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ORIGINAL ARTICLE

Dehydrin, alcohol dehydrogenase, and central metabolite levels are associated with cold tolerance in diploid strawberry (*Fragaria* spp.)

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Abstract The use of artificial freezing tests, identification of biomarkers linked to or directly involved in the low-temperature tolerance processes, could prove useful in applied strawberry breeding. This study was conducted to identify genotypes of diploid strawberry that differ in their tolerance to low-temperature stress and to investigate whether a set of candidate proteins and metabolites correlate with the level of tolerance. 17 *Fragaria vesca*, 2 *F. nilgerrensis*, 2 *F. nubicola*, and 1 *F. pentaphylla* genotypes were evaluated for low-temperature tolerance. Estimates of temperatures where 50 % of the plants survived (LT_{50}) ranged from −4.7 to −12.0 °C between the genotypes. Among the *F. vesca* genotypes, the LT_{50} varied from −7.7 °C to −12.0 °C. Among the most tolerant were three *F. vesca* ssp. *bracteata* genotypes (FDP821, NCGR424, and NCGR502), while a *F. vesca* ssp. *californica* genotype (FDP817) was the least tolerant (LT_{50} −7.7 °C). Alcohol dehydrogenase (ADH), total dehydrin expression, and content of central metabolism constituents were assayed in select plants acclimated at 2 °C. The LT_{50} estimates and the expression of ADH and total dehydrins were highly negatively correlated ($r_{adh} = -0.87$, $r_{dehyd} = -0.82$). Compounds related to the citric acid cycle were quantified in the leaves during acclimation. While several sugars and acids were significantly correlated to the LT_{50} estimates early in the acclimation period, only galactinol proved to be a good LT_{50} predictor after 28 days of acclimation ($r_{galact} = 0.79$). It is concluded that ADH, dehydrins, and galactinol show great potential to serve as biomarkers for cold tolerance in diploid strawberry.

Keywords Galactinol · Hierarchical clustering · Lethal temperature 50 · Metabolite profiling · Raffinose pathway · Survival analysis

Abbreviations

ABA	Abscisic acid
ADH	Alcohol dehydrogenase
CBF	C-repeat/dehydration responsive element binding factor
FDP	*Fragaria* diploid project

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Journal : Large 425 | Dispatch : 17-9-2012 | Pages : 13
Article No. : 1771 | ☐ LE | ☐ TYPESET
MS Code : Planta-2012-07-0465 | ☑ CP | ☑ DISK

GC–MS	Gas chromatography and mass spectrometry
LT_{50}	Temperature where 50 % of the plants are killed
NCGR	National Clonal Germplasm Repository
PCA	Principal component analyses
PPFD	Photosynthetic photon flux density

Introduction

In areas where strawberry is grown in a perennial growing system, the plants have to survive through the winter. In Scandinavia, the majority of the strawberry produce comes from a perennial production system, and winter survival is a major limiting factor for the strawberry industry. A typical annual yield reduction is 20 %, with an occasional total loss (Davik et al. 2000).

The survival of strawberry plants in areas with low temperatures is affected by several physiological responses, e.g., growth cessation, effective cold hardening in autumn, and the response to growth stimulation in periods of temporarily increased temperatures. Abiotic stresses like ice encasement, desiccation, and soil heaving add to the problem. With a range of biotic and abiotic factors contributing and interacting, disentangling the full story of winter survival has proven difficult. However, low-temperature stress per se is one important aspect of winter survival, and in particular during periods with little or no snow cover. The cycles of freezing and thawing during wintertime have been shown to be particularly harmful to strawberry plants. In such cases, the use of insulating cover, either snow or ice, has a significant impact on both the yield and the quality of the yield (Nestby et al. 2000).

Differences among cultivars in winter survival have been known to the industry and also experimentally confirmed both for octoploid *Fragaria* × *ananassa* (Nestby and Bjørgum 1999) and diploid *F. vesca* genotypes (Sønsteby and Heide 2011). Hence, selecting for winter survival is a prime objective for strawberry breeding programs. Given the complexity of the trait and the often fluctuating winter weather, extensive field testing over many years would be required to gain reliable results. More rapid laboratory tests are therefore required. Testing for frost tolerance under controlled conditions to grade genotypes has been used in several plant breeding programs to identify superior genotypes, e.g., in wheat (Gusta et al. 1997) and oilseed rape (Teutonico et al. 1993). This approach could also be a valuable alternative for the strawberry breeder.

In addition to the use of artificial freezing tests, identification of biomarkers linked to or directly involved in low-temperature tolerance processes could prove useful in applied strawberry breeding. During acclimation, plants from temperate and cold climates develop increased tolerance to subsequent low-temperature exposure, and changes in expression of hundreds of genes have been demonstrated in *Arabidopsis thaliana* (Kaplan et al. 2007). In strawberry vegetative tissue, metabolite profiles are totally reconfigured as a result of the low-temperature impact (e.g., Rohloff et al. 2012). The metabolic cold response results in increased levels of compatible solutes such as free amino acids, amines, polyols, and mono-, di-, and trisaccharides as described for the model *Arabidopsis thaliana* (Korn et al. 2010). The molecules' osmo-protective role is based on their properties to stabilize and prevent proteins, membranes (as reviewed by Kaplan et al. 2007), and nucleic acids (Kurz 2008) from the damaging effects of freezing temperatures. Moreover, secondary metabolism is also strongly affected leading to the up-regulation of photoprotective flavonoids (Hannah et al. 2006). The prominent role of the raffinose pathway (Rohloff et al. 2009) and central carbohydrate metabolism is documented in several studies (Guy et al. 2008), and a significant correlation between freezing tolerance and carbohydrate content and accumulation during acclimation has been demonstrated in *A. thaliana* (Hannah et al. 2006).

In other species, expression of alcohol dehydrogenase (ADH) is known to increase under various stresses, including low temperature, drought, abscisic acid (ABA), and salinity (Christie et al. 1991; Jarillo et al. 1993; Dolferus et al. 1994; de Bruxelles et al. 1996; Lindlöf et al. 2007; Diab et al. 2008). In particular, ADH genes are among the most commonly found cold-induced genes in cereal crops and *Arabidopsis* (Lindlöf et al. 2007). Our own preliminary observations have shown a high correlation between ADH levels and cold tolerance in the octoploid strawberry.

Another group of candidate marker proteins are the dehydrins. Dehydrins comprise a family of proteins that are produced in response to low temperatures and drought stress. Dehydrins are often regulated by the CBF cold-responsive pathway and are among the most commonly reported proteins accumulating in plants in response to cold stress (Close 1996). Dehydrins are well conserved in the plant genera, and homologs are readily identified by sequence similarity and occurrence of the dehydrin consensus sequence (Close 1997). In Rosaceous species, dehydrins have been identified that have high similarity to *Arabidopsis* dehydrins (Artlip et al. 1997; Bassett et al. 2009; Garcia-Bañuelos et al. 2009) and in strawberry (*F.* × *ananassa*) Koehler et al. (2012) identified two dehydrin-like proteins (COR47-like, XERO2-like) that were regulated by cold exposure.

Indirect selection using a marker-assisted approach could enhance the efficiency of cultivar development.

