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Dehydrins and Soluble Sugars Involved in Cold Acclimation of *Rosa wichurana* and Rose Cultivar ‘Yesterday’

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Abstract: Rose is the most economically important ornamental plant. However, cold stress seriously affects the survival and regrowth of garden roses in northern regions. Cold acclimation was studied using two genotypes (*Rosa wichurana* and *R. hybrida* ‘Yesterday’) selected from a rose breeding program. During the winter season (November to April), the cold hardiness of stems, soluble sugar content, and expression of dehydrins and the related key genes in the soluble sugar metabolism were analyzed. ‘Yesterday’ is more cold-hardy and acclimated faster, reaching its maximum cold hardiness in December. *R. wichurana* is relatively less cold-hardy, only reaching its maximum cold hardiness in January after prolonged exposure to freezing temperatures. Dehydrin transcripts accumulated significantly during November–January in both genotypes. Soluble sugars are highly involved in cold acclimation, with sucrose and oligosaccharides significantly correlated with cold hardiness. Sucrose occupied the highest proportion of total soluble sugars in both genotypes. During November–January, downregulation of *RhSUS* was found in both genotypes, while upregulation of *RhSPS* was observed in ‘Yesterday’ and upregulation of *RhINV2* was found in *R. wichurana*. Oligosaccharides accumulated from November to February and decreased to a significantly low level in April. *RhRS6* had a significant upregulation in December in *R. wichurana*. This study provides insight into the cold acclimation mechanism of roses by combining transcription patterns with metabolite quantification.

Keywords: cold hardiness; LT₅₀; sucrose; oligosaccharides; soluble sugar metabolism; gene expression



Citation: Ouyang, L.; Leus, L.; De Keyser, E.; Van Labeke, M.-C. Dehydrins and Soluble Sugars Involved in Cold Acclimation of *Rosa wichurana* and Rose Cultivar ‘Yesterday’. *Horticulturae* **2021**, *7*, 379. <https://doi.org/10.3390/horticulturae7100379>

Academic Editor: Anastasios Darras

Received: 30 August 2021

Accepted: 1 October 2021

Published: 8 October 2021

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1. Introduction

Rose is the most economically important ornamental plant. For garden roses grown in northern regions (Northern Europe, North China, Russia, the northern regions of the US and Canada), winter temperatures restrict the geographical distribution and affect winter survival, growth, and ornamental quality of garden roses. Breeding for improved cold hardiness would allow roses to survive wintry temperatures without extra protection, an important selling point for gardeners in colder regions. The study of the mechanisms involved in cold acclimation can help future varietal development in breeding programs as well as the selection of cold-hardy garden roses. Woody plants have evolved the ability to adapt to low temperatures and develop cold hardiness. Cold hardiness has a seasonal dynamic characterized by three phases: cold acclimation during autumn–winter, mid-winter hardiness (maximum level of cold hardiness), and deacclimation (loss of tolerance) during winter–spring. Evaluation of cold hardiness using electrolyte leakage analysis is a reliable method for the stems of woody plants [1,2]. Low temperatures can lead to the loss of membrane integrity, resulting in cellular damage and solute leakage across the membrane. The level of electrolyte leakage is related to the freezing temperatures and cold tolerance of plants.

Cold acclimation, the process by which the plant achieves cold hardiness/freezing tolerance, is associated with a wide spectrum of physiological and biochemical changes that occur in response to the decrease of photoperiod, light intensity and temperatures [3–6]. The most prominent changes during cold acclimation include growth reduction, a decrease in tissue water content, changes in membrane lipid compositions, induction of stress proteins, accumulation of osmolytes (soluble sugars, proline, betaine, etc.), and enhancement of the antioxidant system [7–11]. These changes are the results of genetic adjustments starting from the signal perception and transduction to transcriptional regulation, and finally to downstream stress-responsive gene expression [12,13].

Dehydrins are highly hydrophilic proteins which belong to the late embryo-abundant (LEA and LEA-like) II group of proteins. They play multiple roles in enhancing freezing tolerance, including cryoprotection of enzymes, stabilization of cell membranes, protection of cellular components, and scavenging reactive oxygen species [14,15]. It is assumed that they repair the rigidity of the membrane by forming amphipathic α -helices to stabilize cell membranes [16,17]. Dehydrins may also prevent freeze-induced dehydration by interacting with soluble sugars in the cells [18]. This interaction may be due to the information of stable glasses [19]. The accumulation of both soluble sugars and cold stress proteins is necessary to achieve the maximum cold hardiness [20].

