

Article

Preliminary Study of the Genetic Response of Grapevine Buds to a Preventive Natural Polysaccharide-Based Biogel Under Simulated Late Frost Conditions

Alessandra Zombardo ^{1,†} , Simone Garavelloni ^{1,†} , Chiara Biselli ^{2,*} , Agostino Fricano ³ , Paolo Bagnaresi ⁴ , Marco Ammoniaci ¹  and Mauro Eugenio Maria D'Arcangelo ¹ 

¹ Council for Agricultural Research and Economics, Research Centre for Viticulture and Enology, Viale Santa Margherita 80, 52100 Arezzo, AR, Italy; alessandra.zombardo@crea.gov.it (A.Z.); simone.garavelloni@crea.gov.it (S.G.); marco.ammoniaci@crea.gov.it (M.A.)

² Council for Agricultural Research and Economics, Research Centre for Forestry and Wood, Viale Santa Margherita 80, 52100 Arezzo, AR, Italy

³ Council for Agricultural Research and Economics, Research Centre for Genomics and Bioinformatics, Via San Protaso 302, 29017 Fiorenzuola d'Arda, PC, Italy; agostino.fricano@crea.gov.it

⁴ Council for Agricultural Research and Economics, Research Centre for Olive, Fruit and Citrus Crops, Via La Canapona, 1 bis, 47121 Forlì, FC, Italy; paolo.bagnaresi@crea.gov.it

* Correspondence: chiara.biselli@crea.gov.it; Tel.: +39-0575-353021

† These authors contributed equally to this work.

Abstract

Late spring frosts represent a major threat to grapevine (*Vitis vinifera* L.), a risk increasingly exacerbated by climate change-driven shifts in phenology. To explore sustainable strategies for frost mitigation, this study investigated the effect of a natural polysaccharide-based biogel, derived from carob (*Ceratonia siliqua* L.), on the molecular response of grapevine buds exposed to severe cold stress. To this aim, a preliminary RNA-Seq analysis was carried out to compare the transcriptomes of biogel-treated frozen buds (BIOGEL), untreated frozen buds (NTF), and unstressed controls (TNT). The transcriptomic analysis revealed extensive reprogramming of gene expression under freezing stress, highlighting the involvement of pathways related to membrane stabilization, osmotic adjustment, and metabolic regulation. Interestingly, the biogel treatment appeared to attenuate the modulation of several cold-responsive genes, particularly those associated with membrane functionality. Based on these preliminary transcriptomic data, twelve candidate genes, representative of the functional classes affected by biogel treatment, were selected for qRT-PCR validation. The expression patterns confirmed the RNA-Seq trends, further suggesting that biogel application might mitigate the typical transcriptional activation induced by frost, while supporting genes involved in cellular protection and integrity maintenance. The overall analyses suggest that the biogel may act through a dual mechanism: (i) providing a physical barrier that reduces cold-induced cellular damage and stress perception, and (ii) promoting a selective adjustment of gene expression that restrains excessive defense activation while enhancing membrane stability. Although further field validation is required, this natural and biodegradable formulation represents a promising and sustainable tool for mitigating late frost injuries in viticulture.

Keywords: cold acclimation; cold hardiness; freezing stress; natural products; transcriptionally altered genes; sustainability; *Vitis vinifera*



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1. Introduction

Climate change has a strong impact on viticulture because of the shifting of seasonal patterns, and global warming causes an advance in grapevine phenological phases and the upheaval of the whole growing cycle [1,2]. In recent years, the top wine regions worldwide have experienced the severity of late frost impacts, with up to 30% of yield losses [3–6], and risk is expected to increase in the future under many climate projections [7].

Warmer winters and false springs lead to the premature onset of critical growth stages, such as budburst [8,9]. Once the dormancy halts, the vines are highly vulnerable to sudden temperature drops. In fact, late frost events (below 0 °C) occurring after bud break are a real danger that determines serious injuries to developing shoots and swelling buds, which are vital for fruit development. Even a single frost event can reduce yields and compromise grape quality in the current year and the following if the latent buds are also hit, leading to heavy economic losses for entire wine districts [4,10–12].

Drastic passive protection methods need to be implemented, like site selection of new vineyards (e.g., slopes without cool air pooling) and planting late-budding cultivars to mitigate the effects of late frosts [13,14]. In the short term, however, these are not feasible settlements, especially in areas with steady wine production. Therefore, given the lack of robust prediction models [11], winegrowers are employing a range of active strategies to modify the vineyard climate and create safe environments that shield the vines from freezing temperatures, such as the installation of wind machines, over-vine sprinklers, and air heaters [12,15]. All these practices involve high costs, environmental impacts, and logistical challenges, which are not to be underestimated. Thus, the stakeholders show increasing interest in more affordable solutions, such as sprayable products or cryoprotectants [16], if possible, sustainable and nature-based.

