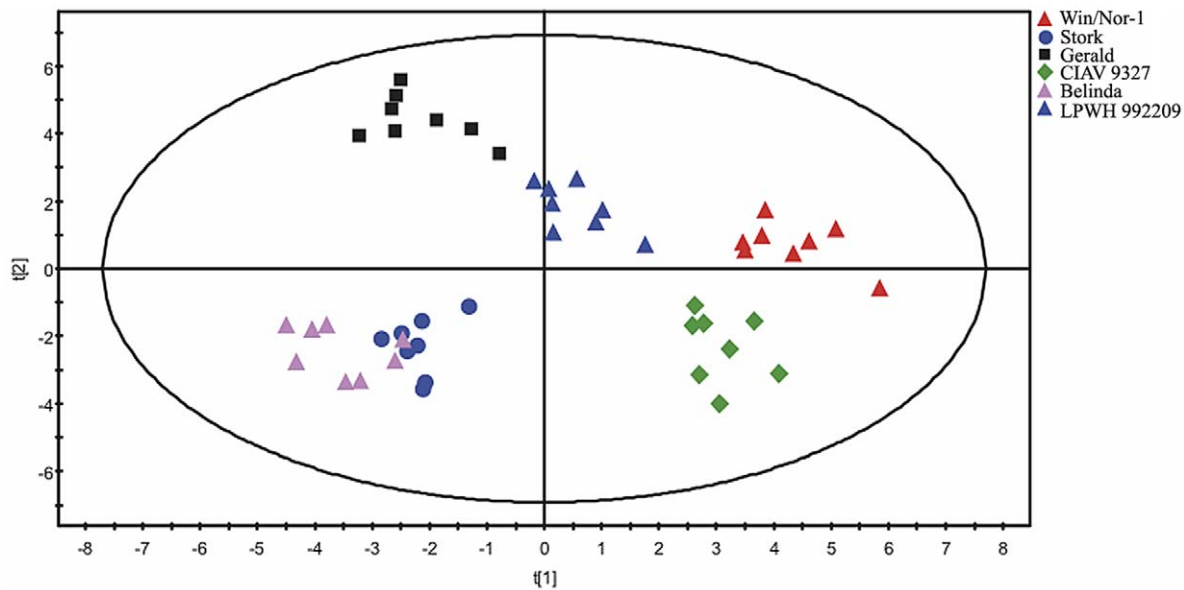


Figure 7. OPLS-DA. OPLS-DA coloring on genotype. Individual replicates for each sample are shown separately. The model had eleven significant components (5 predictive and 6 orthogonal), and the proportions of explained variation in the X-matrix (R^2X) and Y-matrix (R^2Y) were 0.80 (predictive 0.25, orthogonal 0.55) and 0.90, respectively, while the predictive ability according to 7-fold cross-validation (Q^2Y value) was 0.65. doi:10.1371/journal.pone.0029792.g007

Although Win/Nor-1 has been previously reported to have good winter hardiness [31], in our field study it did not perform as well as some of the other selected lines. One reason for this could be, to both survive the winter and to grow well the season after, requires a simultaneous tolerance to several stresses like temperature dynamics, snow cover on the field, biotic stress, soil fertility, water availability, water lodging, drought, and light conditions when the ground is still cold [36,37]. The fact that the agronomic characteristics of all the surviving lines during all years tested never reached 9, including the mildest winter of 2006–2007, indicates that winter survival with full vigour is biologically complex and that the surviving plants need to have a strong adaptation capacity if they are to be useful in agriculture. Inconsistencies in the winter kill between the tested lines were also observed during the four-year period, where in one year some of the lines performed better while in the next year others were better, again reflecting variation in the myriad of different stress factors. On the other hand, consistent differences in survival between lines could be detected, demonstrating that genetic factor has a significant contribution towards winter hardiness.