Soluble sugars contribute to the plant's cold hardiness level by protecting cells from freezing injury in a number of ways. Soluble sugars that function as compatible solutes (osmolytes) can stabilize the osmotic potential of cells and prevent an excessive water loss to the apoplast space, resulting in the enlargement of ice crystals. An accumulation of soluble sugars increases solute concentration and thus drops the freezing point of cells [7]; this has been observed in both herbaceous and woody plants during cold acclimation [21–24]. Soluble sugars can act as cryoprotectants, protecting cell membranes by interacting with lipid molecules [25] and protecting specific enzymes during cold-induced dehydration. Sugars may also stabilize the cell membranes by interacting with lipid molecules [26]. Several essential genes involved in the metabolism of soluble sugars were found to be responsive to cold stress: *sucrose synthase* (SUS), *sucrose-phosphate synthase* (SPS), and *invertase* (INV) in the sucrose metabolism pathway [27,28]; *raffinose synthase* (RS) and *galactinol* (Gals) in the RFOs (raffinose family oligosaccharides) synthesis pathway [29–31].

In the present study, two garden roses with distinct genetic backgrounds (one rose species (*R. wichurana*) and one rose cultivar (*R. hybrida* 'Yesterday')) were selected to study cold acclimation under natural conditions. This study revealed both biochemical and molecular mechanisms involved in cold acclimation of two different rose genotypes, with a main focus on the role of dehydrins and cryoprotective soluble sugars.

2. Materials and Methods

2.1. Plant Material and Experimental Field Condition

R. wichurana is one of the wild rose species. *R. hybrida* 'Yesterday', bred by Harkness (United Kingdom, 1974), is one of the modern rose cultivars and belongs to the rose type Polyantha. Both genotypes are diploid roses. The coldest USDA (United States Department of Agriculture) plant hardiness zone of *R. wichurana* is 5b (−26.1 to −23.3 °C); the hardiness zone for 'Yesterday' is 4b (31.7 to −28.9 °C) (<http://www.helpmefind.com/rose/index.php>; accessed on 4 October 2021). Plant material for *R. wichurana* and 'Yesterday' started as rooted cuttings in summer 2014. The experiment was conducted at ILVO (Melle, Belgium, 51°0' N, 3°48' E). The roses were planted outdoors in February 2015 in light sandy loam soil (pH_{KCl} 5.67, the organic matter 1.35%) and pruned in March 2015 to allow new shoots to grow. A randomized block design was arranged in two blocks; each block included 30 plants per genotype. Stems that emerged during that season were sampled on 19 November 2015, 14 December 2015, 20 January 2016, 19 February 2016, 14 March 2016, and 18 April 2016. Sampled stems were transferred on ice to the laboratory.

2.2. Controlled Freezing Test

Cold hardiness as evaluated by LT₅₀ was conducted using a controlled freezing test ($n = 5$). Internodal stem segments (0.5 cm long) were taken from the middle part of the current-year stem. Stem segments were placed in a cryostat (Polystat 37, Fisher Scientific, Merelbeke, Belgium) from 0 °C to seven target temperatures (−5, −10, −15, −20, −25, −30, −35, and −80 °C) at a cooling rate of 6 °C h^{−1} (0.1 °C min^{−1}). The detailed protocol of the controlled freezing test was described by Ouyang et al. [32]. Index of injury (It) based on electrolyte leakage (EL) values were calculated according to Flint et al. [33] and transformed into the adjusted It value taking into account the It at −80 °C [34]. LT₅₀ values were calculated from the injury versus temperature plot using logistic regression.

2.3. Soluble Sugars

Stem tissue samples of each replicate representing a balanced mix of the apical, median, and basal zone were ground in liquid nitrogen with a mill (IKA® A11 Basic Analytical Mill, Staufen, Germany). The analysis was done in five replicates for each genotype. Soluble sugars were extracted as described in Ouyang et al. [32]. Sucrose, hexoses (glucose and fructose), and oligosaccharides (raffinose and stachyose) were quantified via high-performance anion-exchange chromatography with pulsed amperometric detection (ACQUITY UPLC H-Class, Waters, Milford, MA, USA) using a CarboPac PA-20 analytical column and companion guard column of Dionex (Thermo Fisher Scientific, Sunnyvale, CA, USA) and an eluent of 50 mM NaOH at 22 °C.