Several innovative approaches have been explored to prevent frost damage in crops, including the use of osmoprotectants, biostimulants, or organo-mineral fertilizers, though their effectiveness remains controversial [17–19]. Among emerging strategies, polysaccharide-based biogels of plant origins represent a promising option. These materials combine high viscosity with the ability to form biodegradable coatings that act as physical protective barriers [20]. Traditionally employed in industrial sectors such as food, cosmetics, pharmaceuticals, and textiles, biogels have recently gained attention in agriculture for their potential to enhance soil water retention and serve as carriers for the controlled release of agrochemicals [21–23]. Moreover, recent studies have demonstrated their possible application as seed or plant coatings to mitigate exposure to thermal shocks [24,25]. These hydrophilic polymers, in fact, can form strong interactions with water molecules, helping the water contained in the hydrogel coating remain in a liquid state even at low temperatures [24]. In this context, polysaccharide-based matrices such as cellulose, starch, arabic gum, and above all sodium alginate have already been preliminarily tested with often promising results [24,26]. A product with high potential could be a carob-derived biogel (*Ceratonia siliqua* L.) characterized by high viscosity, strong film-forming and adhesive properties, and excellent water retention capacity [27]. It is also noteworthy from an environmental perspective: it is a plant by-product, fully biodegradable and biocompatible, thus representing a low-impact, sustainable approach in the field against stressful temperatures.

The present research aims to evaluate the preliminary effects of a carob-derived biopolymeric biogel applied to grapevine buds under controlled severe frost conditions. Specifically, the study focuses on understanding the impact of biogel at the molecular level by assessing alterations in gene expression associated with cold tolerance. This approach could help identify and validate the key candidate genes involved in the transcriptional reprogramming of grapevine cold hardiness potentially influenced by biogel application,

providing the basis for a sustainable strategy to mitigate frost damage in viticulture under future climate change scenarios.

2. Materials and Methods

2.1. Plant Materials

The experiment was conducted on grapevine rooted cuttings from *Vitis vinifera* L. cv. Sangiovese. Cuttings were planted in pots with a capacity of 2.4 L ($12 \times 12 \times 20$ cm) inserted into larger capacity pots of 3.6 L ($15 \times 15 \times 20$ cm) coated with polyurethane foam protection and grown in a greenhouse to promote budburst. The trial was carried out on nine selected rooted cuttings that had reached uniform phenological development with buds at the wool stage (brown wool clearly visible), corresponding to BBCH 05 [28]. Three vines per experimental treatment were used for bud sampling for subsequent molecular analysis.

2.2. Product Characteristics, Experimental Treatments, and Cold Stress Protocol

The product selected for this research is a fully biodegradable gel composed of plant-derived polysaccharides, containing 1.8% soluble zinc (LERIGEL[®], GreenApp Srls, Benvenuto, Italy). The formulation is classified as non-hazardous according to European Regulation (1272/2008 CE). Its mechanism of action involves the formation of a thin, persistent film that protects plant organs from the external environment. The biogel-created physical barrier is intended to help mitigate abiotic stress, such as late frost events. For this experiment, the product was prepared at a concentration of 5 g per liter of water.

Among the nine rooted cuttings selected for the experiment, three were designated untreated, non-stressed controls (TNT), with all buds sprayed with distilled water and maintained at room temperature. The remaining six cuttings were divided into two treatment groups: three had their buds sprayed with distilled water (NTF), and three with the polysaccharide-based biogel preparation (BIOGEL). After treatment, the six vines were placed in a climate chamber where the temperature was set at -4 °C, and cold stress was applied for four hours to simulate the conditions of a severe late frost event that may occur in a real vineyard. An ITC 308 plug and play thermostat (Inkbird Tech, Frankfurt am Main, Germany) was used to ensure that the temperature remained constant.

2.3. RNA Extraction and Sequencing

Total RNA was extracted from buds using Plant/Fungi Total RNA Purification Kit (Norgen Biotek, Thorold, ON, Canada) according to manufacturer's protocol and digested with DNase I (Thermo Scientific TM, Waltham, MA, USA) to remove DNA contamination. The concentration and purity of total RNAs were evaluated using Qubit 4 (Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturer's instructions. For each condition, pool RNAs from three biological replicates were sequenced on an Illumina NovaSeq instrument by Genewiz (AZENTA Life Science, Leipzig, Germany) as 150 bp paired-end reads.

2.4. RNA-Seq Analysis and Identification of Transcriptionally Altered Genes

RNA-Seq data were analyzed using nf-core/rnaseq bioinformatics pipeline v 3.18 (<https://nf-co.re/rnaseq/3.18.0>, accessed on 14 November 2024), which allows the automated quality controls of paired-end reads for removing reads with an average quality score below 20 and shorter than 80 nt, trimming of sequences bases with quality below 20 and the alignment of filtered paired end reads against the reference transcriptome of grapevine [29]. Within the nf-core/rnaseq pipeline, STAR aligner was used to map reads against the reference transcriptome of grape, while normalized read counts and transcripts

per millions (TPM) were computed using Salmon. Since the sequencing was performed on pooled biological samples, to identify transcriptionally altered genes (TAGs) in the three pairwise comparisons—BIOGEL vs. NTF, BIOGEL vs. TNT, and NTF vs. TNT—differential expression between conditions was assessed based on fold-change (FC) values, obtained by comparing the normalized read counts in different samples. Loci with $|\log_2\text{FC}| > 1$ were considered. Candidate genes were selected according to their relative expression differences and subsequently validated by quantitative real-time PCR (qRT-PCR).