Journal : Large 425 | Dispatch : 17-9-2012 | Pages : 13
Article No. : 1771 | LE | TYPESET
MS Code : Planta-2012-07-0465 | CP | DISK

Although rudimentary linkage maps have been emerging (Rousseau-Gueutin et al. 2008; Sargent et al. 2009), commercially grown strawberry cultivars are difficult to disentangle in genetic studies due to their octoploid genome. To understand the molecular basis for low-temperature stress and develop molecular markers linked to stress tolerance, we chose a model system using diploid *Fragaria* species in a screening for diverging genotypes. The octoploid strawberry progenitors *F. virginiana* and *F. chiloensis* are believed to be diploidized allopolyploids, each descending from four diploid ancestors. The ancestry of *F. virginiana* and *F. chiloensis* is not fully known, but *F. vesca*, *F. iinumae*, *F. nubicola*, and *F. orientalis* have been suggested by some authors (Potter et al. 2000; Folta and Davis 2006), while Rousseau-Gueutin et al. (2009) have found evidence for *F. vesca*, *F. mandshurica*, and *F. iinumae* being strong candidates. So, there appears to be a consensus among the authors that at least *F. vesca* is one of the early ancestors.

Diploid strawberry species have several features that make them attractive as model species. The plants are easily grown and propagated both through seeds and runners, and they are relatively easy to transform genetically (Oosumi et al. 2006). Moreover, the *F. vesca* genome is relatively small (~240 Mb) and has recently been sequenced (Shulaev et al. 2011). Finally, a high degree of macrosynteny and collinearity between diploid and octoploid strawberry exist, and no major chromosomal rearrangements seem to have occurred (Rousseau-Gueutin et al. 2008). This conserved organization within the *Fragaria* genus supports the use of diploid *Fragaria* as a model system to gain genetic knowledge that subsequently can be transferred to the more complex and economically important octoploid *F.* × *ananassa* (Davis and Yu 1997; Sargent et al. 2004).

This study was conducted to identify genotypes of diploid strawberry that diverge in their tolerance to low-temperature stress and investigate whether a set of candidate proteins and metabolites show correlation with the level of tolerance. The work presented here is part of a project where the main goal is to gain basic knowledge about the genetic variation of winter survival of strawberry. The development of molecular markers useful in the amelioration of strawberry cultivars with improved winter survival rate is our long-term goal.

Materials and methods

Plant material and multiplication

The plants were either collected as runners in Norway (Alta, Bukammen, and Haugastøl) or obtained as seeds from the National Clonal Germplasm Repository (NCGR-accessions) in Corvallis, OR, USA, and East Malling Research (FDP-accessions), UK. Seeds were propagated and one single plant was collected from each of the accessions mentioned in Table 1, hereafter called 'genotype' or 'genotypes', even though we retain the original label. Multiplication of each of the genotypes was subsequently done by runnering, aiming for uniform test plants. The plants were then raised in a heated greenhouse for 5 weeks maintained at 20 ± 2 °C and 20-h photoperiod. Throughout the experiments, the plants were grown in 10 cm plastic pots containing a peat-based potting compost (90 % peat, 10 % clay), with the addition of 1:5 (v/v) of granulated perlite. The plants were watered twice a week (and 1 day immediately before harvesting for freezing treatments), sufficient to keep the soil moist at all times. A balanced nutrient solution containing 7.8 mmol N, 1 mmol P, and 4.6 mmol K per liter (used in 1:100 ratio) was applied twice a week.

Freezing experiments

For the LT_{50} determinations, the plants were subsequently acclimated for 6 weeks at 2 °C and 10-h photoperiod. Supplemental light was provided by high-pressure sodium lamps (SON-T) at a PPFD of about 90 µmol quanta m^{-2} s^{-1}. After hardening, the plants were exposed to freezing temperatures ranging from 0 to −27 °C (0, −8, −9, −10, −12, −14, −15, −18, −21, −24, and −27 °C). The freezing was performed in darkness in freeze cabinets initially set at 2 °C. The temperature was immediately lowered to −2 °C, and kept at this temperature for 12 h to ensure that the soil in the pots was frozen. The temperature was then lowered by 2 °C/h until the target temperature was reached where it was held for 4 h, before raising the temperature by 2 °C/h to 2 °C and holding for 10 h. Control plants were exposed to 0 °C in darkness for 12 h. After completion of the freezing exposure, the plants were moved into a greenhouse maintained at 18 ± 2 °C and 20-h photoperiod for 5 weeks before survival was scored (dead or alive).

Setup and statistical analysis of the freezing experiments

Six freezing experiments were performed under identical conditions with the 22 genotypes presented in Table 1. In each experiment, we used 12 clonally propagated plants from each genotype in each of the temperature treatments. Occasionally, and for some genotypes, only nine plants were used due to the great variation in stolon formation between the genotypes. For the same reason, some genotypes were represented in four experiments, while one was represented only once. On average, each genotype was represented 2.5 times in one of the six experiments. However, statistical connectivity between the experiments

Journal : Large 425 Dispatch : 17-9-2012 Pages : 13
Article No. : 1771 LE TYPESET
MS Code : Planta-2012-07-0465 CP DISK

Table 1 Strawberry genotypes included in this study

Accession ID/genotype	Species subspecies	Origin	Altitude (m a.s.l.)	LT_{50} (°C) ± SE
FDP821/NCGR546	*F. vesca* ssp. *bracteata*	Wyoming, USA	1,200	−12.0 ± 1.2
NCGR1428	*F. vesca*	Bolivia	n/a	−12.0 ± 1.7
Alta	*F. vesca* ssp. *vesca*	Alta, Norway	50	−11.6 ± 1.2
NCGR1603	*F. vesca*	Rakitovo, Bulgaria	1,070	−11.1 ± 1.3
NCGR424	*F. vesca* ssp. *bracteata*	Oregon, USA	1,300	−11.1 ± 1.5
NCGR1309	*F. vesca*	Italy	1,200	−11.0 ± 1.5
NCGR1364	*F. vesca*	Epinel, Italy	1,300	−11.0 ± 1.5
Haugastøl	*F. vesca* ssp. *vesca*	Haugastøl, Norway	1,080	−10.4 ± 2.0
NCGR198	*F. vesca*	Hawaii, USA	2,135	−10.4 ± 2.0
FDP815	*F. vesca* ssp. *vesca*	Inbred from Baron Solemacher	n/a	−10.3 ± 1.7
NCGR502	*F. vesca* ssp. *bracteata*	New Mexico, USA	2,500	−10.3 ± 1.7
Bukammen	*F. vesca* ssp. *vesca*	Stjørdal, Norway	250	−9.8 ± 1.5
NCGR1780	*F. vesca*	Ukraine	n/a	−9.6 ± 1.3
NCGR1001	*F. vesca*	Ecuador	2,460	−9.2 ± 1.5
NCGR1848	*F. vesca*	Hokkaido, Japan	180	−8.9 ± 1.3
NCGR522	*F. nubicola*	Kohistan, Pakistan	2,400	−8.4 ± n/a
FDP701	*F. pentaphylla*	Wolong Preserve, Sichuan, China	2,400	−8.3 ± 1.6
NCGR1363	*F. vesca*	Bolivia	n/a	−8.2 ± 1.2
FDP301	*F. nubicola*	Uttar Pradesh, Pakistan	n/a	−7.7 ± 1.7
FDP817/NCGR371	*F. vesca* ssp. *californica*	California, USA	28	−7.7 ± 0.5
NCGR1825	*F. nilgerrensis*	Yunnan, China	2,100	−6.1 ± 1.9
NCGR1188	*F. nilgerrensis*	Guizhou, China	1,550	−4.7 ± 3.2