2.4. RNA Extraction and Reverse Transcription

Stem tissue samples of each replicate representing a balanced mix of the apical, median, and basal zone were ground in liquid nitrogen. The analysis was done in three replicates for each genotype. Each replicate of the RNA sample was extracted from 100 mg of the ground tissue sample in 700 µL extraction buffer using a CTAB protocol. The RNA quality was controlled and tested by the NanoDrop (ND-1000) spectrophotometer (Isogen Life Science, Utrecht, The Netherlands). The RNA quality was further determined by the Experion™ Automated Electrophoresis System and RNA StdSens Chips (Bio-Rad Laboratories N.V., Temse, Belgium) with a random selection of about 10% of total samples spread over the two genotypes and sampling points. RNA samples (starting from 550 ng of RNA) were converted to single-stranded cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories N.V., Temse, Belgium). Detailed protocols were based on Luybaert et al. [35].

2.5. Gene Isolation and Expression

Candidate genes associated with dehydrins and soluble sugar metabolism were selected according to the literature (Table 1). These homologous sequences were locally BLASTed against the ILVO *Rosa hybrida* transcriptome database in CLCbio. This transcriptome database was built based on transcriptomic data of *R. wichurana* and ‘Yesterday’. BLASTx [36] was used to confirm fragment identity. Several dehydrins were found, but only *RhDHN5* and *RhDHN6* were expressed and thus retained for further study. Four key genes involved in the soluble sugar metabolism were studied—including *RhSPS1*, *RhSUS*, and *RhINV2* in the sucrose metabolism pathway, and *RhRS6* in the RFOs (raffinose family oligosaccharides) synthesis pathway (Table 1). RT-qPCR primers of target genes were designed using Primer3Plus software [37] (Table 2). The RT-qPCR analysis was performed as described in Luybaert et al. [35]. Candidate reference genes (*PGK*, *RPS18c*, *2-UBC9*, *APT1*, *ACT*, *CAB*, *HMG1*, *HSP81*, *MDHC1*, *RBCS1A*, and *TUB*) were chosen from Pipino [38]. GeNorm analysis was conducted based on Vandesompele et al. [39], and gene-specific amplification efficiencies were determined by LinRegPCR according to Ruijter et al. [40] (Table 2). A normalization factor based on three validated reference genes (*PGK*, *PR518c*, and *2-UBC9*) was used for the calculation of calibrated normalized relative quantities (CNRQ) in the qbase+ software (Biogazelle, Ghent, Belgium) [41]. CNRQ values were exported to Microsoft Excel. Biological replicates were averaged geometrically.

Table 1. List of candidate genes in other species used to identify the putative homologue and isolate from the *Rosa* spp. transcriptome database.

Genes in Roses	Functional Annotation	Species	Acc. No.
<i>RhDHN5/6</i> *	Dehydrin	<i>Prunus persica</i>	U34809
<i>RhSPS1</i>	Sucrose-phosphate synthase	<i>Camellia sinensis</i>	KF696388
<i>RhSUS</i>	Sucrose synthase	<i>Camellia sinensis</i>	KF921302
<i>RhINV2</i>	Invertase	<i>Camellia sinensis</i>	KP053402
<i>RhRS6</i>	Raffinose synthase	<i>Camellia sinensis</i>	KP162174

* *RhDHN5/6* were chosen according to Artlip et al. [42], and other four candidate genes (*RhSPS1*, *RhSUS*, *RhINV2*, and *RhRS6*) were chosen from Yue et al. [43].

Table 2. List of RT-qPCR primer sequences and product size for *Rosa* spp. target gene fragments and reference genes.

Gene	Acc. No.	F or R	Primer Sequence 5'–3'	Amplicon Size (bp)	PCR Efficiencies
<i>RhDHN5</i>	MH249069	F R	GGTCAACAAGGACGATCCCTA CCCTTATGCTCTTGGTGCTC	86	1.886
<i>RhDHN6</i>	MH249070	F R	CCGTGAGAATAAGGGAGTGG GCCGTAACCCGGTGTAGTAG	106	1.914
<i>RhSUS</i>	MH249072	F R	AGACCCTTCTCACTGGGACA GCGATCAAGGTTGGAGACA	142	1.798
<i>RhINV2</i>	MH249073	F R	TCTGTGGCAACTGATGTTGTT TTGTTTCGTCCACCTTGAGC	130	1.893
<i>RhRS6</i>	MH249076	F R	CATTAGTGGCGGACCTGTTT CCGTCCGGCAATACTATCTT	84	1.912
<i>RhPGK</i> *	EC586265.1	F R	GCCAAAGTCATCTTGGCTTC CCACTCCAAGGAGCTCAGAC	101	1.869
<i>RhRPS18c</i> *	BI977264.1	F R	ATCTCGAGCGGTTGAAGAAG TGCGACCAGTAGTCTTGGTG	97	1.890
<i>Rh2-UBC9</i> *	EC586612.1	F R	GACCCAAATCCTGATGATCC CGTACTTCTGGGTCCAGCTC	104	1.903

* These genes were selected from Pipino [38].