2.5. Gene Ontology and Enrichment Analyses

For exploring the ontology content of the TAGs, the whole proteome consisting of the translated coding sequences (CDSs) extracted from *V. vinifera* reference sequence PN40024.v4 [29] was functionally annotated. Briefly, CDSs were blasted using diamond blast as provided by omicsbox version 3.4, using the following parameters: sensitive mode, viridiplantae as taxonomy group, expect value of 1×10^{-3} and a high-scoring pair (HSP) length cutoff of 33. CDSs were blasted against NCBI non-redundant database or NCBI Reference Sequence database, both released in December 2024. GO terms obtained from these matches were checked for taxonomy consistency, that is, against the viridiplantae, which allowed GO to avoid artefacts and were later used for GO enrichment analysis.

Enrichment analyses of GO terms were conducted in the R statistical environment using the R package ‘TopGO’ (v2.59.0) [30] for identifying GO terms that were over- and under-represented in the set of TAGs. For the GO enrichment analysis, the whole set of grape CDSs was used as baseline, whereas the over- and under-represented GO terms were investigated in pairwise sets of TAGs using the ‘weight01’ algorithm implemented in ‘TopGO’ for considering GO hierarchy and selecting the most stringent subset of over-represented and under-represented GO terms. Bar plots reporting GO terms and the corresponding *p*-value were generated using the package ‘ggplot2’ (v3.5.1) in the R statistical environment (R Developmental Core Team, 2015) [31].

2.6. Primer Design and qRT-PCR Analyses

Among the TAGs identified through the RNA-Seq analysis, twelve genes previously reported in the literature as involved in plant responses to abiotic stress, particularly cold stress, were selected for validation by qRT-PCR. For genes characterized in species other than grapevine, the *V. vinifera* homologs were used (Table 1). Reverse transcription was carried out using the Ominiscript RT Kit (Qiagen, Milan, Italy) and oligo(dT)₁₈ primers, following manufacturer’s instructions. The resulting cDNA samples were quantified using both a NanoDrop spectrophotometer and a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and subsequently diluted to a concentration of 1 ng/μL. Primer design was performed using Primer3web (version 4.1.0), and primer specificity was verified through BLAST analysis on grapevine genome reference PN40024.v4 [29] using blast software version 2.6.0.

The QuantiNova™ SYBR® Green PCR Kit (QIAGEN N.V., Venlo, NL, USA) was used to perform qRT-PCR on 2 ng of cDNA per sample. Three technical replicates were conducted. Reactions were carried out on a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The $2^{-\Delta\Delta C_t}$ method [32] was used to calculate fold change (FC) values, and relative expression levels were normalized to the grapevine reference gene poly-ubiquitin (*VvUBI*, VIT_219s0177g00040) [33].

Table 1. List of forward and reverse primer sequences used for qRT-PCR validation of transcriptionally altered genes (TAGs) identified through RNA-Seq analysis.

Gene	Forward Primer Sequence	Reverse Primer Sequence
<i>AtChi</i>	AGCTTATTGGTGTGTCAGTAGT	TGCCCTTAACACTGGCCTATT
<i>Isp5</i>	CACACATGCATGGCTCAGGAAGA	CAGCCAGCGGCTTGGAGCTA
<i>DIR-6</i>	ATGCGGTAAGTGGCATCCGC	GCCTTTGCTCGTGGTCTTGCT
<i>ERF80</i>	CGGAGAGGATCGAGGGTATG	CAGCTTCAAGGGGAAATTG
<i>WAK</i>	ACGCAACAAAGGAAACTCAAGCCA	GGGTGAACATGAGGGAGACATTGGG
<i>TPP</i>	TGATCTGCTGTTCTTCAGGATTCA	ATCGGGTACCCCTCTCCTCG
<i>ICE4</i>	GCTCCTTGAAGATGCCCAT	TGAAAGAGCTCCTAGAGAAAATCAA
<i>COR78</i>	GAAGGTGGCAGAAGCAGGAA	CTTTCCGAACCAAGTGCCTTG
<i>WRKY33</i>	AGCCCCAACTTCAGTCACCA	AGGATCCAGCGGGAAACTGT
<i>ERD6</i>	TGAGCCGGGAGTCCTCATGC	GACTGGGCCAAACACGCTGC
<i>4CL</i>	TCATTGGAGGTTTACCCGATCGTT	GGAGTGGGTTTTTAAATAACTGGGC
<i>AAA</i>	CCACTCTGACTTTTTGCGCCC	TCAGAAATCGGCAGCGGAAGC

3. Results

3.1. Preliminary RNA-Seq Analysis Allowed to Identify Candidate Genes Underlying the Protective Effect of Biogel

RNA-Seq on pooled samples has been suggested to be useful in identifying candidate genes to be validated using qRT-PCRs [34]. To identify candidate genes involved in cold stress response whose modulation is affected by biogel treatment, a preliminary RNA-Seq analysis was conducted on grapevine samples treated with biogel (BIOGEL), cold-stressed controls incubated at 4 °C for 4 h (NTF), and untreated controls maintained at room temperature (TNT). The sequencing of libraries generated a total of 31.4 Million (M), 30.0 M, and 31.9 M paired-end reads for the BIOGEL, NTF, and TNT samples, respectively.