One spring cultivar (Stork), one winter hardy control Hampus (barley) and 15 winter lines were subjected to sub-zero temperatures under controlled conditions and tested in the EL assay both before and after cold acclimation. In all cases, cold acclimated plants performed better than the non-acclimated ones. On the other hand, the ranking of the winter lines based on the EL assay did not entirely correlate to how well they did in the field. The Win/Nor and CIAV lines, which are, of American origin, performed better than all the German lines except CIAV9346. Moreover, Win/Nor-1 performed the best, showed an impressive freezing tolerance and was almost as tolerant as the winter barley control. However, all the German lines did better in the field. One possible reason for this, could be that the German lines are better adapted to the European climate, with poor snow cover and frequent variations between sub-zero and above-zero temperatures. The American lines, on the other hand, are selected under winter conditions, that are colder and stable than in Europe, with

plants often covered in snow. Therefore, the American lines would be expected to be cold harder than the German ones, which is corroborated in the EL test.

To test the temperature factor in combination with crown regeneration capacity, we performed a freezing experiment on dissected crowns from 2 spring varieties, 2 intermediate and 2 of the best performers in the EL test. After incubating the different crowns under identical conditions at -12°C and after a recovery period for 16 hours at $+4^{\circ}\text{C}$ in dark, the crowns were replanted and moved to normal growth conditions. Under the conditions used, the 2 spring cultivars were killed while the winter lines survived, although their recovery was different (Figure 3), again showing that temperature is a critical selection factor in the field. Win/Nor-1 performed the best, as it did in the EL test. However, LPWH992209, which was superior in the field, outperformed CIAV9347, which performed better than LPWH992209 in the EL-test (Figure 2). Clearly, the fact that the crowns have to be regenerated adds an extra dimension to this assay and obviously LPWH992209 is better adapted to this selection.

Expression analysis of the vernalisation gene, *AsVRN1*, showed a strong correlation between expression pattern and freezing tolerance characteristics. It was expressed at all time points in Stork and Belinda, detectable after 2 weeks of cold acclimation in Gerald and CIAV9327, but much delayed in LPWH992209 and Win/Nor-1, where it was not fully expressed until after 6 weeks of cold acclimation. This indicates that, monitoring of *AsVRN1* gene expression gives an indication of the winter survival potential for the selected lines. Furthermore, flowering time, and the influence of vernalisation on the flowering time, was estimated for the six lines. This showed that, flowering time correlated well with the expression pattern of *AsVRN1*, since spring cultivars Belinda and Stork had delayed flowering upon vernalisation, whereas, the remaining lines showed earlier flowering upon vernalisation. Thus, both the *AsVRN1* expression and flowering time analysis indicate that the winter lines have a vernalisation requirement.

To further identify differences between the spring and winter lines, levels of early-induced metabolites under cold stress were