2.6. Statistical Analysis

The homoscedasticity of data was checked by Levene's test ($p \geq 0.01$) before performing a one-way analysis of variance (ANOVA). LT₅₀ and soluble sugars were analyzed with a one-way ANOVA and the accompanying Scheffé's post-hoc test ($p = 0.05$). CNRQ values were log-transformed. Gene expression was analyzed using one-way ANOVA with a Scheffé's post-hoc test at a 0.05 significance level; if homoscedasticity of data was not fulfilled, a Kruskal–Wallis test was performed ($p = 0.05$). Statistics were analyzed in SPSS Statistics 24.0, and all figures were performed in SigmaPlot 13.0. Gene expression graphs are made according to non-log transformed data. Correlation analysis between LT₅₀ and the concentration of sugars and between LT₅₀ and gene expression were conducted by Spearman's two-tailed test ($p = 0.05$).

3. Results

3.1. Air Temperature and Day Length Condition

Cold hardiness of woody plants is a seasonal dynamic process including three phases: acclimation, mid-winter hardiness and deacclimation. As the process is mainly influenced by changes in temperature and photoperiod, the mean temperature and day length were recorded seven days before the sampling points. The air temperature was monitored on location at 30-min intervals by a sensor integrated into a weather station (HortiMaX, Maasdijk, The Netherlands) and installed near the trial field at ILVO (Melle, Belgium, 51°0' N, 3°48' E) on the greenhouse roof, 5 m above the ground level. Data for day length at Melle was based on information found at <https://www.timeanddate.com> (accessed on 4 October 2021).

The average temperatures dropped from 9.9 °C in November to 5.5 °C in December and dropped further to below zero in January (−2 °C) and February (−1.8 °C). In March the mean temperature increased to −0.1 °C then rose sharply to 5.2 °C in April. Negative minimum temperatures were noted in January, February, and March. Day length shifted from 8 h 57 min in November to 8 h 2 min in December and then lengthened to 13 h 48 min at the end of April.

3.2. Cold Hardiness

Seasonal changes in cold hardiness as estimated by LT₅₀ values (i.e., the temperature that causes 50% of injury) are given in Figure 1. LT₅₀ values decreased from November to December/January, remained relatively low in February–March, and increased to April. A significantly lower LT₅₀ value of −26.4 °C was found in December for ‘Yesterday’ ($p < 0.05$) compared to that in other months. This indicates a strong and fast acclimation pattern, with the highest cold hardiness achieved in early winter. In contrast with ‘Yesterday’, *R. wichurana* developed maximum cold hardiness later in January and reached a lower mid-winter hardiness of −20.1 °C. It can be concluded that ‘Yesterday’ has a fast acclimation and is relatively more cold-hardy than *R. wichurana*. A certain degree of deacclimation of both genotypes was observed during January–March with a strong deacclimation observed in April.

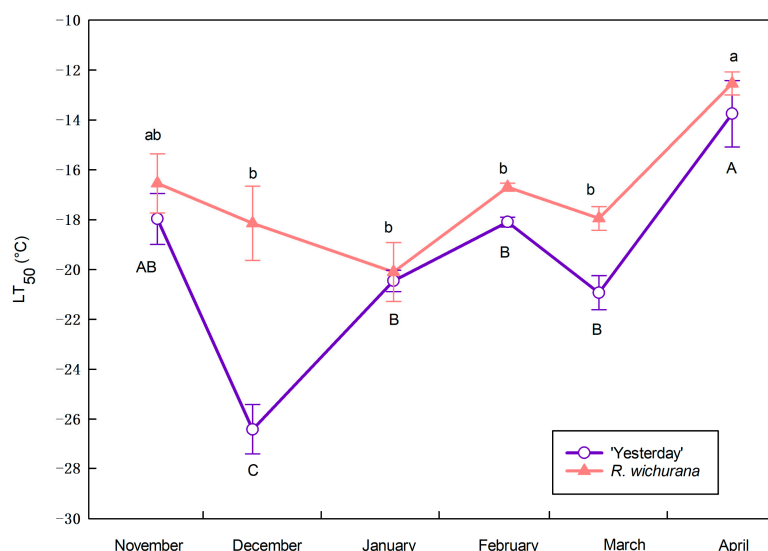


Figure 1. Seasonal changes of cold hardiness of stems expressed as LT₅₀ (temperature of 50% relative electrolyte leakage) of two rose genotypes (*Rosa hybrida* ‘Yesterday’ and *R. wichurana*). Different letters indicate significant differences among sampling time points within each genotype ($p = 0.05$). Values are means \pm SE ($n = 5$).

