The role of raffinose in the cold acclimation response of *Arabidopsis thaliana*

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Abstract In many plants raffinose family oligosaccharides are accumulated during cold acclimation. The contribution of raffinose accumulation to freezing tolerance is not clear. Here, we investigated whether synthesis of raffinose is an essential component for acquiring frost tolerance. We created transgenic lines of *Arabidopsis thaliana* accessions Columbia-0 and Cape Verde Islands constitutively overexpressing a galactinol synthase (GS) gene from cucumber. GS overexpressing lines contained up to 20 times as much raffinose as the respective wild type under non-acclimated conditions and up to 2.3 times more after 14 days of cold acclimation at 4 °C. Furthermore, we used a mutant carrying a knockout of the endogenous raffinose synthase (RS) gene. Raffinose was completely absent in this mutant. However, neither the freezing tolerance of non-acclimated leaves, nor their ability to cold acclimate were influenced in the RS mutant or in the GS overexpressing lines. We conclude that raffinose is not essential for basic freezing tolerance or for cold acclimation of *A. thaliana*.

Keywords: Cold acclimation; Freezing tolerance; Galactinol synthase; Raffinose family oligosaccharide; Raffinose synthase; *Arabidopsis thaliana*

1. Introduction

Compatible solutes are synthesized by many organisms ranging from bacteria to animals and plants, in response to desiccation, osmotic stress or low temperature (see [1,2] for reviews). The functional role of sugars in cellular stress tolerance, however, has recently been challenged [3]. Soluble sugars of the raffinose family have been implicated in plant responses to abiotic stress conditions. In many species, raffinose family oligosaccharides (RFO) are accumulated during the process of cold acclimation, when plants acquire increased frost tolerance [4–8]. During seed maturation, RFO are accumulated concurrently with the reduction of tissue water content and the development of desiccation tolerance of seeds (see [9] for a review). It has been proposed that raffinose (Raf) and sucrose (Suc) are involved in cytoplasmic vitrification in dry seeds [10], thereby stabilizing sensitive macromolecular structures (see [11] for a review). In addition, RFO interact with membranes in the dry state by replacing water molecules in the hydration shell of the lipid headgroups, thereby preventing deleterious lipid phase transitions [12]. The molecular mechanisms by which Raf may influence cellular freezing tolerance are not clear, but it has been shown previously that Raf can stabilize isolated chloroplast thylakoid membranes during a freeze–thaw cycle [13].

RFO are synthesized from Suc by subsequent addition of activated galactose moieties donated by galactinol [14]. The synthesis of galactinol from *myo*-inositol and UDP-galactose is considered a key regulatory step in RFO synthesis [15] and enzyme activity of galactinol synthase (GS) is increased at low temperatures [6,16]. In *Arabidopsis*, this increase is correlated with increased transcript abundance [17], which results from cold induction of one of the seven GS genes [18]. This induction is mediated by CBF/DREB1 transcription factors, which control a complex set of plant responses to low temperatures [19,20]. Overexpression of CBF3/DREB1A causes increased frost tolerance and the accumulation of Raf in transgenic *Arabidopsis* plants, indicating physiological relevance of the GS induction [7].

Functional evidence for a role of RFO in plant abiotic stress tolerance was obtained for transgenic *Arabidopsis* that accumulate high levels of Raf due to constitutive overexpression of GS [18] and for transgenic petunia plants with a reduced α-galactosidase activity [21]. These plants contained elevated levels of Raf under normal growth conditions and showed higher drought and freezing tolerance, respectively, than wild-type plants.

In the present study, we used a knockout mutant of the raffinose synthase (RS) gene of *Arabidopsis thaliana*, which was not able to accumulate Raf, to investigate whether synthesis of Raf during cold acclimation is an essential component for acquiring frost tolerance. In addition, we compared wild-type plants with transgenic lines overexpressing GS and thus accumulating higher amounts of Raf than the wild type. Surprisingly, although the investigated genotypes differed widely in their Raf content, both in the non-acclimated (NA) and the acclimated (ACC) state, they did not show differences in freezing tolerance.