Origin and altitude of collection site, the estimated temperatures for 50 % survival (LT_{50}), and the corresponding standard errors 5 weeks after low-temperature exposure are presented

n/a not available

was ensured by replicating some genotypes across experiments. To analyze the unbalanced survival data (dead/alive), the following logistic model was used.

$$\pi_{ijkt} = P(y_{ijkt} = 1 | E_j, (E\alpha)_{ij}) = P(y = 1)$$
$$= P(\text{a plant survives}) = \frac{e^{\beta_0 + \alpha_i + \beta_1 \cdot t + E_j + (E\alpha)_{ij}}}{1 + e^{\beta_0 + \alpha_i + \beta_1 \cdot t + E_j + (E\alpha)_{ij}}}$$

where β_0 is an unknown constant, α_i is the main effect of the genotype i ($i = 1$,…, 22), β_1 is the coefficient that estimates the effect temperature (t) has on plant survival, E_j is the effect of experiment or run j ($j = 1$,…, 6), k denotes a clonal plant from each genotype in a given experiment, $k = 1$,…, 12, t is the temperature plant k is exposed to ($t = -15$ °C to 0 °C), $(E\alpha)_{ij}$ is the interaction between genotype i in experiment j, and π_{ijkt} is the observation [alive (1)/dead (0)] made on plant k from genotype i, in experiment j, exposed to temperature t.

The LT_{50} for genotype i was estimated as

$$\hat{E}(LT_{50}) = -\frac{\hat{\beta}_0 + \hat{\alpha}_i}{\hat{\beta}_1}.$$

The *Glimmix* procedure in SAS® was used to implement this model. The standard errors for the estimated LT_{50} values were computed from the covariance matrix using the delta method (Coles 2001).

Protein extraction, Western blot, and quantitative analysis

For the SDS-PAGE and subsequent blot analyses, a subset of ten genotypes from Table 1 was used (Alta, Bukammen, FDP817, FDP821, NCGR424, NCGR522, NCGR1363, NCGR1603, NCGR1780, and NCGR1848). Plant cultivation was carried out as described previously. Cold treatment was performed at 2 °C for a 10-h photoperiod at 90 µmol m^{-2} s^{-1} for 0, 1, 2, 14 and 42 days. Tissue from crowns was harvested, immediately shock frozen in liquid nitrogen and stored at −80 °C until processing. Control samples (0 day) were harvested prior to the transfer to the cold room. Each time point was represented by tissue samples from 12 crowns (4 crowns per replicate).

Total protein extracts were isolated from cold-treated and control crown tissues. Tissue samples (200 mg FW) were ground to a fine powder in liquid nitrogen and then extracted with homogenizing buffer composed of 1.5 M Tris (pH 8.8), 2 % glycerol, 2 % SDS w/v, 2 %

Journal : Large 425 | Dispatch : 17-9-2012 | Pages : 13
Article No. : 1771 | LE | TYPESET
MS Code : Planta-2012-07-0465 | CP | DISK

mercaptoethanol, and 1× Complete Roche Protease inhibitors. The homogenates were then centrifuged for 5 min at 10,000*g* and the supernatants were stored at −80 °C. Loading of the SDS-PAGE was normalized by adding equivalent amounts of protein in each lane. Protein concentration was estimated using the Amido Black method (Kaplan and Pedersen 1985).

Proteins extracted from crown tissue (5 µg) were separated by 12 % SDS-PAGE and transferred to nitrocellulose membranes overnight at 0.2 constant Amps at 4 °C. Membranes were blocked and then probed in PBS/5 % non-fat milk (pH 7.4) with either anti-dehydrin antibody (1:2000 supplied by Tim Close, UC Riverside, CA, USA) or anti-alcohol dehydrogenase (ADH) (Agrisera, Vannas, Sweden) followed by peroxidase-labeled goat anti-rabbit (1:4,000 Sigma®, St Louis, MO, USA). SuperSignal® West Dura (Thermo Scientific, Rockford, IL, USA) was used to visualize chemiluminescence on a ChemiDoc™ XRS Molecular Imager (Bio-Rad). Image analysis and densitometry were performed with ImageJ (NIH IMAGE, http://rsbweb.nih.gov/ij/).

Since the anti-dehydrin antibody had not been used previously in strawberry, experiments confirming specificity, using K-peptide competition, were performed (Suppl. Fig. S1).

These data were analyzed statistically and plotted using the *Reg* and the *Sgscatter* procedures in SAS®.

Metabolite experiment

Since strawberries are propagated by stolons from the crown, a most efficient breeding strategy would be to screen non-essential tissues rather than to destroy the propagule. Thus, we wanted to investigate the potential of using metabolite profiles from leaf tissues to predict low-temperature tolerance. For this experiment, a subset of ten *F. vesca* genotypes (Table 1) with contrasting freezing tolerance was selected (Alta, Bukammen, FDP817, FDP821, Haugastøl, NCGR1363, NCGR1428, NCGR1603, NCGR1780, and NCGR1848). Twelve-week-old runner-propagated *Fragaria* plants, raised on fertilized soil in plug trays (3 × 6 cells) in a greenhouse at 18 ± 2 °C under natural light and long-day conditions, were transferred to a cold storage room at 2 °C under artificial light (fluorescent tubes, 90 µmol m^{-2} s^{-1}) for a period of 4 weeks. Three mature leaves were sampled from individual plants ($n = 3$ per genotype and time point) at the following time points: 0, 1, 2, 14, and 28 days. The control samples (0 day) were harvested before cold exposure. Samples were immediately shock frozen in liquid nitrogen and stored at −80 °C prior to further processing. A modified extraction and derivatization protocol (Roessner et al. 2001) was utilized, based on mechanical sample crushing using a handheld high-speed mixer (300 mg FW). A lyophilized aliquot (300 µl) was further processed using methoxyamine and trimethylsilyl derivatization. Samples were transferred to 1.5 ml autosampler vials with glass inserts and stored at −20 °C prior to analysis by gas chromatography and mass spectrometry (GC–MS).

An Agilent 6890/5975 GC–MS was used for all analyses. Sample volumes of 1 µl were injected with a split ratio of 25:1. GC separations were carried out on an HP-5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm). The injection temperature was 230 °C and the interface was set to 250 °C. The carrier gas was helium at a constant flow rate of 1 ml/min. The GC temperature program was held isothermically at 70 °C for 5 min, ramped from 70 to 310 °C at a rate of 5 °C/min, and finally held at 310 °C for 7 min (analysis time: 60 min). The MS source was adjusted to 230 °C and a mass range of *m/z* 50–700 was recorded. All mass spectra were acquired in EI mode. Chromatogram visualization and peak area integration were carried out using the Agilent ChemStation software. For mass spectra evaluation and peak identification, the AMDIS software (v. 2.64) was used in combination with the following mass spectral libraries: NIST05 database and a target library containing MS spectra of trimethylsilylated (TMS) metabolites (Hummel et al. 2010). Numerical analysis was based on peak area integration being corrected for FW variation, using the internal standard ribitol (normalized response). For the statistical analyses, the ribitol-corrected peak areas within each time point were standardized to zero mean and a standard deviation of one for each metabolite.