3.3. Expression Analysis of Dehydrins

The expression of *RhDHN5* and *RhDHN6* was induced from November to January in both genotypes (Figure 2). High transcript abundance was observed in the more cold-hardy genotype ‘Yesterday’ during this period, indicating a higher induction when compared to that during February–April. The expression of *RhDHN5* and *RhDHN6* showed a similar seasonal pattern in *R. wichurana*, although transcript levels were lower than in ‘Yesterday’. In addition, the expression levels of *RhDHN5* and *RhDHN6* in ‘Yesterday’ from November to January were much higher than those in *R. wichurana*, which corresponds to the stronger development of cold hardiness (lower LT₅₀ value) observed in ‘Yesterday’ during the same period. No significant correlations between gene expression of dehydrins and LT₅₀ were observed in either genotype.

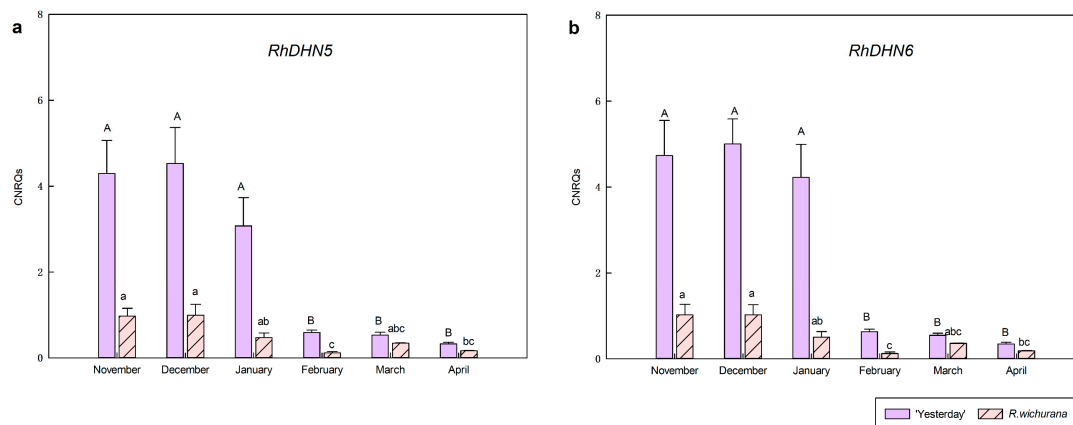


Figure 2. Seasonal changes in expression of dehydrins including *RhDHN5* (a) and *RhDHN6* (b) in the more cold-hardy genotype (*Rosa hybrida* 'Yesterday') and the less cold-hardy genotype (*R. wichurana*). Data were assessed by one-way ANOVA and a Scheffé post-hoc test ($p = 0.05$). Different letters (A, B, etc.; or a, b, etc.) indicate significant differences between time points within each genotype. Normalized relative quantities (CNRQs, non-log-transformed) are presented as geometric means \pm SE ($n = 3$).

3.4. Soluble Sugars

Sucrose represented the largest proportion of total soluble sugars in the test season (Figure 3). In 'Yesterday', the proportion of sucrose was highest in November and December at around 75%, followed by a slight decrease to 66.3–69.8% during January–February. After a recovery in March, the proportion of sucrose dropped to 53.5% in April. In *R. wichurana*, the proportion of sucrose increased sharply from 41.3% in November to 62.0% in December and remained relatively stable during December–April, varying between 61.9–65.2%. A negative correlation between sucrose and LT_{50} value was detected in 'Yesterday' ($r = 0.42$, $p < 0.05$).

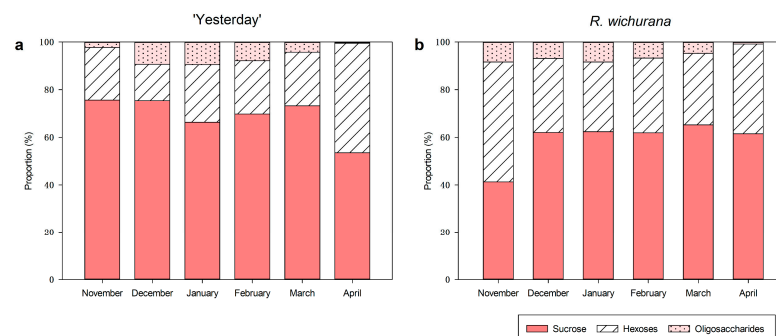


Figure 3. Proportions of sucrose, hexoses (glucose + fructose), and oligosaccharides (raffinose + stachyose) in total soluble sugars of the more cold-hardy genotype (*Rosa hybrida* 'Yesterday') (a) and the less cold-hardy genotype (*R. wichurana*) (b) within each sampling points. Values are means ($n = 5$).