2. Materials and methods

2.1. Plant material

The *A. thaliana* plants used were from the accessions Columbia-0 (Col-0) and Cape Verde Islands (Cvi). The RS mutant RS14 in the

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Col-0 background was obtained from the GABI-Kat collection (line 106F01). The mutant carries a 5.8 kb T-DNA insert in the RS gene (At5g40390) at position 720 (relative to the start codon). Homozygous mutants were identified by PCR using two different primer pairs: a fragment of the wild-type allele of At5g40390 was amplified with the primers AtRS5′ (5′-GAGCCATGTGACTAGAACCATGTA) and AtRS3′ (5′-GATGGATGCCACGTGGTGGC), which give a fragment of 614 bp with the intact gene only. The mutant allele was traced with the AtRS5′ and the T-DNA-3′ primer (5′-CCCATTTGGACGTGAATGTAGACAC) that amplified a 865 bp fragment.

To create *Arabidopsis* lines with elevated Raf content, a GS cDNA from cucumber (*Cucumis sativus* L.) was isolated from a library constructed in the Lambda Zap Express phage using cucumber leaf RNA as template. From one of the five clones carrying a 1586 kb insert, the coding region of 993 bp as well as 135 bp 5′ and 163 bp 3′ UTR was excised and cloned into the binary vector pBin19 [22] containing the cauliflower mosaic virus 35S promoter/OCS terminator cassette. The GS overexpressing lines were created by *Agrobacterium* mediated gene transfer using the floral dip method [23].

All plants were grown in a greenhouse at 16 h day length with light supplementation to reach at least 200 $\mu Em^{-2}\ s^{-1}$ and a temperature of 20 °C during the day, 18 °C during the night until bolting. This was between 46 and 53 days after sowing. For cold acclimation, plants were transferred to a 4 °C growth cabinet at 16 h day length with 90 $\mu Em^{-2}\ s^{-1}$ for an additional 14 days.

2.2. Carbohydrate analysis

Leaf samples from three different plants were frozen in liquid nitrogen immediately after sampling and homogenized using a ball mill "Retsch MM 200" (Retsch, Haan, Germany). The homogenate was extracted twice in 1 ml of 80% ethanol at 80 °C. Extracts were dried and dissolved in 1 ml of distilled water. Afterwards the samples were deionized (AG 501-X8 resin, Bio-Rad). Glucose (Glc), fructose (Fru), Suc, and Raf were analyzed by high-performance anion exchange chromatography (HPAEC) using a CarboPac PA-100 column on a Dionex DX-500 gradient chromatography system coupled with pulsed amperometric detection by a gold electrode [12]. For the determination of galactinol, a MA-1 column was used on the same Dionex system. All measurements represent pools of three plants and were replicated five times. Samples were taken from the same plants used for the freezing experiments. Statistical analysis was performed using unpaired *t* tests.

2.3. Freezing experiments

Freezing experiments were performed as described recently [24]. Briefly, three rosette leaves taken from three individual plants were placed in a glass tube containing 200 µl of distilled water. Tubes were transferred to a programmable cooling bath set to −1 °C, a control was left on ice during the entire experiment. After 30 min of temperature equilibration at −1 °C, ice crystals were added to the tubes to initiate freezing. After another 30 min, the samples were cooled at a rate of 2 °C/h. Over a temperature range of −1 to −12 °C for NA plants and −1 to −18 °C for acclimated plants, samples were taken from the bath and thawed slowly on ice. After thawing, leaves were immersed in distilled water and placed on a shaker for 16 h at 4 °C. Electrolyte leakage was determined as the ratio of conductivity measured in the water before and after boiling the samples [24]. The temperature of 50% electrolyte leakage (LT_{50}) was calculated as the log EC_{50} value of sigmoidal curves fitted to the leakage values using the software GraphPad Prism3. Regression curves were calculated using the mean of six replicates, each of which consisted of leaves from three plants. Statistical analysis of the differences in LT_{50} was performed using unpaired *t* tests.