After filtering and trimming, 31.1 M, 29.9 M, and 31.7 M paired-end reads were obtained for BIOGEL, NTF, and TNT samples, respectively. Alignment of filtered reads against the reference transcriptome of *V. vinifera* PN40024.v4 [29] showed that 88.7%, 92.2% and 90.1% of paired-end reads were properly mapped for BIOGEL, NTF, and TNT samples, respectively.

Three pairwise comparisons (BIOGEL vs. NTF, BIOGEL vs. TNT, and NTF vs. TNT) were performed to identify TAGs and evaluate the impact of the polysaccharide-based biogel on grapevine transcriptional responses to cold stress. The lowest number of TAGs was detected between BIOGEL and TNT (6755 TAGs, including 2808 induced genes and 3947 repressed loci), whereas the highest amounts of modulated genes were observed in the comparisons involving NTF (9678 TAGs, of which 3125 were upregulated and 6553 downregulated, in BIOGEL vs. NTF, and 9028 TAGs, 5622 and 3406 of which were up- and downregulated in NTF vs. TNT, respectively) (Table S1).

TAGs were grouped into expression clusters based on their presence across the three pairwise comparisons, as determined by the Venn diagram analysis (Figure 1A).

Within each cluster, TAGs were further subdivided into subclusters according to common expression trends shown in the pairwise comparisons (Table 2).