The second-largest proportion of total soluble sugars were hexoses, measured in the range of 15.3–46.0% in 'Yesterday' and 30.0–50.3% in *R. wichurana* (Figure 3).

Oligosaccharides, including raffinose and stachyose, were the least abundant sugars ($< 10\%$) in both genotypes (Figure 3). In 'Yesterday', oligosaccharides accumulated during December–February, measured at 7.8–9.5%. In *R. wichurana*, a higher proportion of oligosaccharides (6.7–8.4%) was also observed in November–February. In April, the oligosaccharide fraction was only 0.5% and 0.8% in 'Yesterday' and *R. wichurana*, respectively. Furthermore, oligosaccharides showed a significant negative correlation with LT_{50} value for both 'Yesterday' ($r = -0.70$, $p < 0.01$) and *R. wichurana* ($r = -0.68$, $p < 0.01$).

3.5. Expression Analysis of Sugar Metabolism-Related Genes

Sucrose is synthesized by SPS in the cytosol and is degraded either by SUS or by INV into hexoses or derivatives. The expression pattern of *RhSPS1*, *RhSUS*, and *RhINV2* is given in Figure 4a–c, respectively. For ‘Yesterday’, the expression of *RhSPS1* was upregulated from November to January; however, transcripts of *RhSPS1* were hardly detectable in *R. wichurana*. *RhSUS* transcripts were low during November–January for the two genotypes but increased towards April. This upregulation of *RhSUS* was pronounced for the cold-hardy genotype of ‘Yesterday’. For *R. wichurana*, the expression of *RhINV2* was induced during November–December and decreased after January. In contrast, for ‘Yesterday’ the expression of *RhINV2* remained relatively stable during cold acclimation. *Raffinose synthase* (RS) is a critical gene in the RFOs (raffinose family oligosaccharides) pathway. However, the four-fold upregulation of *RhRS6* was found only in the less cold-hardy genotype *R. wichurana* in December as compared to November and January (Figure 4d). No significant correlations were found between the expression levels of sugar metabolism-related genes and LT₅₀ values in ‘Yesterday’ and *R. wichurana*.

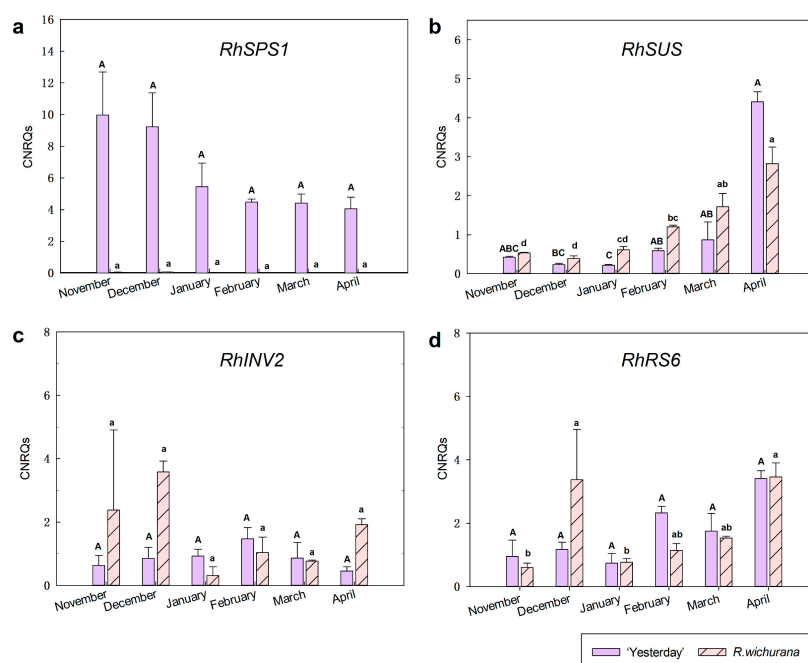


Figure 4. Seasonal changes in gene expression of *RhSPS1* (a), *RhSUS* (b), and *RhINV2* (c) in sucrose biosynthesis and *RhRS6* (d) in RFOs (raffinose family oligosaccharides) biosynthesis in the more cold-hardy genotype (*Rosa hybrida* ‘Yesterday’) and the less cold-hardy genotype (*R. wichurana*). Data were assessed by one-way ANOVA and a Scheffé post-hoc test ($p = 0.05$) except for *RhSUS* of ‘Yesterday’ by the Kruskal–Wallis test ($p = 0.05$). Different letters (A, B, etc.; or a, b, etc.) indicate significant differences between sampling time points within each genotype. Normalized relative quantities (CNRQs, non-log-transformed) are presented as geometric means \pm SE ($n = 3$).