2.4. Proline measurements

Proline measurements followed the method of [25], with the modifications described recently [24]. Statistical analysis was performed using unpaired *t* tests.

2.5. Analysis of gene expression

RNA was prepared from approximately 200 mg of rosette leaf tissue following the method described in [26]. For gel blot analysis, 20 µg of RNA was denatured in a buffer containing 34% formamide, 4% formaldehyde and 30 $\mu g\ ml^{-1}$ ethidum bromide. Denaturing gel electrophoresis using 1.5% agarose gels was carried out as described in [27] and gels were blotted on nylon membrane (Hybond N, Amersham, Braunschweig, Germany) by capillary transfer. Membranes were prehybridized for 4 h at 42 °C in a buffer containing 40 mM sodium phosphate (pH 6.8), 5× Denhardt's solution, 1% (w/v) SDS, 100 µg/ml salmon sperm DNA, 50% formamide and 5× SSC, and hybridized with radioactively labeled probes (Ready Prime, Amersham) for 16 h at 42 °C. The probe specific for the RS of *A. thaliana* was amplified by PCR with the following primers: Atrafsyn 5′: GAGCCATGTG-ACTA-GAACCATGTA, Atrafsyn 3′: GATGGATGCCACGTGGTGGC. After hybridization, membranes were washed for 20 min at 60 °C in 2× SSC/0.5% (w/v) SDS and for two times 20 min at 60 °C in 1× SSC/0.5% (w/v) SDS, before exposing them to phosphoimaging screens (Fuji, Berlin, Germany).

The expression level of GS in wild-type and transformants was determined by quantitative real-time RT-PCR essentially as described previously [24]. The primers were designed using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) to be specific for the cucumber gene: CsGS-F: TGCCCCAGAAAGTGCCATT, CsGS-R: ATTACCAGCCAAGAACGTCACG. PCRs were performed in a 5700 Sequence Detection System from Gene Amp, using the QuantiTect SYBR Green RT-PCR kit (Quiagen). Threshhold cycle numbers were directly compared between wild-type and transformants, without the use of a reference gene.

2.6. DNA extraction and Southern blot analysis

Genomic DNA was prepared from whole plants following the method of [28]. Fifty micrograms of high molecular weight DNA was digested with the restriction enzyme *Dra*I and size fractionated by agarose gel eletrophoresis. Transfer onto nylon membranes and hybridization was as described for RNA analysis. The probe used for hybridization spanned the region of T-DNA insertion into the RS gene. A probe was designed specific for a 865 bp region, including the insertion site of the T-DNA within the RS gene with the following primers: Atrafsyn 5′: GAGCCATGTGACTAG-AACCATGTA and T-DNA 3′: CAATTGTAAATGGC-TTCA.

3. Results

3.1. Characterization of the A. thaliana RS mutant RS14 and GS overexpressors

Eighty-two plants grown from seeds of the GABI-Kat batch 106F01 were screened for homozygous mutants of the RS gene by PCR. The primer combination AtRS5′/T-DNA-3′, which is specific for the mutated allele, yielded the expected product in 3/4 of the plants. The wild-type allele, which was amplified with the AtRS5′/AtRS3′ combination, was absent in half of the plants (data not shown). From these homozygous mutants, line RS14 was chosen for further experiments. Southern blotting experiments indicated only one T-DNA insertion in the genome of RS14 (data not shown). To establish that line RS14 really carries a knockout mutation in the RS gene, we determined RS transcript levels by Northern blotting. Fig. 1 shows that the RS gene is cold induced in the wild-type Col-0 plants, but that the transcript is not detectable in the mutant RS14 plants, both under NA and ACC conditions.

Soluble sugars and galactinol in NA as well as ACC wild-type and RS14 mutant plants were determined by HPAEC (Fig. 2). Raf was completely absent in the mutant, while a peak at the retention time of galactinol was strongly amplified, especially in the acclimated RS14. For quantification of sugars and galactinol, different HPAEC columns had to be used to achieve better separation of sugar alcohols. The results are summarized in Table 1 for NA and in Table 2 for ACC plants. In addition, the determination of proline levels in wild-type and RS14 plants under NA and ACC conditions showed no evidence for a compensatory accumulation of proline in the mutant (data not shown).