A multivariate regression approach was taken to model the LT_{50} estimates using the metabolite data at 28 days of acclimation. *Proc Reg* (SAS Institute Inc. 2008) with the *stepwise* option was used for this. In order to reveal structures in the metabolite data that could be associated with the impact of the acclimation period or with the specific genotype, we used principal component analyses (PCA) including all the 13 compounds observed at time points 0 and 28. The SAS® *Princomp* procedure was used for the PCA, and the *Sgplot* procedure was used for generating the PC loading plot (SAS Institute Inc. 2008). Finally, heat maps were made to visualize structures and metabolic responses to cold acclimation. For this, the *heatmap.2* function in *R* (http://www.r-project.org) was used.

Results

Freezing tests of 22 genotypes

Typical results of the freezing tests are shown in Fig. 1 where one of the low-temperature-tolerant genotypes (*F. vesca* ssp. *bracteata*, NCGR424) and one low-temperature-sensitive

Journal : Large 425 | Dispatch : 17-9-2012 | Pages : 13
Article No. : 1771 | LE | TYPESET
MS Code : Planta-2012-07-0465 | CP | DISK

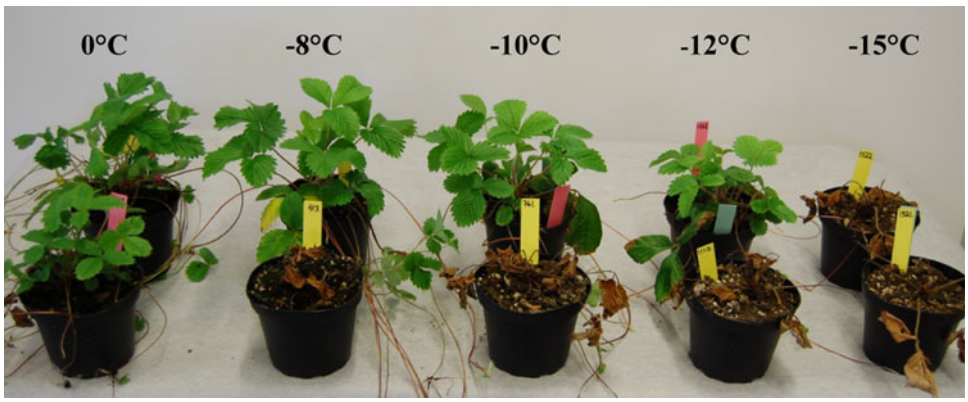

Fig. 1 Typical result from low-temperature stress experiments. A frost-tolerant accession (NCGR424, rear, *F. vesca* ssp. *bracteata*) and a frost-susceptible (FDP 817, front, *F. vesca* ssp. *californica*) accession of *F. vesca* exposed to five levels of freezing stress. The plants had been grown at 18 °C in the greenhouse for 5 weeks after low-temperature exposure when the picture was taken

genotype (*F. vesca* ssp. *californica*, FDP817) are presented. The estimated LT_{50} values (temperature at which 50 % of plants survived) and their corresponding standard errors are presented in Table 1.

In general, there was a negative ($r = -0.47$) and significant ($P = 0.04$) correlation between LT_{50} estimates and geographical latitude. The correlation to altitude was, however, not significant.

The LT_{50} estimates have a range from −4.7 (NCGR1188) to −12.0 °C (FDP821 and NCGR1428). NCGR1188 is an *F. nilgerrensis*, while the two most tolerant are *F. vesca* species. In general, the *F. vesca* genotypes seem to be more low-temperature tolerant than the other species tested. In particular, the three *F. vesca* ssp. *bracteata* genotypes were all in the low-temperature-tolerant side of the distribution (Table 1), while the two *F. nilgerrensis* genotypes appeared on the susceptible side of the same distribution, to some extent together with the *F. nubicola* genotypes. Regarding their average LT_{50} values, *F. vesca* ssp. *bracteata* (Avg LT_{50} −11.1) differ significantly from the value of the one *F. vesca* ssp. *californica* genotype (LT_{50} −7.7, $P = 0.003$), the average of the two *F. nilgerrensis* genotypes (Avg LT_{50} −5.4, $P < 0.0001$), and from the average of the two *F. nubicola* genotypes (Avg LT_{50} −8.1, $P = 0.0002$). Finally, the *F. nilgerrensis* average also differs significantly ($P = 0.0003$) from the *F. vesca* ssp. *vesca* average (LT_{50} −10.5).

The NCGR1363 is another low-temperature susceptible *F. vesca* genotype (LT_{50} −8.2). Pair-wise tests showed that Alta (LT_{50} −11.6) was significantly different from both FDP817 (LT_{50} −7.7) and NCGR1363. Hence, these are excellent candidates for parent mapping populations.

Alcohol dehydrogenase and dehydrin levels

Western blotting and probing with anti-dehydrin (K-segment specific) for the full time course sample series (noncold-acclimated treated control, 1, 2, 14, and 42 days cold) was carried out for eight *F. vesca* genotypes with three biological replicates each (Fig. 2). Dehydrins were not detected in the untreated control or in the 1-day and 2-day cold-treated crowns. Interestingly, dehydrins in the leaves could not be detected at any time points (data not shown). However, three bands were first observed at 14 days, which accumulated to much higher levels at 42 days (Fig. 2). This should be considered a relatively slow cold response, particularly relative to *Arabidopsis* where dehydrin levels are readily detected by 2 days and are at near maximum at 4–6 days after initiation of cold treatment. Competition experiments (Suppl. Fig. S1) showed that all bands represented true dehydrins as they were competed by the K-peptide. Four distinct patterns of dehydrin expression were observed in the genotypes and were exemplified by FDP821, Alta, NCGR522, and NCGR1603 (Suppl. Fig. S2). The dehydrin masses were extrapolated from the competition experiment (Suppl. Fig. S1). Bioinformatic analysis identified seven distinct dehydrins (Suppl. Fig. S3). Application of antibodies specific to *Arabidopsis* dehydrins revealed multiple polypeptides, confounding identification of specific *Fragaria* orthologs (Suppl. Fig. S2). The total dehydrin content (obtained by summing all K-peptide antibody-reactive bands) after 14 days of cold acclimation was not correlated to the LT_{50} values (data not shown); however, a strong correlation was evident at 42 days ($r = -0.81$, $P < 0.0001$; Fig. 3a).