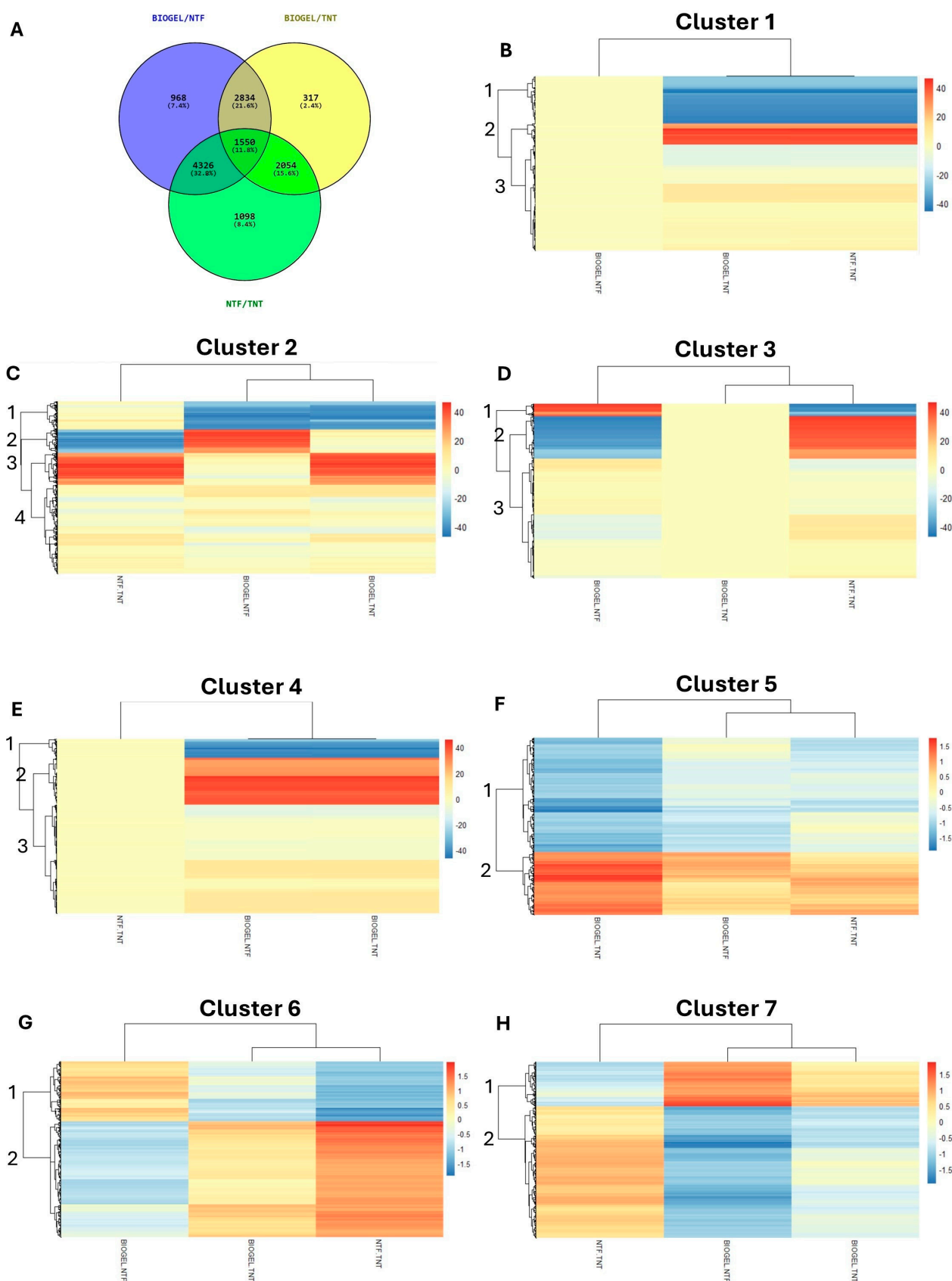


Figure 1. Schematic representation of TAG classification. **(A)** A Venn diagram illustrating the common TAGs identified in the three pairwise comparisons. **(B–H)** Heat maps of the TAG clusters identified by the Venn diagram. For each heat map, FCs in each pair-wise comparison are shown according to a color scale: red = upregulation; blue = down-regulation. For each cluster, the subclusters depicted by the hierarchical clustering are indicated by numbers.

Table 2. Summary of the subclusters detected for clusters 1, 2, 3, and 4. For each subcluster, the corresponding comparisons, number of TAGs, and type of modulations are reported. The percentage of total number of TAGs in each cluster is indicated in brackets.

Cluster	Comparisons	Subcluster	n. of TAGs	Modulation
1	BIOGEL vs. TNT NTF vs. TNT	1_1	939 (45.7%)	Strongly induced in both comparisons
		1_2	535 (26.1%)	Strongly repressed in both comparisons
		1_3	580 (28.2%)	Weakly regulated in both comparisons
2	BIOGEL vs. NTF BIOGEL vs. TNT NTF vs. TNT	2_1	325 (20.9%)	Strongly repressed in BIOGEL vs. NTF and BIOGEL vs. TNT; weakly modulated in NTF vs. TNT
		2_2	289 (18.6%)	Strongly induced in BIOGEL vs. NTF; strongly repressed in NTF vs. TNT; weakly modulated in BIOGEL vs. TNT
		2_3	493 (31.81%)	Strongly induced in BIOGEL vs. TNT and BIOGEL vs. TNT; weakly modulated in BIOGEL vs. NTF
		2_4	443 (28.58%)	Weakly modulated in the three comparisons
3	BIOGEL vs. NTF NTF vs. TNT	3_1	387 (8.95%)	Strongly induced in BIOGEL vs. NTF; strongly repressed in NTF vs. TNT
		3_2	1391 (32.15%)	Strongly repressed in BIOGEL vs. NTF; strongly induced in NTF vs. TNT
		3_3	2548 (58.90%)	Weakly modulated in both comparisons
4	BIOGEL vs. NTF BIOGEL vs. TNT	4_1	468 (16.51%)	Strongly repressed in both comparisons
		4_2	1644 (58%)	Strongly induced in both comparisons
		4_3	722 (25.48%)	Weakly modulated in both comparisons

Cluster 1 (Figure 1B) contained 2054 (15.6%) TAGs shared between BIOGEL vs. TNT and NTF vs. TNT. Interestingly, each gene showed similar expression trends and fold changes (FCs) in two comparisons. For this cluster, TAGs were classified into three different subgroups:

- Cluster 1_1 (939 loci, 45.7% of Cluster 1) included genes strongly repressed under cold stress in both treatments ($\log_2$ FCs from -36.16 to -27.90);
- Cluster 1_2 (535 genes, 26.1%) comprised highly upregulated genes in response to cold ($\log_2$ FCs from 28.90 to 34.50 in BIOGEL vs. TNT and from 28.89 to 34.07 in NTF vs. TNT);
- Cluster 1_3 (580 loci, 28.2%) included moderately regulated genes ($\log_2$ FCs from -3 to 3.59 in BIOGEL vs. TNT and from -3.59 to 4.32 in NTF vs. TNT).

For the 1550 (11.8%) TAGs of Cluster 2 (Figure 1C), found in all three comparisons, four distinct expression subgroups were identified:

- Cluster 2_1 (325 genes, 20.9% of Cluster 2) comprises genes strongly repressed by biogel treatment, regardless of cold exposure (BIOGEL vs. NTF: $\log_2$ FCs from -36.55

to -29.89 ; BIOGEL vs. TNT: $\log_2$ FCs from -34.65 to -28.89), while showing weak modulation in NTF vs. TNT ($\log_2$ FCs from -3.17 to 4.64);

- Cluster 2_2 (289 TAGs, 18.6%) showed a strong repression under cold stress in NTF samples ($\log_2$ FCs from -34.96 to -28.83), but a much milder response in BIOGEL-treated samples ($\log_2$ FCs from -3.92 to 5.26), resulting in significantly higher expression in BIOGEL vs. NTF ($\log_2$ FCs from 27.58 to 37.51);
- Cluster 2_3 (493 loci, 31.81%) included genes induced by frost regardless of treatment ($\log_2$ FCs: 28.90 – 37.91 in BIOGEL vs. TNT; 28.90 – 36.56 in NTF vs. TNT) and only slightly modulated in BIOGEL vs. NTF ($\log_2$ FCs: -6.47 to 7.80);
- Cluster 2_4 includes 443 (28.58%) TAGs with moderate regulation in the three comparisons.