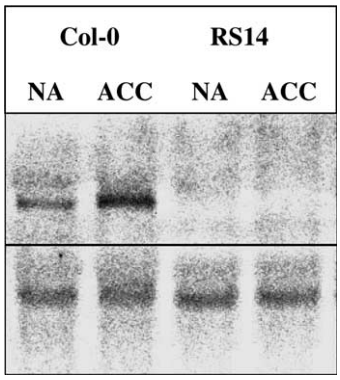

Fig. 1. Northern blot analysis of the expression of the raffinose synthase gene in leaves of *Arabidopsis thaliana* wild-type (Col-0) and RS knockout mutant (RS14) plants. Plants were either harvested after growth under NA conditions, or after 14 days at 4 °C (ACC). The upper panel shows the band corresponding to RS transcripts, the lower panel shows the band for 18S ribosomal RNA as a loading control.

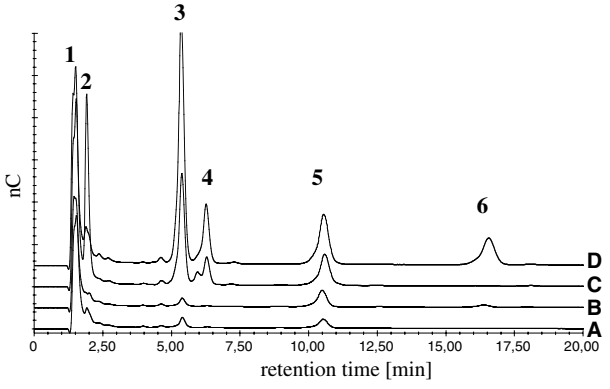

Fig. 2. HPAEC chromatogram of leaf extracts from NA RS14 (A), NA Col-0 wild-type (B), cold acclimated RS14 (C) and cold acclimated Col-0 wild-type (D). Peak labels are: (1) sugar alcohols including *myo*-inositol; (2) galactinol; (3) glucose; (4) fructose; (5) sucrose; (6) raffinose.

Of the 58 transgenic Col-0 lines screened for GS expression by analysis of soluble carbohydrates, three lines with the highest Raf content were selected for expression analysis. In the accession Cvi, 38 transgenic lines were analyzed for their composition of soluble carbohydrates. From these, three lines with the highest Raf content were chosen for further expression analysis. Quantitative real-time RT-PCR using primers specific for the cucumber GS clearly showed elevated expression of the GS gene compared to the respective wild-type in all three Col-0 and Cvi transgenic lines (data not shown). The difference in threshhold cycle number was between 15.3 and 12.7 for Cvi and between 15.7 and 5.6 for Col-0. There was, however, no direct correlation between gene expression level and Raf or galactinol content.

The GS overexpressing lines in the Col-0 background contained between 2 and 3.5 times as much Raf as the wild-type under NA conditions, but only 1.6 times more after cold exposure (Tables 1 and 2). Due mainly to differences in SEM, not all differences were statistically significant. Galactinol was significantly elevated in some lines under NA conditions, but not after cold acclimation, presumably due to an increased expression of RS, which channelled galactinol onto Raf. The relative increases in Raf content were even higher in transformants in the Cvi background. In CsG13, Raf content was 20-fold higher than in the wild-type under NA conditions and 2.3-fold higher in ACC plants. The absolute values, however, were always much lower in the Cvi plants than in the Col-0 plants.

The Raf content of RS14 plants was always below the detection limit. The galactinol content, on the other hand, was 8.7-fold higher in RS14 than in the wild-type Col-0 plants after cold acclimation, while it increased 4.8-fold in NA plants. For the other sugars, changes were less pronounced, except for a 2- to 3.5-fold reduction for Suc, Glc and Fru in the acclimated RS14 (Tables 1 and 2).