Our own preliminary observations in the octoploid *F.* × *ananassa* indicated that there was a high correlation between alcohol dehydrogenase (ADH) levels and cold tolerance as indicated by LT_{50} values (Koehler et al. 2012). In the present experiments with *F. vesca*, ADH levels were very low in control crowns (not shown), but strongly induced in the cold-treated crowns. For some genotypes and in particular the ones that turned out to possess the highest tolerance to cold, a 200-fold increase in ADH protein levels was observed after 42 days of cold treatment, relative to the controls (Figs. 3b, 4). The correlation between the estimated LT_{50} values and the ADH

Journal : Large 425 | Dispatch : 17-9-2012 | Pages : 13
Article No. : 1771 | LE | TYPESET
MS Code : Planta-2012-07-0465 | CP | DISK

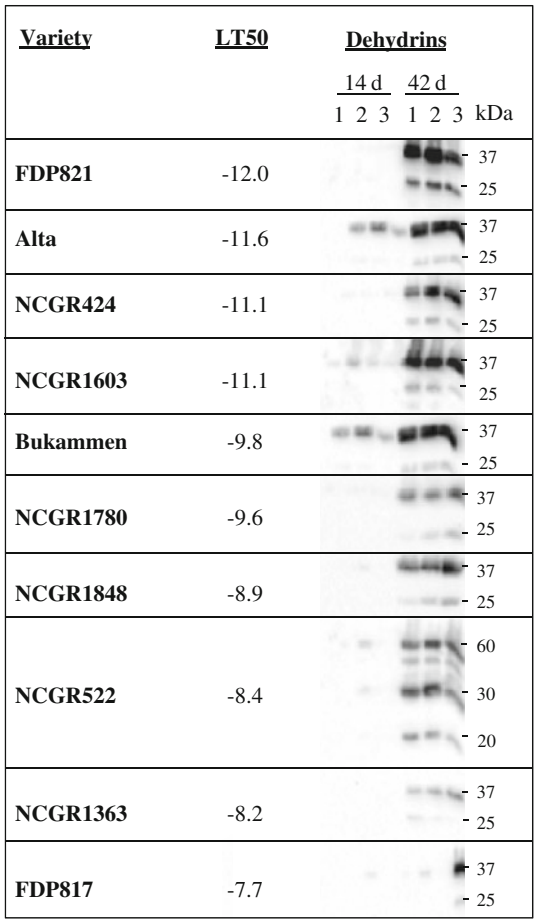

Fig. 2 Dehydrin levels in ten *Fragaria* genotypes. Extracts of crowns in three biological replicates from plants treated for 0 h, and 1, 2, 14, and 42 days at 2 °C were separated on 12 % SDS-PAGE and probed with anti-K peptide. Probing and visualization were done in two groups. Antibody-reactive bands appeared only for the 14 and 42 days cold-treated samples, and only blot sections with these samples are shown

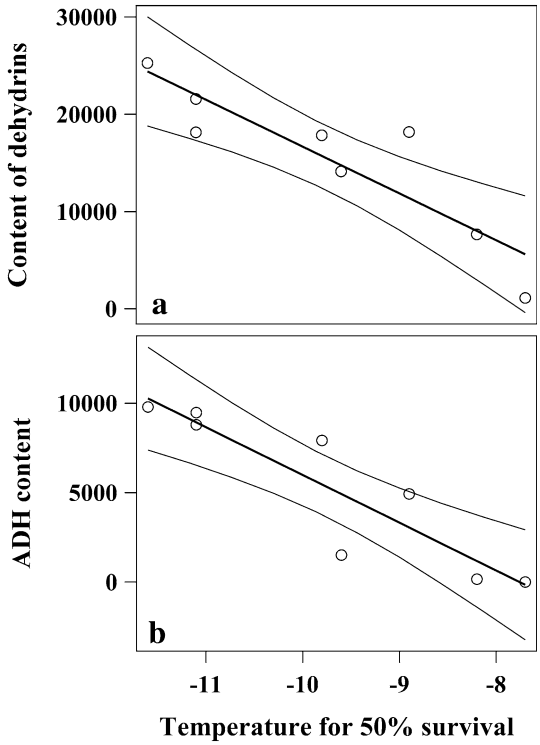

Fig. 3 Linear regression line fit between dehydrin content (**a**, $R^2 = 0.67$, $r_{dehyd} = -0.82$, $P < 0.0001$), alcohol dehydrogenase content (**b**, $R^2 = 0.74$, $r_{adh} = -0.87$, $P < 0.0001$), and the LT_{50} estimates. The 95 % confidence intervals are indicated. Protein levels are expressed as dimensionless arbitrary values. Each data point is the average of three measurements. Only *F. vesca* genotypes were used for these correlations. When all the genotypes were included in the regression analyses, the R^2 values fell to $R^2_{dehyd} = 0.24$ ($r = -0.49$, $P = 0.0034$) and $R^2_{adh} = 0.47$ ($r = -0.69$, $P < 0.0001$)

expression levels after 6 weeks of cold acclimation was significant with an $r = -0.86$ ($P < 0.0001$; Fig. 3b).

The relationship between dehydrins or ADH levels and cold tolerance in *F. vesca* genotypes revealed by linear regression (Fig. 3) did not include two of the genotypes tested in the Western blots (Figs. 2, 4). *F. nubicola*, while of interest for its low cold tolerance and dehydrin expression, represented a distinct species from *F. vesca*. FDP821, a distinct subspecies which did not produce any fertile hybrids when used as a parent in hybridization experiments with *F. vesca* (not shown), suggesting significant chromosomal differences or an efficient incompatibility system between FDP821 and all the other *F. vesca* genotypes, was also not included in the regression analysis. It was interesting; however, that FDP821, the most cold tolerant of the tested genotypes, showed the highest levels of dehydrin accumulation, but relatively low expression of ADH, though not as low as the least cold-tolerant genotypes. *F. nubicola* (NCGR522), a cold-susceptible genotype, had a moderate but distinctive dehydrin expression pattern and no detectible ADH. However, when these genotypes were included in the regression analyses, the R^2 obtained were $R^2_{dehyd} = 0.24$ ($r = 0.49$, $P = 0.0034$), and $R^2_{adh} = 0.47$ ($r = 0.69$, $P < 0.0001$).

Metabolite profiling

From the table of means (Table 2), there seem to be different patterns of leaf metabolic responses across the time points. Metabolites like fumaric acid, aspartic acid, glutamic acid, asparagine, citric acid, galactose, sucrose, and raffinose by and large show an increase in content during the whole acclimation period. Others in general decrease toward the last time point (succinic acid, malic acid, fructose, and glucose), and finally there are metabolites that do not seem to change much as the acclimation proceeds (galactinol). These general patterns are, however, frequently broken by local peaks or troughs, e.g., the galactose content at day 14 (Table 2).

Journal : Large 425 Dispatch : 17-9-2012 Pages : 13
Article No. : 1771 LE TYPESET
MS Code : Planta-2012-07-0465 CP DISK

Some of the metabolites show significant positive correlations to the LT_{50} estimates (Table 3). A positive correlation would indicate that at the particular time point, the content of the metabolite is lower for the more cold-tolerant genotypes. The significant correlations observed for succinic acid at four of the five time points is to a large extent caused by the relatively high content of succinic acid in the low-tolerant genotype, FDP817 (Suppl. Table S1).

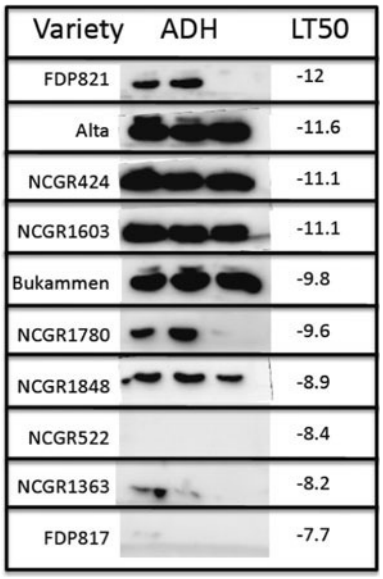

Fig. 4 Alcohol dehydrogenase (ADH) protein levels in *F. vesca* genotypes. Extracts of crowns from plants treated for 6 weeks at 2 °C were separated on 12 % SDS-PAGE and then probed with anti-ADH. Since bands were not visible for controls (0 h at 2 °C) at this exposure, they are not shown. Gels were all blotted onto the same nitrocellulose paper and thus probed simultaneously with antibodies. For each variety, triplicates are shown

The lack of correlation between the raffinose content and LT_{50} estimates is notable, but consistent across all time points (Table 3). On the other side, both sucrose and galactinol correlate well with LT_{50}, at least at some of the early time points.