As expected, the majority of TAGs (4326, 32.9%) belonged to Cluster 3 (Figure 1D) and were specific to NTF compared to the other conditions. All these TAGs exhibited opposite expression trends in NTF vs. TNT and BIOGEL vs. NTF and could be subdivided into three subclusters:

- Cluster 3_1: 387 (8.95% of Cluster 3) TAGs strongly induced in BIOGEL vs. NTF ($\log_2$ FCs: 28.93 – 37.34), whereas strongly repressed in NTF vs. TNT ($\log_2$ FCs from -37.92 to -28.86);
- Cluster 3_2: 1391 (32.15%) TAGs strongly repressed in BIOGEL vs. NTF ($\log_2$ FCs from -37.76 to -28.90), while strongly activated in NTF vs. TNT ($\log_2$ FCs: 28.90 – 37.76);
- Cluster 3_3: 2548 (58.90%) TAGs with slighter regulation in BIOGEL vs. NTF and NTF vs. TNT ($\log_2$ FCs ranging, respectively, from -5.76 to 6.31 and from -5.65 to 5.13).

The presence of such a high number of TAGs belonging to this cluster supports the hypothesis that biogel alleviates frost-induced stress responses at the transcriptomic level.

Moreover, Cluster 4 (Figure 1E) included 2834 TAGs (21.6%) that were exclusively regulated in BIOGEL compared to both TNT and NTF, with no differential expression between the two control conditions, and showing the same behavior in the two comparisons in terms of both regulation trend and FC values. This cluster could be divided into three subclusters:

- Cluster 4_1, represented by 468 (16.51%) TAGs strongly repressed by the biogel application with $\log_2$ FCs ranging from -36.93 to -28.90 in BIOGEL vs. NTF and from -36.45 to -28.90 in BIOGEL vs. TNT;
- Cluster 4_2 contained 1644 (58%) TAGs strongly induced by the biogel treatment with $\log_2$ FCs from 27.57 and 36.64 in both comparisons;
- Cluster 4_3, including 722 (25.48%) TAGs showing lower regulation than the other two subgroups, with $\log_2$ FCs from -4.17 to 7.54 in BIOGEL vs. NTF and from -3.69 to 6.96 in NTF vs. TNT.

As noted, some TAGs were exclusively modulated in a single comparison, nonetheless exhibiting relatively modest changes in expression: Cluster 5 (Figure 1F) was the poorest cluster, characterized by 317 (2.14%) TAGs in BIOGEL vs. TNT with ranged from -1.99 to 2 ; Cluster 6 (Figure 1G) included 1098 (8.4%) TAGs in NTF vs. TNT, with -1.93 to 2 ; while Cluster 7 (Figure 1H) represented 968 (7.4%) TAGs modulated in BIOGEL vs. NTF with $\log_2$ FCs -2 to 1.94 .

3.2. Gene Ontology (GO) Enrichment Analysis

GO enrichment analysis of TAGs across the three pairwise comparisons revealed distinct differences such as NTF tissues enriching for stress-related GO terms, while BIOGEL tissues are associated with membrane stability (Table S1).

In the NTF vs. TNT comparison, TAGs were enriched in structural constituent of ribosome (GO:0003735), heme binding (GO:0009733), regulation of transcription (GO:0003700, GO:0003677, and GO:0006355), protein kinase activity (GO:0004672), translation (GO:0006412), response to stress (GO:0006950), cellular response to stimulus (GO:0051716), defense response (GO:0006952), membrane (GO:0016020), and cellular anatomical (GO:0110165). On the other hand, the significant GO terms detected for BIOGEL vs. TNT were mainly related to membrane (membrane—GO:0016020 and plasma membrane—GO:0005886), suggesting a potential protective role of biogel in maintaining membrane integrity under cold stress.

Further GO classification was conducted for TAGs within each specific regulatory cluster identified through Venn diagram analysis (Figure S1).

Considering the clusters related to basal response to cold, Cluster 1, which includes TAGs modulated by frost in both treated and control conditions (BIOGEL vs. TNT and NTF vs. TNT), was enriched in GO terms related to DNA regulation, heme binding (GO:0020037), and membrane function (GO:0016020, GO:0005886), while Cluster 6, specific to NTF vs. TNT, was enriched in post-transcriptional regulation processes, such as DNA-binding transcription factor activity (GO:0003700), mRNA binding (GO:0003729), sequence-specific DNA binding (GO:0043565), ribosome (GO:0005840), and ribonucleoprotein complex (GO:1990904).

Biogel-specific transcriptional responses were evident in Cluster 4 (BIOGEL vs. NTF and BIOGEL vs. TNT), which included TAGs involved in membrane-related processes (GO:0016020 and GO:0005886) and cellular anatomical entity (GO:0110165), as well as Cluster 5, including BIOGEL vs. TNT-specific TAGs and showing similar GO-enriched classes as Cluster 4.