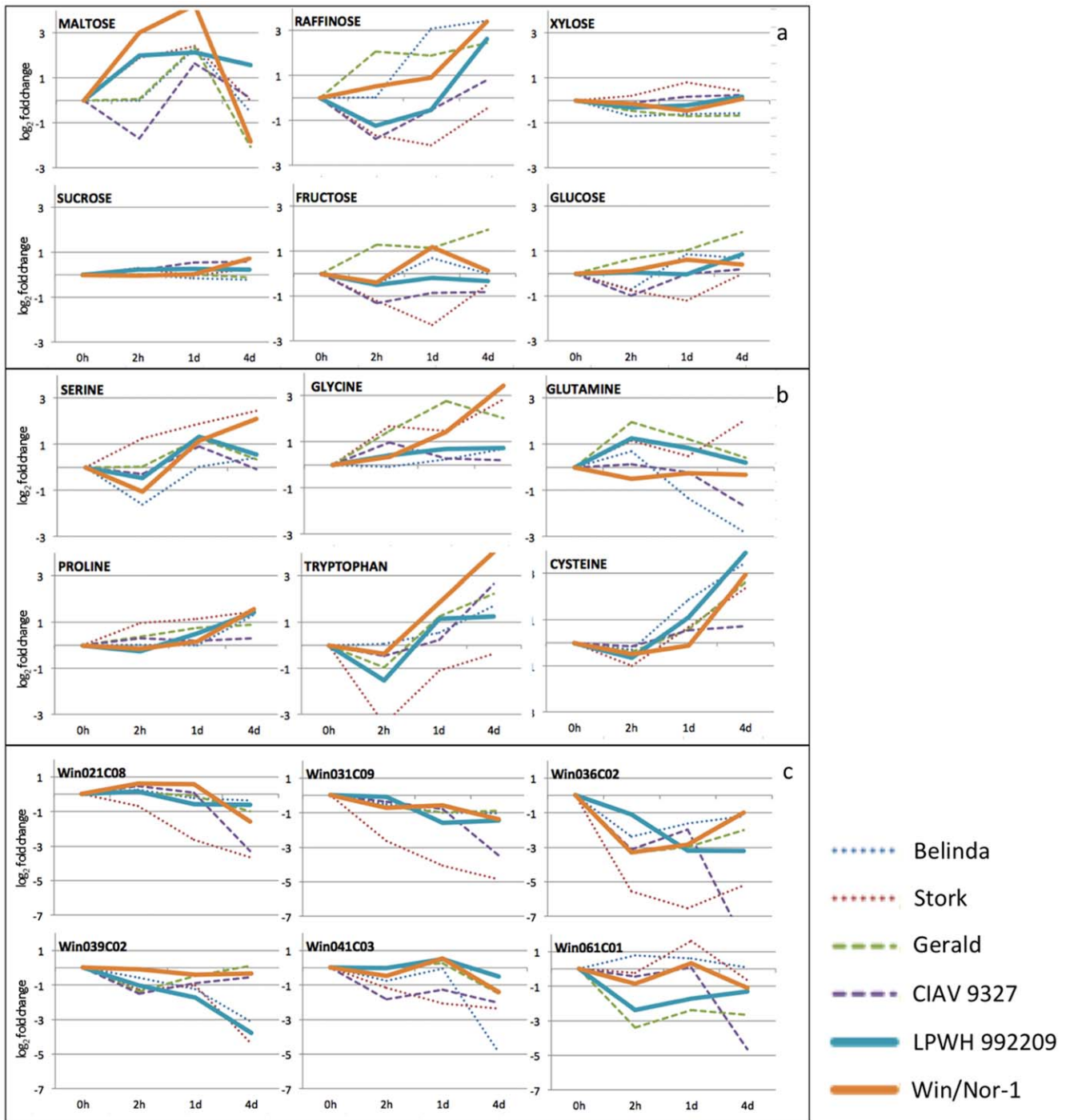


Figure 8. Expression profiles of metabolites in log₂ fold change. 8a: Sugars; 8b: Amino acids; 8c: Unknown down-regulated metabolites. Unknowns are named after the ids they were given during data processing in MATLAB. doi:10.1371/journal.pone.0029792.g008

analysed. Since, in this case, the aim was to confirm the oat winter model system, leaf tissue was used for practical reasons and for a better comparison to previous experiments in Arabidopsis. To even out differences between samples, several different leaves from each plant were pooled. By metabolic analysis, 245 metabolites and MSTs were identified with varied expression under cold stress. By two-way ANOVA, 73 metabolites were identified that showed significant interactions with the cultivar parameter. Metabolic

profiles showed that, the sugars - maltose and raffinose were amongst the most highly altered metabolites. Amino acids including tryptophan, glycine, cysteine and proline levels were also differentially expressed. Additionally, the levels of several MSTs were altered in different time points. A more detailed analysis of the sucrose metabolism pathway showed that maltose, galactinol and raffinose were the probable primary products while xylose levels were unchanged or slightly decreased. This indicates