3.2. Freezing tolerance of RS mutant and GS overexpressors

To assess freeze-induced cell damage of Arabidopsis leaf tissue differing in Raf content, we measured electrolyte leakage from rosette leaves frozen to temperatures varying from −1 to −18 °C [24]. The LT_{50} values for the various lines in the NA and ACC state are shown in Fig. 3. We recorded a difference in the LT_{50} of NA vs. ACC leaves of about 4 °C in the Col-0 wild-type, demonstrating a substantial effect of cold acclimation on freezing tolerance, as reported for this accession before (see [24] and references therein). Cvi only acclimated by 1.9 °C,

Table 1
Soluble carbohydrates in NA leaves of wild-type *A. thaliana*, accessions Col-0 and Cvi, transgenic plants overexpressing GS from cucumber (CsG26, 32, 58 in the Col-0 background; CsG13, 28, 33 in the Cvi background) and the RS mutant RS14 in the Col-0 background

	Galactinol	Glc	Fru	Suc	Raf
Col-0	0.02 ± 0.00	0.24 ± 0.06	0.08 ± 0.05	1.19 ± 0.15	0.02 ± 0.01
CsG26	**0.03 ± 0.00**	0.24 ± 0.07	0.09 ± 0.04	0.75 ± 0.09	0.04 ± 0.01
CsG32	**0.05 ± 0.00**	0.18 ± 0.04	0.04 ± 0.02	0.74 ± 0.07	**0.07 ± 0.01**
CsG58	0.04 ± 0.01	0.23 ± 0.03	0.07 ± 0.02	0.99 ± 0.11	0.06 ± 0.03
Col-0	0.03 ± 0.00	0.48 ± 0.07	0.03 ± 0.01	0.73 ± 0.22	0.09 ± 0.03
RS14	**0.14 ± 0.01**	0.28 ± 0.04	0.02 ± 0.01	0.58 ± 0.04	n.d.
Cvi	0.02 ± 0.01	0.55 ± 0.30	0.09 ± 0.07	0.84 ± 0.14	0.01 ± 0.00
CsG13	**0.08 ± 0.02**	0.21 ± 0.04	0.02 ± 0.01	1.00 ± 0.11	**0.20 ± 0.05**
CsG28	0.02 ± 0.01	0.20 ± 0.04	0.03 ± 0.01	0.91 ± 0.07	**0.04 ± 0.01**
CsG33	0.02 ± 0.00	0.21 ± 0.04	0.02 ± 0.01	0.96 ± 0.07	**0.05 ± 0.01**

The experiments with the overexpressors and the mutant were carried out independently and therefore independent Col-0 wild-type controls are shown. Values are given in μmol g^{-1} FW and represent means ± SEM of six measurements on biological pools of three plants. Numbers in boldface indicate values that are significantly higher than in the corresponding wild-type ($P < 0.05$). n.d.: not detectable.

Table 2
Soluble carbohydrates in cold acclimated leaves of wild-type *A. thaliana*, accessions Col-0 and Cvi, transgenic plants overexpressing GS from cucumber (CsG26, 32, 58 in the Col-0 background; CsG13, 28, 33 in the Cvi background) and the RS mutant RS14 in the Col-0 background