Cluster 2, comprising TAGs shared among all three comparisons, was particularly enriched for signal transduction and stress-related pathways, including protein kinase activity (GO:0004672), serine/threonine kinase activity (GO:0004674), iron ion binding (GO:0005506), response to stimulus (GO:0050896), and redox homeostasis-related functions such as heme binding (GO:0020037), monooxygenase activity (GO:0004497), and oxidoreductase activity (GO:0016705). Interestingly, within this cluster, a total of 245 TAGs were associated with the GO term membrane (GO:0016020), of which 83 loci were strongly repressed by biogel in both cold and control conditions (Cluster 2_1). In addition, for another subset of 71 membrane genes in Cluster 2_2, the strong frost-induced downregulation was mitigated by biogel treatment.

3.3. qRT-PCR Validation of Candidate Genes Underlying the Protective Effects of Biogel

Based on the preliminary set of genes identified with RNA-Seq analysis, a subset of twelve TAGs, known from prior studies to be associated with abiotic stress responses, especially cold stress, was chosen for qRT-PCR validation. All specific information on the ten genes, their involvement in the response to environmental stresses, and details on the mechanism of action are illustrated in Table 3.

The qRT-PCR validation confirmed that several target genes exhibited significant regulation under cold stress conditions, with clear differential expression patterns. Interestingly, genes previously associated with abiotic stress response showed consistent up- or down-regulation after cold stress application (Table 4).

A substantial agreement between the RNA-Seq and qRT-PCR data was observed, providing strong validation for the overall experiment. According to the results, within Cluster 2_1, *AtChi*, *DIR6*, and *TTP* genes resulted strongly downregulated in both BIOGEL vs. NTF and BIOGEL vs. TNT, with the highest differences in BIOGEL vs. NTF, while they were induced by frost in controlled conditions. Similarly, *IspS*, *ERF80*, and *WAK* were

repressed in the presence of BIOGEL treatment in comparison to both the control NTF and TNT, with, however, the strongest effect in BIOGEL vs. TNT. These genes were almost unregulated in NTF vs. TNT.

Table 3. Overview of ten selected TAGs specifically involved in cold stress response.

Gene/Protein	Gene ID (<i>Vitis vinifera</i>)	Function/Role	Stress Conditions	Mechanism/ Molecular Targets	Supporting References
<i>AtChi</i> Chitinase	Vitvi16g01978	Defense protein involved in stress responses	Environmental stresses	Targeting of glycolipids; possible role in signaling	[35]
<i>DIR6</i> Dirigent protein 6	Vitvi18g00895	Hormonal regulation and membrane integrity under stress	Various stresses	Membrane integrity maintenance	[36]
<i>TPP</i> Trehalose-6- phosphate phosphatase	Vitvi15g00992	Stabilizes membranes and promotes isoprene accumulation	Abiotic stress	Membrane stabilization; involved in terpene metabolism	[37,38]
<i>IspS</i> Isoprene Synthase	Vitvi12g00576	Isoprene biosynthesis pathway	Cold stress	Downregulation of isoprene production	[39]
<i>ERF80</i> Ethylene- Responsive Factor 80	Vitvi18g00895	Transcription factor regulating hormone signaling	Cold stress	Synthesis of ethylene	[40]
<i>WAKs</i> Wall-Associated Kinases	Vitvi10g00955	Hormone signaling, development, and stress responses	Biotic and abiotic stress, including cold	Links cell wall to cytoplasm	[41]
<i>ERD6</i> Early Response to Dehydration Six-Like Transporter	Vitvi10g02222	Sugar (hexoses) transporter across the tonoplast	Drought, cold stress	Upregulated under stress	[42]
<i>4CL</i> 4-coumarate-CoA ligase	Vitvi14g01588	Activation of phenylpropanoid pathway	Cold stress (chilling)	Flavonoids, lignin, coumarin production and accumulation	[43,44]
<i>AAA</i> AAA-type ATPase	Vitvi14g03042	Core cellular functions (proteolysis, secretion, mitochondrial activity, etc.)	Cold, drought, salt stress	Upregulated under stress	[45]
<i>ICE4</i> Inducer of CBF expression 4	Vitvi07g02613	Transcription factor regulating cold stress genes	Cold stress (freezing)	Hormone- mediated signaling, sugar accumulation	[46]

Table 3. Cont.

Gene/Protein	Gene ID (<i>Vitis vinifera</i>)	Function/Role	Stress Conditions	Mechanism/ Molecular Targets	Supporting References
COR78 Cold-regulated gene 78	Vitvi16g01022	Signaling pathway (ICECBFCOR cascade)	Cold stress (freezing)	Stabilization of cell membranes	[46]
WRKY33 WRKY DNA-binding protein 33	Vitvi15g01003	Transcription factor regulating hormone signaling	Cold stress	Synthesis of ethylene, calcium and receptor kinase signaling, co-expressed with ERF family genes	[47]

Table 4. Comparison of RNA-Seq and qRT-PCR results. For each gene tested, the corresponding cluster and log₂ FCs in the pair-wise comparisons, obtained by both techniques, are indicated.