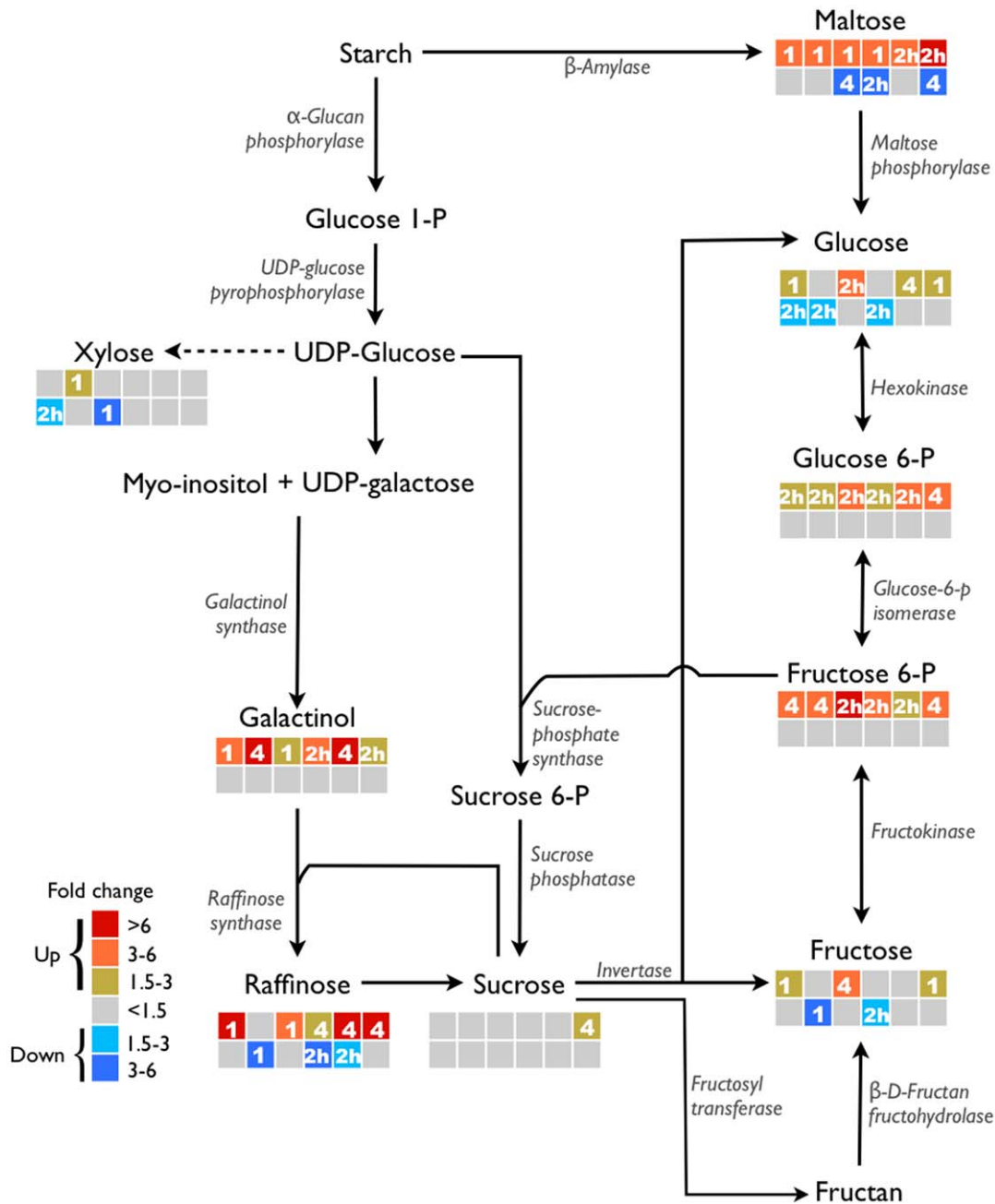


Figure 9. Sucrose metabolism pathway. Colors indicate fold change thresholds; numbers indicate time-points - 2 hrs, 1day and 4days. For each row, each square indicates a different cultivar - from left: Belinda, Stork, Gerald, Ciav-9327, LPWH 992209 and Win/Nor-1. Top row: higher levels; bottom row: reduced levels. Dotted line represents involvement of multiple steps. doi:10.1371/journal.pone.0029792.g009

that, raffinose accumulation is an important step in the cold protection process. Sucrose levels were slightly changed only in Win/Nor-1 and at 4 days, indicating that raffinose hydrolysis and sucrose accumulation is the preferred pathway in the later stages of cold stress.