	Galactinol	Glc	Fru	Suc	Raf
Col-0	0.20 ± 0.04	9.36 ± 1.89	2.12 ± 0.29	3.28 ± 0.37	1.68 ± 0.20
CsG26	0.27 ± 0.03	13.72 ± 3.31	2.87 ± 0.81	3.66 ± 0.37	**2.54 ± 0.14**
CsG32	0.29 ± 0.04	8.80 ± 1.79	1.87 ± 0.57	2.53 ± 0.28	2.70 ± 0.42
CsG58	0.28 ± 0.03	9.37 ± 2.21	2.78 ± 1.02	3.11 ± 0.29	2.65 ± 0.43
Col-0	0.24 ± 0.03	12.18 ± 1.64	2.49 ± 0.52	3.48 ± 0.27	1.69 ± 0.25
RS14	**2.12 ± 0.12**	3.42 ± 0.34	0.73 ± 0.25	1.65 ± 0.29	n.d.
Cvi	0.05 ± 0.02	2.49 ± 0.64	0.24 ± 0.04	2.81 ± 0.32	0.42 ± 0.08
CsG13	**0.15 ± 0.01**	4.47 ± 0.91	0.77 ± 0.22	2.81 ± 0.14	**0.99 ± 0.05**
CsG28	0.08 ± 0.01	4.59 ± 1.04	0.95 ± 0.38	2.80 ± 0.26	0.55 ± 0.05
CsG33	**0.10 ± 0.00**	**6.45 ± 0.85**	**1.44 ± 0.35**	3.23 ± 0.34	**0.77 ± 0.05**

The experiments with the overexpressors and the mutant were carried out independently and therefore independent Col-0 wild-type controls are shown. Values are given in μmol g^{-1} FW and represent means ± SEM of six measurements on biological pools of three plants. Numbers in boldface indicate values that are significantly higher than in the corresponding wild-type ($P < 0.05$). n.d.: not detectable.

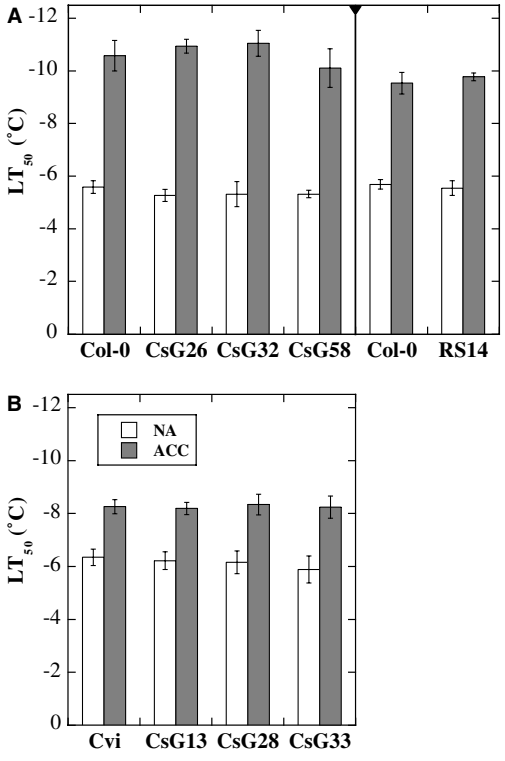

Fig. 3. Freezing tolerance of *Arabidopsis thaliana* leaves determined as the temperature that causes 50% electrolyte leakage after a freeze–thaw cycle (LT_{50}). (A) Data from experiments with Col-0 wild-type plants, and corresponding plants either overexpressing GS (CsG26, 32, 58), or carrying a T-DNA insertion in the RS gene, resulting in a knockout mutation. The experiments with the overexpressors and the mutant were carried out independently and therefore independent Col-0 wild-type controls are shown. (B) The results of experiments with Cvi wild-type plants and corresponding GS overexpressors (CsG13, 28, 33). The SEM is indicated for each bar.

very similar to the accession C24, which we investigated previously [24].

In contrast, differences between the different genotypes were very small and always below the significance threshold. Most interestingly, the RS14 mutant was indistinguishable from the wild-type, both under ACC and NA conditions.