One of our goals in the current work was to identify compounds that could be correlated to low-temperature tolerance (LT_{50} estimates). We expected plants to be fully acclimated after 28 days of low-temperature exposure and choosing this time point for our multivariate data analyses seemed natural. In the multiple regression analysis approach using data from day 28, only the galactinol content was retained as the only significant ($R^2 = 0.63$, $P < 0.0001$) explanatory variable for the variation in low-temperature tolerance (LT_{50}). The content of raffinose showed no such co-variation with the LT_{50} estimates. The linear regression lines for the raffinose and the galactinol contents are presented in Fig. 5.

The PCA of the metabolite data from before the acclimation started (day 0) and at the end of the acclimation period (day 28) showed that five components were required to account for 90 % of the total variation in the metabolite data matrix. The first principal component had contributions from most of the metabolites, but not from succinic acid, malic acid, fructose, and glucose. The component loadings varied between 0.36 and 0.42. The second principal component was dominated by glucose, fructose, and galactinol.

The plot of the two first PC axes is given in Fig. 6. At the starting point (day 0), all the genotypes cluster relatively well together. After 28 days of cold acclimation, their metabolite profiles had become much more heterogeneous and spread in various directions. This response

Table 2 Leaf metabolite changes averaged across ten diploid *Fragaria* genotypes during acclimation at 2 °C

Metabolite	Abbrev.	Days in cold acclimation					
		0	Content of metabolite relative to day 0				
		µg g^{-1} FW	0	1	2	14	28
Succinic acid	SucA	44.7 ± 5.0	100	46	58	33	36
Fumaric acid	FumA	23.7 ± 3.9	100	73	84	100	224
Malic acid	MalA	1,913 ± 171	100	150	86	77	55
Aspartic acid	AspA	19.5 ± 5.4	100	204	344	590	806
Glutamic Acid	GluA	74.7 ± 16.7	100	111	180	475	576
Asparagine	Asp	6.5 ± 3.5	100	52	866	2,616	4,526
Citric acid	CitA	1,824 ± 93	100	164	109	144	247
Fructose	FruS	1,130 ± 75	100	159	119	83	63
Galactose	GalS	7.9 ± 3.5	100	1,329	4,000	7,089	3,177
Glucose	GluS	574 ± 24	100	170	113	87	79
Sucrose	SucS	16,202 ± 490	100	452	121	126	184
Galactinol	Galact	141 ± 10	100	257	112	140	116
Raffinose	RafS	309 ± 24	100	249	128	389	520

Metabolite content at initiation was set to 100 % and percent increases/decreases are relative to these initial values. Actual contents in µg g^{-1} FW and the corresponding standard errors at day 0 are also presented. An extended table of the metabolite contents is given in Suppl. Table S1

Journal : Large 425 Dispatch : 17-9-2012 Pages : 13
Article No. : 1771 LE TYPESET
MS Code : Planta-2012-07-0465 CP DISK

Table 3 Pearson correlation coefficients (r) of selected metabolites versus LT_{50} values for ten diploid *F. vesca* genotypes from cold acclimation trials at 2 °C over a period of 4 weeks (sample material: leaf)

Metabolite	0	1 day	2 days	14 days	28 days
Succinic acid	0.63***	0.63***	0.62***	0.52**	0.15
Fumaric acid	0.46*	0.12	0.23	0.40*	0.02
Malic acid	0.05	0.25	0.17	0.29	0.31
Aspartic acid	0.04	−0.23	−0.17	−0.11	−0.14
Glutamic acid	−0.01	−0.14	−0.14	−0.11	−0.13
Asparagine	−0.01	−0.30	0.19	−0.02	−0.25
Citric acid	0.51**	0.35	−0.15	0.27	−0.13
Fructose	−0.09	−0.14	−0.03	−0.01	0.31
Galactose	−0.28	−0.14	−0.09	0.08	0.21
Glucose	−0.03	0.10	−0.03	0.03	0.53**
Sucrose	0.51**	0.68***	0.42*	0.29	0.29
Galactinol	0.52**	0.49**	0.30	0.27	0.79***
Raffinose	−0.04	−0.16	−0.22	0.29	0.24

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

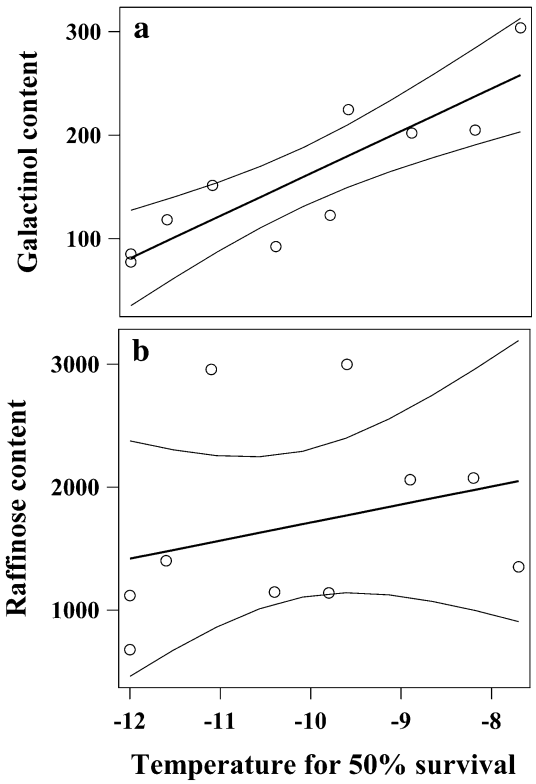

Fig. 5 Linear regression line fit between the LT_{50} estimates and galactinol (**a**, $R^2 = 0.63$, $r_{galact} = 0.79$, $P < 0.0001$) and raffinose content (**b**, $R^2 = 0.06$, $r_{raff} = 0.24$, $P = 0.24$) after 28 days of cold acclimation at 2 °C. The 95 % confidence intervals are indicated. Each data point is an average of three measurements and the sugar content unit is µg/g FW. Only *F. vesca* genotypes were used for these regression plots

due to acclimation is also illustrated in the heat maps of the two time points (Suppl. Fig. S4). FDP817 and NCGR1780 appear to respond in a similar way, while the remaining genotypes form a more or less diffuse cluster (Fig. 6). The one exception is possibly the Alta genotype. It seems to behave differently from the other genotypes by showing little movement or regrouping caused by acclimation (Fig. 6).