Galactinol levels were shown to have increased upon low temperature exposure in several studies [19,21,22,23,27], and the correlation between galactinol accumulation and freezing tolerance was shown to be high [23]. Here, by two-way ANOVA analysis, we show that there is a significant interaction between galactinol and the cultivar parameter, suggesting its importance in

freezing tolerance in oats. In Belinda, raffinose levels were more than 6 fold increased just after 1 day in cold, while in the two winter hardy lines, it reached the 6 fold threshold after 4 days (Figure 9). On the other hand, in spite of the high levels, raffinose shows no significant interaction with the cultivar parameter in the two-way ANOVA test. Previous reports, on the role of raffinose in increased freezing tolerance have also been contradictory, since, in some studies, a strong correlation was found between the two [20,38], while, in other studies, the correlation was less convincing [24,29]. Our conclusion is, in oat, raffinose level do not correlate with freezing tolerance to a significant extent.

A deeper understanding of molecular processes involved in freezing tolerance in oats will be essential for the development of winter oat. From the results of field studies and controlled freezing experiments, it is clear that a number of different genetic markers needs to be identified for the further development of an oat, that will not only survive the Swedish winters, but will also keep up its vigour during the following growing season. In this work, by a non-targeted metabolomics approach, both previously known metabolites and new, unknown ones with potentially important roles in cold acclimation, were identified. However, a comparative metabolomic study between leaves and crowns as well as a more in-depth studies on various compounds involved in the metabolic pathways of carbohydrates and other metabolites needs to be carried out.

In a previous study, the expression levels of 947 genes in winter and spring wheat were monitored and 65 genes, differentially expressed in the two cultivars were identified [18]. Since DNA sequencing costs have been reduced multifold in the past decade, massive parallel sequencing to identify differentially expressed ESTs is now a good and complementary approach to metabolomics. Here, we have developed an excellent oat winter model system that allows such comparative studies. Later we will also take advantage of a recently published oat TILLING-population [35] to screen for mutations in essential cold acclimation regulating genes.

Conclusion

No winter oat cultivar exists that can survive Scandinavian winters and produce a high-yielding crop the following season. Therefore, oat is not grown as a winter crop in Scandinavia. With the goal to build a model system to study winter oat, we collected oat lines from winter oat breeding programs and screened them for winter survival in the field in south Sweden. From the field studies, 14 particularly promising lines were selected for further analysis. By controlled crown freezing assays we show that the German line LPWH992209 and the North American line Win/Nor-1 showed excellent freezing tolerance although the German line performed better in the field, most likely due to a superior adaptability to European winter field conditions. A comparative metabolic study, of the most winter hardy, intermediate, and spring oats confirmed the usefulness of these lines as a model and gave an insight into the differences in metabolic profiles during cold acclimation. Additionally, several MSTs showed differences in their expression levels and will now be further characterised to add to the current understanding of cold acclimation in oats.

Methods

Field experiments

In year 2003, 294 accessions were kindly provided by Dr. Harold Bockelman (USDA-ARS (NSGC)), Dr. Igor Loskutov (Vavilov institute), Dr. R. Premkumar (uniform oat winter hardiness nurseries), and Per Sepstrup (Lochow-Petkus GmbH). Commercially grown Swedish spring oat Stork, winter barley Hampus, and winter triticale Fidelio were included as controls. The accessions initially tested thus originated from several different oat growing areas while the accessions selected for further testing were mainly of German and US origin. Seven additional accessions were introduced for testing in 2005–2006 trials.

The field trials were conducted near the town Svalöv, located in south of Sweden. Breeding lines were planted using a Hege plot drill. Depending on seed availability, two to six 1.5 m long rows were drilled using approximately 2 grams of seed per row. For

practical reasons, the winter oat experiments were planted at the same time as other winter cereals, in this case in late September to mid-October, which probably was 2–3 weeks later than the optimal sowing date for the less winter-hardy winter oats. Nitrogen fertiliser application corresponded to approximately 120 kg/ha. Herbicide, if applied, was in early spring. The herbicide ‘Cougar’ that was applied in the winter wheat plots could not be used on winter oats due to expected phytotoxicity.