4. Discussion

Using transgenic *A. thaliana* overexpressing a cucumber GS gene, we showed that the elevated levels of Raf we achieved in this study did not significantly increase the freezing tolerance of leaf tissues in the NA or ACC state. Increased drought tolerance of *Arabidopsis* plants overexpressing an endogenous GS gene has been demonstrated [18]. This could indicate that Raf is only important for drought, but not for freezing tolerance of plants. In transgenic petunia, however, the accumulation of Raf was associated with increased freezing tolerance of the leaves [21]. Increased Raf and stachyose accumulation was induced by antisense inhibition of the expression of the α-galactosidase gene. Also, overexpression of this gene resulted in reduced Raf and stachyose content and reduced freezing tolerance of the leaves. These data could be interpreted in different ways. Either freezing tolerance in petunia and *Arabidopsis* is affected differently by Raf, or the stachyose that is also accumulated in petunia, but not in *Arabidopsis*, is the active agent in petunia freezing tolerance, or α-galactosidase has additional effects on freezing tolerance that are unrelated to effects on RFO accumulation. The last assumption seems reasonable, since it has been shown that α-galactosidases have a variety of substrates besides RFO (see [14] for a review). A conclusive answer, however, could only be provided by manipulating the α-galactosidase gene expression in Arabidopsis and/or overexpressing the GS gene in petunia. In addition, it should be mentioned that the amounts of Raf that our transgenic plants accumulated were significantly lower than the amounts reported in [18] and [21]. Therefore, the possibility cannot be excluded that higher amounts of Raf would also improve the freezing tolerance of *Arabidopsis* leaves.

In *Arabidopsis*, the content of soluble sugars increased strongly during cold acclimation, largely due to an accumulation of Glc. A strong rise in Glc levels has previously been described in the *Arabidopsis* accession Wassilewskija-2 and can also be observed in transgenic plants overexpressing the cold-response regulator CBF3/DREB1A [7]. However, monosaccharides are far less effective than disaccharides in protecting proteins or membranes against damage during freezing [29], and exogenously applied Glc was shown to be incapable of increasing freezing tolerance of *Eucalyptus* cell cultures [30]. Instead of a specific adaptive response, the in-

crease in Glc may thus more likely reflect metabolic changes at low temperatures.

Suc was the second most abundant sugar in the acclimated Col-0 and Cvi plants and it increased substantially in concentration during cold acclimation, as did Fru. However, we showed in a recent publication that although Glc, Fru and Suc accumulate during cold acclimation in different *Arabidopsis* genotypes, there is no correlation between the concentration of these substances and leaf freezing tolerance. The only correlation we found in this study was between Raf content and freezing tolerance [24].

Nevertheless, neither the basic freezing tolerance of NA leaves nor their ability to cold acclimate was influenced in the RS mutant, demonstrating that Raf accumulation is dispensable for both components of freezing tolerance. In the case of NA plants, this may be expected, because of the low concentration of Raf. The lack of an effect of the RS mutation on the ability of the plants to cold acclimate, however, is astonishing. The RFO pathway is strongly induced in leaves during cold acclimation through cold-inducibility of one of the seven GS genes [18,19]. In CBF3/DREB1A overexpressing plants, in which multiple physiological processes associated with cold acclimation are already operating under NA conditions, Raf levels are elevated about 4-fold and further increase during cold acclimation to a level 7-fold higher than in wild-type plants [7]. These plants show enhanced freezing tolerance including a higher acclimation capacity. This also correlates with transcriptional induction of pyrroline-5-carboxylate synthase, a key enzyme in proline biosynthesis, implying a concerted induction of the synthesis of different compatible solutes.

It therefore seemed possible that the RS mutant would compensate for its lack of Raf by increasing levels of other sugars or proline, but this was clearly not the case. Only galactinol was substantially accumulated in the mutant. Reports on a possible role of galactosyl cyclitols in plant abiotic stress tolerance are rare. They are accumulated, sometimes together with RFO, in the seeds of many plant species [14]. However, proof of function is missing and data for a role in frost tolerance are not available. A compensatory role of galactinol in the RS14 mutant during cold acclimation can, nevertheless, not be ruled out. This would, however, not negate our conclusion that Raf itself is not required for cold acclimation.

Taken together, our data demonstrate that Raf is not an essential component of basic freezing tolerance and cold acclimation in *A. thaliana*. Since overexpression of GS did not lead to the large increase in Raf concentration observed in CBF3/DREB1A overexpressing plants, it will be interesting to study whether upregulating RS gene expression could further increase Raf levels and whether this would improve acclimation capacity.

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