4. Discussion

Breeding of roses with a strong freezing tolerance is necessary for application in northern climates. Cold acclimation, the ability to adapt to seasonal changes in temperature, is a prerequisite for perennial plants in temperate and boreal climate zones. Cold acclimation depends on various biochemical adaptations. The present study focused on two crucial metabolic pathways: cryoprotective dehydrins and soluble sugars.

The cold hardiness of ‘Yesterday’ and *R. wichurana* conforms to the seasonal dynamic shown in most plants. It is characterized by three phases: cold acclimation, mid-winter hardiness, and deacclimation. Two patterns of cold acclimation were observed in the roses under study. ‘Yesterday’ had a fast acclimation, reaching its highest cold hardiness level

in December, despite the relative lack of sub-zero temperatures. In contrast, *R. wichurana* reached its highest cold hardiness level in January when the average minimum temperature was below zero, suggesting that freezing events are needed for the full development of cold hardiness in this less cold-hardy genotype. The observation that ‘Yesterday’ with a higher maximum hardiness level is more cold-hardy than *R. wichurana*, conforms the published cold hardiness (USDA zone) information. The rate of deacclimation is reported as being relatively faster than cold acclimation [44], which was also confirmed in our study: the rising temperature in March–April resulted in a sharp rise of LT₅₀ and loss of cold hardiness. A faster deacclimation was found in the more cold-hardy genotype ‘Yesterday’. Rapid deacclimation has also been observed in other woody ornamentals, indicating that the pace of deacclimation is not correlated to maximum cold hardiness [45,46].

The first *dehydrin* (*ppdhn1*) found in peach (*Prunus persica*) has higher transcript levels in bark tissue during autumn and early winter [42]. We have identified *RhDHN5/6*, which are homologues of *ppdhn1*, in rose. As in peach, our results also show significant upregulation in rose during the period from November to January (Figure 2). Dehydrins in rose respond strongly to low temperatures, as also found in the bark tissue of apple (Rosaceae family) where 5 °C induced transcript levels ranging from 15-fold (*MdDhn3*) to a maximum of 122-fold (*MdDhn1*) [47]. From February to April, the decrease of *RhDHN5* and *RhDHN6* was associated with deacclimation; this correlation has also been observed in blueberry and Scots pine [48,49]. The seasonal pattern of dehydrin genes/proteins is also reported in different tissues (leaves, stem, and floral buds) of woody plants (e.g., apple, peach, birch, *Rhododendron*) during the overwintering process [50–54]. The observed seasonal dynamic of dehydrins in rose showed a positive relation to the changing pattern of cold hardiness level (determined by LT₅₀), suggesting that dehydrins might be closely associated with the freezing tolerance of rose. In Scots pine, several dehydrin genes belong to the top 50 genes. Dehydrins correlate significantly with cold hardiness of Scots pine, and were therefore selected as a marker candidate for frost tolerance in this species [55].

Soluble sugars act as osmoregulators/osmolytes under cold stress. They stabilize osmotic potential, reduce cellular dehydration, protect macromolecules, and serve as scavengers of reactive oxygen species [5,21].

Sucrose is the principal agent in cell membranes protection, as its hydroxyl groups replace water in the phospholipid groups of the membrane. The accumulation of sucrose is essential for cold acclimation [18,56]. For ‘Yesterday’, the proportion of sucrose is relatively higher in the months of November and December and much lower in April, consistent with their cold hardiness pattern. For *R. wichurana*, the proportion of sucrose gradually increased to a maximum in December, with a slight decrease noted from January to April. This slower increase and decrease of sucrose proportion are also in accordance with the rather late response of cold acclimation and deacclimation found in *R. wichurana*. The correlation between sucrose and LT₅₀ values in ‘Yesterday’ also indicate the important role of sucrose in the development of cold hardiness. The proportion of hexoses showed a reverse seasonal pattern compared to sucrose in both rose genotypes: hexoses decreased during cold acclimation and increased during deacclimation (especially in April). Compared to sucrose and raffinose, hexoses have a lower cryoprotective efficiency [57]. Oligosaccharides act as osmoprotectants and are strongly associated with the development of cold hardiness in many woody plants [58–60]. In both rose genotypes, although oligosaccharides (raffinose + stachyose) were the least abundant sugars (<10%), the accumulation of oligosaccharides during cold acclimation was prominent, while these sugars were hardly detectable in April showing a strong association with cold hardiness (Figure 3). In addition, the prominent correlation between oligosaccharides and LT₅₀ values in both genotypes showed that oligosaccharides are closely associated with cold hardiness.