Hourly temperatures in Svalöv, for the entire test period, were obtained through Mesoscale Analysis system, from Sveriges meteorologiska och hydrologiska institut (SMHI; www.smhi.se), Sweden. The daily maximum, minimum and average temperatures were extracted from the hourly data with a Perl script. The start of the winter was considered as the first day when the average temperature was below zero in November, December or January. The end of the winter, was considered as the last day in February or March, when the daily average below zero was followed by above zero temperatures for at least 4 days in a row.

Rating of material in the field for survival

The lines were graded for winter survival (0–100%) and agronomic value (1–9). Winter survival was graded as the percentage of established plants still viable after winter. Survival was graded, when plants started to grow in spring (March–April), and a clear differentiation could be made between alive and dead plants. Agronomic value is an estimate of the overall yield capacity of the line that survived the winter. A high value indicates a higher estimate for yield. Main traits included in the agronomic value were panicle density per square meter, time of maturity and lodging resistance.

Controlled growth conditions

Regular treatment. Seeds were germinated in 2-litre pots filled with soil. Plants were grown in climate chambers under metal halogen lamps with a photon flux density of 240 $\mu\text{mol}/\text{m}^2/\text{sec}$. The photoperiod was set to long days of 16 hrs, the day/night temperature was 20/18°C, and the relative humidity approx. 70%. The plants were watered as needed.

Cold treatment: Two week old seedlings were moved to growth chambers set at constant temperature of +4°C with a 16 h photoperiod at a photon flux density of 200 $\mu\text{mol}/\text{m}^2/\text{sec}$. The plants were watered as needed.

Crown freezing assay

Crown freezing assay was done as described [28]. Briefly plants were grown in 10×10×7.5 cm pots under the ‘‘Regular treatment’’ conditions as above. Two weeks old seedlings were then cold acclimated for 48 hours under the ‘‘Cold treatment’’ conditions as above. After this, seedlings were removed from the soil, leaves and roots trimmed to 13 cm length (denoted as crowns) and placed in transparent re-sealable plastic bags. Ice chips were added, bags were sealed, and then rolled. Samples were then frozen in the dark at –12°C for 135 minutes in a freezing chamber (Model No. LT-36VL, Percival). After freezing, the samples were allowed to thaw for 16 hours at +4°C in dark, removed from the bags, replanted in the soil and allowed to recover under the ‘‘regular treatment’’ conditions. Plants were photographed after 20 days of recovery under ‘‘regular treatment’’.

Electrolytic leakage assay

Seedlings were grown as described under ‘‘Regular treatment’’. For cold-acclimation, two-weeks old seedlings were moved to cold

chamber set at conditions as described under “Cold treatment”. EL assay for cold-acclimated and non-acclimated plants was done as described [39] with modifications. 3–5 leaves pooled from the seedlings were placed in a glass test tube (16×125 mm) containing 100 μ l distilled water and the samples were then cooled to -2°C for 30 min in a low temperature bath (Model No. 1187P, VWR International). Ice nucleation was induced by the addition of ice chips to the tubes, which were further incubated at -2°C for 90 min. The temperature was then reduced to -8°C with a decrement of 1°C every 10 min. Samples were removed from the bath once the temperature reached -8°C , were allowed to thaw on ice for 3 hours, and transferred to fresh test tubes containing 7 ml-distilled water and incubated at RT for 24 hours on the lab bench. Electrolyte leakage was measured with a conductivity meter. The solution was then removed, samples were frozen in -80°C for 1 hour, followed by thawing on ice for 3 hours, and re-incubating with the original solution to obtain the 100% EL for each sample. The percentage of EL for each sample was then calculated from the ratio of EL to 100% EL.

Total RNA preparation & RT-PCR

Pooled leaf samples were taken from seedlings undergoing cold treatment at time intervals 0 min (Pre-cold treatment), 4 days, 2 weeks, 4 weeks and 6 weeks. Plants were then moved to growth chamber set at regular treatment conditions and allowed to recover for 2 weeks before collecting the 8 weeks leaf samples. RNA was extracted from pooled leaves by RNeasy[®] plant mini kit (cat no. 74904, Qiagen). On column DNase digestion was performed using RNase-Free DNase set (cat no. 79254, Qiagen), as suggested by the kit protocol. RNA was suspended in RNase free water supplied with the kit, quantified spectrophotometrically at OD260, and re-suspended to a final concentration of 50 ng/ μ l.