Since Fig. 6 only depicts two of the five axes necessary to account for the bulk variation (>90 %) in the metabolite data, a more nuanced illustration is provided by the heat map in Fig. 7. Here, the simultaneous hierarchical clustering of the two time points is presented as a heat map. There appear to be two genotype clusters that mainly consist of genotypes before acclimation (0 days) and those after acclimation (28 days). However, a couple of exceptions are notable. The Alta genotype appears in the same cluster both before and after acclimation, in agreement with Fig. 6, indicating that the acclimation results in only subtle changes in this genotype's metabolite composition. Alta originates from the very northern parts of Norway and has been shown to respond differently also in other traits, e.g., in response to flowering stimuli (Heide and Sønsteby 2007). Moreover, at time point 28, the NCGR1848 and FDP817 are clustered together with the bulk of the time point 0 entries. Figure 7 indicates that these entries do not respond typically to the acclimation. For instance, they lack the accumulation of aspartic acid, glutamic acid, and asparagine observed after the acclimation period in the majority of the entries (Fig. 7). Finally, NCGR1780 also responds atypically (Figs. 6, 7). The implication is that there are varying responses to acclimation at the metabolic level, and given the diverse origin of the accessions, this may not be surprising.

As for the metabolites, there seem to be two or three structures in their responses to the cold acclimation (Fig. 7). The first cluster consists of fructose, glucose, succinic acid, malic acid, and galactinol, and their content is reduced toward the end of the acclimation period. A second cluster consists of aspartic acid, glutamic acid, citric acid, and asparagine, and these metabolites are in

Journal : Large 425 Dispatch : 17-9-2012 Pages : 13
Article No. : 1771 LE TYPESET
MS Code : Planta-2012-07-0465 CP DISK

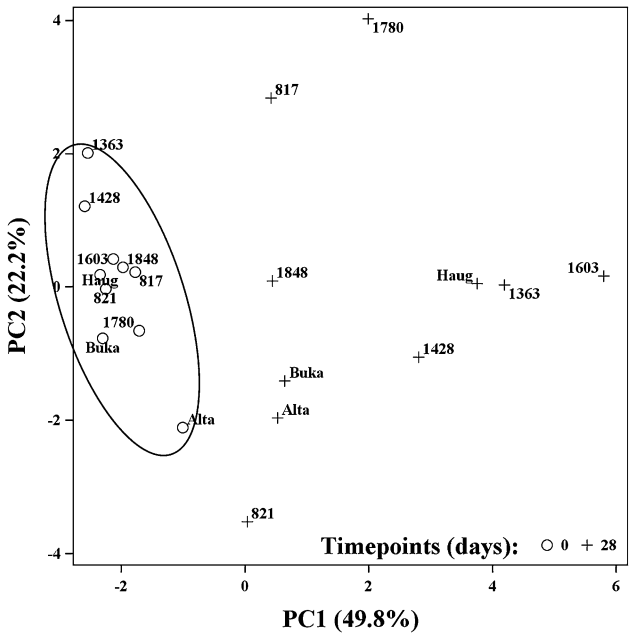

Fig. 6 The plot of the two first principal component axes spanning a total of 72.0 % of the total variation in the metabolite data matrix before (time point 0, *open circles*) and after acclimation (time point 28 days, *plus sign*). All the metabolites presented in Table 3 were included in the PC analysis. For improving readability, three or four letters/digits are used for the genotype identifications in the plot, e.g., 'Buka' refers to Bukammen, '1428' refers to NCGR1428, and 817 refers to FDP817 (Table 1) and so forth

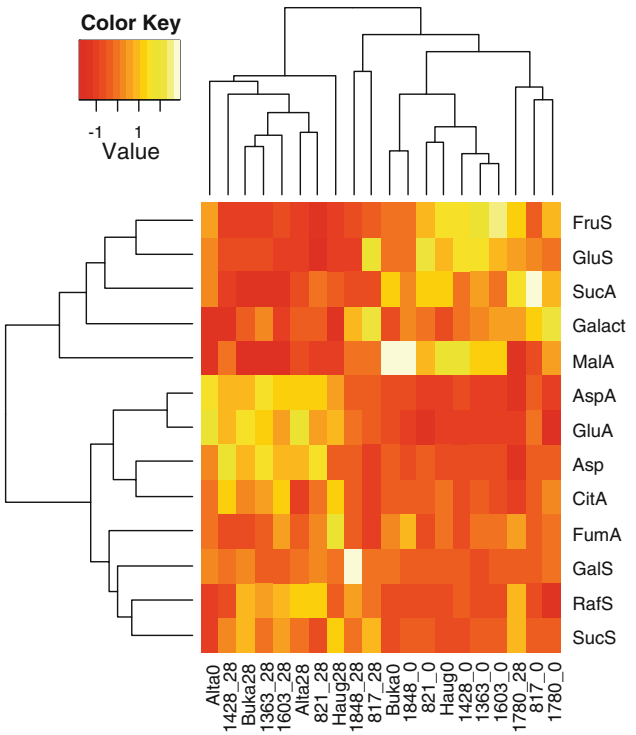

Fig. 7 Hierarchical clustering of the metabolite contents in ten genotypes before (0) and after (28 days) cold acclimation at 2 °C. Refer to Fig. 6 for identification of the genotypes and to Table 2 for the metabolites. Measurements within each time point were standardized to zero mean and a standard deviation of one for all metabolites and Euclidian distances was used for the clustering. The *points' colors* indicate the deviation from the mean for that particular metabolite × genotype × time point combination. *Red* indicates the lowest values and *white* the highest

general accumulated during acclimation. For the third cluster consisting of fumaric acid, galactose, raffinose, and sucrose, the response appears more diffuse, but there is an accumulation of these metabolites during acclimation.

Discussion

Genotypic differences in the LT_{50} estimates

One of our approaches to the study of low-temperature stress is to develop molecular markers and identify genomic regions of importance for the regulation of this trait. Identifying parents that differ significantly is a prime objective at this stage and pair-wise comparisons were therefore performed. In particular, we were interested in using Alta as one parent for mapping purposes. Alta is collected in the north of Norway and has been studied extensively in day-length × temperature experiments (Heide and Sønsteby 2007). We did expect it to be on the robust side of the response distribution. Another robust parental candidate is FDP821, which turned out to be as low-temperature tolerant as Alta. FDP821 was collected from Wyoming, USA. Our previous hybridization experiments, however, revealed that developing F_2 mapping populations with FDP821 as one of the parents proved difficult, as F_1 hybrid plants do not set viable seed. FDP817 (*F. vesca* ssp. *californica*), which had the highest LT_{50} estimates among the *F. vesca*, survives the winter poorly even in southern England according to anecdotal information.

To our knowledge, only two papers have been published on low-temperature stress tolerance in diploid *Fragaria* species (Sønsteby and Heide 2011; Rohloff et al. 2012). One of the genotypes previously tested was also tested in our work, namely Alta. Although previous work did not compute LT_{50} values, they observed surviving Alta plants even at −24 °C. These temperatures are well below that observed as the survival limit, and also Sønsteby and Heide (2011) found that all non-hardened plants survived at −6 °C. One possible explanation for such differences compared to our observations could be that Sønsteby and Heide (2011) hardened the plants under different acclimation conditions. In fact, they state that deep cold hardening in woodland strawberry requires several months of exposure to temperatures slightly above 0 °C to develop

Journal : Large 425 Dispatch : 17-9-2012 Pages : 13
Article No. : 1771 LE TYPESET
MS Code : Planta-2012-07-0465 CP DISK

deep cold hardiness. The 6 weeks we used as acclimation may thus be too short to attain the full level of hardiness. We considered the ranking of the genotypes to be our major goal and, although we could not dismiss the possibility of genotypes interacting with other environmental factors resulting in shifts in ranking, we expect that such possible shifts would be of minor importance.