SPS (sucrose-phosphate synthase) is involved in the biosynthesis of sucrose in the cytosol. The upregulation of *RhSPS1* was clearly detected in the more cold-hardy genotype ‘Yesterday’ during acclimation. Increased activities of SPS were also observed during cold acclimation in poplar [61]. Consistent with these observations, the induction of *RhSPS1*

is also associated with the apparent increase of sucrose proportion in the stem tissue of ‘Yesterday’ during November–February (Figure 3a). SUS (sucrose synthase) is associated with both sucrose synthesis and degradation but its main function is the cleavage of sucrose [62]. A similar expression pattern of *RhSUS* was found in both genotypes: it was suppressed during cold acclimation and increased steadily from February to April (Figure 4b). This regulation of *RhSUS* may help the plants to maintain a higher level of sucrose during cold acclimation (Figure 3). INVs (invertases) can cause the cleavage of sucrose into glucose and fructose and are classified into three forms based on their subcellular location, namely cell wall invertases, cytoplasmic invertases, and vacuolar invertases [28]. According to the BLASTx result, *RhINV2* might be vacuolar invertase. The upregulation of *RhINV2* in *R. wichurana* might enable the less cold-hardy genotype to maintain sufficient monosaccharide levels to support different functions in the cell. However, the lack of upregulation of *RhINV2* in ‘Yesterday’ may help the cold-hardy genotype to keep higher sucrose levels (Figure 3a). RS encodes the enzyme of the rate-limiting step in raffinose biosynthesis, and its regulation precedes the biosynthesis of raffinose [63]. However, the strong upregulation of *RhRS6* was only observed in *R. wichurana* in December, and the regulation pattern of *RhRS6* cannot fully reflect the accumulation of oligosaccharides which may be due to either a long sampling period or temperature fluctuations in the field. Furthermore, the present study focused on one raffinose synthase gene that was reported to be closely associated with cold hardiness in tea plants [43]. Further study of gene families of RS or other key genes related to the RFOs synthesis pathway might provide better explanation of the prominent accumulation of oligosaccharides during cold acclimation.

5. Conclusions

A seasonal dynamic of cold hardiness (cold acclimation, mid-winter hardiness, and deacclimation) is found in two rose genotypes that showed different patterns of cold acclimation. ‘Yesterday’ acclimated faster and achieved its maximum cold hardiness in December, while *R. wichurana* acclimated relatively slowly, only reaching its highest cold hardiness level in January. The accumulation of dehydrins in two genotypes may be closely associated with their cold acclimation. Dehydrin transcripts (*RhDHN5* and *RhDHN6*) accumulated significantly during November–January, with more pronounced accumulation in the more cold-hardy genotype ‘Yesterday’. The proportion of sucrose and oligosaccharides increased during cold acclimation in both genotypes. However, the accumulation patterns were different, possibly due to the distinct expression patterns of essential genes involved in their pathway. Sucrose and oligosaccharides are involved in cold acclimation and are associated with cold hardiness. The differences in gene regulation in the two genotypes may be due to their distinct genetic backgrounds, which led to different adaptation strategies to cold stress. A better understanding of the underlying biochemical and molecular mechanisms involved in the cold acclimation of roses will help to select hardy roses in breeding programs. In the present study, we found that dehydrins and soluble sugars played an important role in the process of cold acclimation. Sucrose and oligosaccharides are significantly associated with cold hardiness of Yesterday and *R. wichurana*.

Author Contributions: L.O. and M.-C.V.L. planned the experiment. L.O. conducted the experiment and analyzed all the data. L.O. and M.-C.V.L. interpreted the data. L.O. wrote the manuscript. M.-C.V.L., L.L. and E.D.K. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Sichuan Science and Technology Program, grant number 2021JDRC0140 and The Agricultural Science and Technology Innovation Program (34-IUA-05).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All-new research data were presented in this contribution.

Acknowledgments: We acknowledge Pheno Geno Roses. BV (The Netherlands) for providing the rose material, Magali Losschaert for assistance during RT-qPCR analysis, Christophe Petit for technical support, and Miriam Levenson for English style corrections.

Conflicts of Interest: The authors have declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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