Reverse Transcriptase Polymerase Chain Reactions (RT-PCR) were performed on total RNA prepared from collected leaf samples using the SuperScript[™] III One-Step RT-PCR kit (Invitrogen[™]). Primers for AsVRN1 were as described [13], namely PoidVRN1 5'-CACCAAGGGAAAGCTCTAC-3' and AsVRN1R-out 5'-GCAGTCTACTACTTTTYACTGA-3'. AsActin gene expression was used for equal loading and amplification control. It was amplified using primers AsActinF 5'-GCGACAATGGAAGTGGC-3' and AsActinR 5'-GTGGTGAAGGAGTAACCTCTCTCG- 3'. RT-PCR reactions of 20 μ l volume were setup as per the manufacturer's instructions with 75 ng total RNA. The RT-PCR was done using C1000[™] thermal cycler (Bio-rad) as follows: 30-min reverse transcription at 55°C , followed by amplification by PCR (94°C for 30 s, 57°C (AsVRN1/AsActin) for 30 s, extension temperature of 68°C for 1 min). To verify the RT-PCR reactions, equal amounts (30%) of the corresponding RT-PCR reaction mix were loaded on 1% agarose gel containing 1X GelStar[®] (Cambrex Bio Science Rockland, Inc.).

Flowering time

Flowering time was calculated for the six lines as described [13] with modifications. Day 1 was defined as the day when the coleoptile was visible above the soil surface. The heading time was defined as the number of days after day 1 when the inflorescence had entirely emerged out of the flag leaf. Plants were either vernalised or not vernalised. Vernalisation was done by exposing two-weeks old seedlings to cold treatment for 6 weeks under the “Cold treatment” conditions as mentioned earlier and after this the seedlings were moved back to chambers set at “Regular treatment” conditions and allowed to develop until maturity. Non-vernalised samples were grown under the regular treatment

conditions until maturity. Ca 10 plants per cultivar were studied in each replicate.

Metabolic profiling

Tissue preparation. Plants were grown under regular treatment conditions for two weeks after which they were moved to the cold treatment conditions. Pooled leaf tissue samples, including several leaves from each plant, were collected from two plants for each replicate. Tissue sample for the time-point 0 hours was collected just before moving the plants to cold treatment conditions. Under cold treatment conditions, tissue samples at time-points 2 hours, 1 day and 4 days were collected for all six cultivars. All the tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until further processing. Tissue sample were grounded to a fine powder for further analysis.

Metabolite extraction: Samples were extracted and analysed according to the methods described by Gullberg et al. [40], with some minor changes. Briefly, stable isotope reference compounds (15 ng μ l⁻¹ each of [¹³C₃]-myristic acid, [¹³C₄]-hexadecanoic acid, [²H₄]-succinic acid, [¹³C₅, ¹⁵N]-glutamic acid, [²H₇]-cholesterol, [¹³C₅]-proline, [¹³C₄]-disodium α -ketoglutarate, [¹³C₁₂]-sucrose, [²H₄]-putrescine, [²H₆]-salicylic acid and [¹³C₆]-glucose) were added to an extraction mixture consisting of chloroform:MeOH:H₂O (6:2:2). The samples (10 mg each) were then extracted in 1 ml of the extraction mixture, using a vibration mill set to a frequency of 30 Hz s⁻¹ for 3 min, with 3 mm tungsten carbide beads added to each extraction tube to increase the extraction efficiency. The extracts were then centrifuged for 10 min at 14,000 rpm before 200 μ l of each supernatant was transferred to a GC-vial and evaporated to dryness. The samples were then derivatised by shaking with 30 μ l of methoxyamine hydrochloride (15 mg mL⁻¹) in pyridine for 10 min at 5°C prior to incubation for 16 h at room temperature. After this, samples were then trimethylsilylated by adding 30 μ l of MSTFA with 1% TMCS and incubating them for 1 h at room temperature. After silylation, 30 μ l of heptane was added.