The range in the LT_{50} values we found for *F. vesca* is in some agreement with observations in *F.* × *ananassa* (Marini and Boyce 1977, 1979) where normally hardened plants are slightly wounded at −4 °C. Severe injuries occurred at −12 °C, but survival was observed with crown temperatures reaching −20 °C. These authors used a slightly different testing regime, i.e., they only allowed the plants to reach the target temperature and then removed them immediately to thaw in the greenhouse, while we kept the target temperature for 4 h and then slowly, with controlled temperatures, returned plants to above freezing temperatures, and finally to 18 °C growing conditions.

Fragaria dehydrins and alcohol dehydrogenase are associated with low-temperature tolerance

Dehydrins are known to be involved in response to a wide range of abiotic stresses, such as cold, drought, and salt stress (Campbell and Close 1997). Dehydrins are well conserved between the plant genera and homologs are readily identified based on sequence similarity and in particular by the presence of the K-segment, a signature dehydrin consensus sequence. However, Koehler et al. (2012) identified changes in dehydrin transcript levels during cold acclimation in two Norwegian *F.* × *ananassa* cultivars, Frida and Jonsok. These two cultivars differ in their low-temperature tolerance—Jonsok being the most tolerant one. The XERO2-like dehydrin increased in both cultivars during the acclimation period, but to a much larger extent in Jonsok. While the COR47-like dehydrin transcript decreased with acclimation, the level in Jonsok before the acclimation period was much higher than the less cold-tolerant cultivar (Koehler et al. 2012).

In other species within Rosaceae, dehydrins have been identified to have a high similarity to *Arabidopsis* dehydrins. In peach, COR47-like (Bassett et al. 2009) and XERO2-like dehydrins have been found (Artlip et al. 1997), and in apple, an ERD10-like dehydrin (Garcia-Bañuelos et al. 2009). To obtain a better understanding of the dehydrin family of proteins in strawberry, we based our bioinformatic analyses (Suppl. Fig. S3) on the recently published *F. vesca* genomic sequence (Shulaev et al. 2011). We predicted that the *Arabidopsis*-derived dehydrin antibodies used in our present experiments were likely to react with the predicted *Fragaria* dehydrin proteins (Suppl. Figs. S2 and S3).

All dehydrin bands correlate with the LT_{50}; however, for the eight *F. vesca* genotypes the total level of dehydrin at 6 weeks was highly correlated ($r = -0.81$) with LT_{50}. This makes the overall dehydrin content a very good candidate for a freezing tolerance protein marker. That increased dehydrin expression is sufficient to increase frost tolerance was previously shown by transforming a *F.* × *ananassa* cultivar with the wheat dehydrin gene WCOR410. Freezing tolerance, as measured by the electrolyte leakage test, increased by −5 °C compared to the wild type (Houde et al. 2004). In blueberry stem and leaf tissue, two varieties of differing cold hardiness were compared (Danyluk et al. 1994), and in agreement with our results, the most winter hardy variety showed the strongest induction of dehydrin, both at the protein and mRNA levels. A positive correlation between a dehydrin and freezing tolerance was also found in a segregating F_2 population of *Rhododendron* (Lim et al. 1999).

We examined alcohol dehydrogenase as it can enhance stress survival by ameliorating hypoxic conditions brought on by melting snow or ice encasement. Thus by increasing the glycolytic fermentation pathways and shifting the end point away from lactate and toward ethanol (Drew 1997), elevated levels of ADH can prevent accumulation of toxic end products of anaerobic metabolism, preventing injury and thus increasing winter survival. Based on the high correlation of ADH levels with LT_{50} ($r = -0.86$), it is likely that ADH contributes to cold hardiness in *F. vesca*. This protein is thus a very good candidate as a molecular marker for cold stress tolerance.

Central metabolites in the leaf showed correlation to LT_{50}-based freezing tolerance

Sucrose accumulation in response to cold exposure is a common observation and is a result of the increased activity of sucrose phosphate synthase and sucrose synthase (Sasaki et al. 2001). Recently, Schulze et al. (2011) observed significant increases in leaf content of glucose, fructose, and sucrose during cold acclimation of *A. thaliana*, in agreement with other authors (Cook et al. 2004; Kaplan et al. 2007; Guy et al. 2008) and also in accordance with our overall response observations. But a closer look showed that the genotypes responded differently as also reported in our earlier study (Rohloff et al. 2012). If we look at the correlations between these sugars and the survival rate of the plants after cold exposure, the LT_{50} estimates, there is a positive correlation to the sucrose. So, even though on an overall basis there is a significant accumulation of the mono- and disaccharides (i.e., galactose, sucrose, and raffinose), the positive correlation between sucrose levels and LT_{50} at the beginning of the acclimation period indicates that the genotypes with the

Journal : Large 425 Dispatch : 17-9-2012 Pages : 13
Article No. : 1771 LE TYPESET
MS Code : Planta-2012-07-0465 CP DISK

lowest sucrose content are the most low-temperature tolerant ones.

Raffinose is often found up-regulated in other plant species, e.g., *Arabidopsis thaliana*, during cold acclimation (e.g., Korn et al. 2010), and this was also the case with our material (Table 2). However, we did not observe a significant correlation to the LT_{50} estimates at any time point during acclimation (Table 3). It has been shown, however, that raffinose accumulation is neither necessary nor sufficient for the induction of freezing tolerance in *A. thaliana* (Zuther et al. 2004). While our present results (Table 2) and previous ones (e.g., Saito and Yoshida 2011; Rohloff et al. 2012) show that both raffinose and galactinol contents are enhanced during acclimation (i.e., the raffinose pathway), only galactinol content showed a significant correlation to cold stress tolerance in our study (Fig. 5). Moreover, this correlation was positive, implying a relatively lower level of metabolite in the hardiest genotypes.

The majority of studies on low-temperature tolerance have been conducted with *Arabidopsis thaliana*, which survives winter either as a small plantlet (winter annual) or as seed. The strawberry, however, prepares for winter by senescence and translocation of the majority of assimilates to the crown. Could this explain why we, for instance, observe that the most cold-tolerant genotypes exhibit the lowest levels of galactinol after acclimation? Is it because these are the genotypes that most efficiently transport the solutes to the crown in preparation for winter? Our ongoing research addresses these issues.

To examine the natural variation in cold/freezing tolerance, 22 diploid *Fragaria* genotypes were acclimated and then tested to obtain plant survival estimates (LT_{50}). Correlation of plant survival with leaf metabolite profiles and with the expression of dehydrin and alcohol dehydrogenase proteins in the crown during acclimation indicated that the proteins and the sugar alcohol galactinol showed the clearest association with cold tolerance and thus the greatest potential to be developed into biomarkers.

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Conflict of interest The authors declare that they have no conflict of interest.

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Journal : Large 425 | Dispatch : 17-9-2012 | Pages : 13
Article No. : 1771 | ☐ LE | ☐ TYPESET
MS Code : Planta-2012-07-0465 | ☑ CP | ☑ DISK

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