GC-TOF-MS. Samples were analysed, according to Gullberg et al. [40], using GC-TOF MS together with blank control samples and a series of n-alkanes (C₁₂-C₄₀), which allowed retention indices to be calculated [41]. One μ l of each derivatised sample was injected splitless into a gas chromatograph equipped with a 10 m×0.18 mm i.d. fused silica capillary column with a chemically bonded 0.18 μ m DB 5-MS stationary phase. The injector temperature was 270°C , the septum purge flow rate was 20 ml min⁻¹, and the purge was turned on after 60 s. The gas flow rate through the column was 1 ml min⁻¹, the column temperature was held at 70°C for 2 min, then increased by 40°C min⁻¹ to 320°C , and held there for 2 min. The column effluent was introduced into the ion source of a Pegasus III GC-TOF MS. The transfer line and the ion source temperatures were 250°C and 200°C , respectively. Ions were generated by a 70 eV electron beam at an ionisation current of 2.0 mA, and 30 spectra s⁻¹ were recorded in the mass range 50 to 800 m/z. The acceleration voltage was turned on after a solvent delay of 150 s.

Data analysis: All non-processed MS-files from the metabolic analysis were exported from the ChromaTOF (GC/MS; (Leco Corp., St Joseph, MI, USA) software in NetCDF format to MATLAB software R2010b (Mathworks, Natick, MA, USA), in which all data pre-treatment procedures, such as base-line correction, chromatogram alignment, time windows setting and Hierarchical Multivariate Curve Resolution (H-MCR) were performed using custom scripts according to Jonsson et al [42,43]. All manual integrations were performed using ChromaTOF 4.30 software. Metabolites from the GC/MS analysis were

identified by comparing retention indices and mass spectra with data in retention index and mass spectra libraries [41].

The raw data was normalised by dividing with the sample weights and the response of the internal standards. The data was then analysed using the software SIMCA+ 12.0 (Umetrics AB, Umeå, Sweden) and GeneSpring GX 11.5 (Agilent Technologies, Inc., CA, USA). For supervised multivariate analysis OPLS-DA, the data was \log_{10} -transformed, centred and scaled to unit variance using SIMCA. The data was imported to GeneSpring and quantile normalised. Metabolites were filtered on expression, and only those with raw expression values above the 20th percentile of the overall expression were considered. Out of 248 metabolites, 235 passed the filtering criteria and thus analysed further by fold change estimation, Analysis of Variance (ANOVA) and Principal Component Analysis (PCA).

Supporting Information

Figure S1 Temperature data from the Svalöv area from 2003–2007. Daily maximum and minimum temperatures from May 2003 till July 2007 for the fields in Svalöv obtained by Mesoscale analysis system from SMHI.
(TIF)

Figure S2 Sugar analysis by enzymatic assay. Sugar analysis (glucose, sucrose or fructose) in Stork (blue), LPHW992209 (green) and Win/Nor-1 (orange). Pooled leaf samples were collected from two weeks old non-acclimated plants (0 days) and cold acclimated plants (2 and 4 days). Approximately 0.5 g of leaf tissue was lyophilised and incubated for 10 min in

2 ml 80% ethanol in a water bath set at 90°C. The extract was then filtered through 20 μ m nylon mesh. The incubation and filtration was repeated once more on the leaf tissue. Finally, the extract was centrifuged for 5 min at 4000 g. Glucose concentration was determined by Glucose (HK) assay kit (GAHK-20, Sigma Aldrich), sucrose by Sucrose assay kit (SCA-20, Sigma Aldrich) and fructose by fructose assay kit (FA-20, Sigma Aldrich). The samples were analysed within 6 hours of extraction. Error bars are SEM.

(TIF)

Table S1 Expression levels of metabolites and list of metabolites from Two-Way ANOVA.

(XLS)

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Author Contributions

Conceived and designed the experiments: AC OO AJ. Performed the experiments: AC PL TM MB RJ. Analyzed the data: AC PL TM RJ OO. Wrote the paper: OO AC.

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