Cluster	Gene	RNA-Seq			qRT-PCR		
		BIOGEL/NTF	BIOGEL/TNT	NTF/TNT	BIOGEL/NTF	BIOGEL/TNT	NTF/TNT
2_1	<i>AtChi</i>	−33.7144	−29.8986	3.8158	−8.40	−2.82	3.07
2_1	<i>DIR6</i>	−31.8976	−29.8986	1.9990	−5.64	−3.47	1.63
2_1	<i>TPP</i>	−31.4827	−29.8984	1.5843	−5.48	−2.20	2.50
2_1	<i>IspS</i>	−31.8978	−29.8984	1.9994	−3.95	−5.56	−1.41
2_1	<i>ERF80</i>	−31.8976	−29.8986	1.9990	−3.24	−5.78	−1.79
2_1	<i>WAK</i>	−31.4828	−29.8983	1.5846	−2.56	−3.18	−1.24
3_1	<i>ERD6</i>	29.8963	−1.0013	−30.8976	2.20	−3.28	−7.14
3_1	<i>4CL</i>	31.8964	1.9988	−29.8976	5.10	−1.20	−6.17
3_1	<i>AAA</i>	32.3049	2.3308	−29.9740	3.20	−1.14	−3.68
5_1	<i>ICE4</i>	−1.6471	−0.2440	1.4032	2.03	1.42	−1.42
5_2	<i>COR78</i>	1.1811	−0.6344	−1.8155	2.17	1.30	−1.67
5_2	<i>WRKY33</i>	1.5295	0.2214	−1.3082	−1.12	−1.25	−1.12

The genes *4CL* and *AAA-type ATPase* belonged to Cluster 3_1 and, as expected, were repressed by cold in controlled conditions and activated during cold stress by BIOGEL application and not modulated in BIOGEL vs. TNT. *ERD6* showed the same behavior as these two genes in BIOGEL vs. NTF and BIOGEL vs. TNT but was repressed by BIOGEL treatment also at room temperature.

Slight regulations in the three pairwise comparisons were observed for the Cluster 5 genes *ICE4*, *COR78*, and *WRKY33*.

4. Discussion

Deciduous fruit trees, particularly those growing in temperate regions, have evolved a complex network of molecular responses to ensure survival during adverse environmental conditions, including cold acclimation [48]. Grapevine relies on such mechanisms to withstand temperature fluctuations, even if it remains highly vulnerable to late spring frosts, a phenomenon exacerbated by climate change [49]. The main adaptive strategies against cold stress involve the modulation of gene expression, most notably through the ICE-CBF-COR signaling cascade [50], and the accumulation of protective metabolites such as soluble sugars, proline, and antioxidants, which contribute to membrane stabilization and reactive oxygen species (ROS) scavenging. These molecular responses lead to the

accumulation of these metabolites, enhancing freezing tolerance and maintaining cellular homeostasis under low-temperature conditions [51,52].

Given the growing threat of late spring frosts, there is an urgent need for fast-acting, low-cost, and biocompatible protective solutions. In this context, the repurposing of natural-origin formulations, previously used in other applications [53,54], represents a promising strategy to safeguard plants against frost damage.

This research aimed to investigate the protective effect of a natural polysaccharide-based biogel at the molecular level, focusing on the expression of candidate genes in grapevine tissues exposed to severe cold stress. The experimental design simulated an extreme frost scenario that could occur in a vineyard to assess whether a preventive treatment with a high viscosity, strong film-forming carob-derived biogel (*Ceratonia siliqua* L.) could mitigate the extensive transcriptional reprogramming typically induced by sudden and intense freezing.

4.1. Biogel Apparently Reduces the Transcriptional Disruption Caused by Severe Cold Stress

The results of the preliminary RNA-Seq analyses provide indication that the application of the carob-derived polysaccharide-based biogel modulates the gene expression profile of grapevine buds under severe cold stress. The notably lower number of TAGs in the comparison between BIOGEL and TNT (6755), relative to BIOGEL vs. NTF (9678) and NTF vs. TNT (9028), suggests that biogel-treated samples maintained a transcriptomic profile comparable to the unstressed control. This might indicate that biogel has a mitigation effect on the transcriptional disruption typically induced by low temperature, likely through reduced stress perception or attenuation of stress signaling pathways.

Subsequent analysis of TAG clusters revealed distinct regulatory patterns, indicating either a constitutive or treatment-dependent gene expression response. For example, the TAGs in Cluster 1 (Figure 1B) appear to be consistently induced by low temperatures and activated in response to cold stress, regardless of treatment. These genes are likely part of the basal cold acclimation program and play a key role in initiating core protective mechanisms during the early phases of cold exposure [52].

Moreover, the strong induction or repression of genes in Cluster 1 highlights the high sensitivity of younger plant tissues or organs, such as the grapevine buds, to freezing exposure [24]. In contrast, Clusters 2 (Figure 1C) and Cluster 3 (Figure 1D) may be associated with the ability of biogel to modulate stress-induced gene expression. Specifically, Cluster 2_2 included genes that were strongly repressed under cold stress in NTF samples but were significantly less affected in BIOGEL-treated tissues. This suggests that biogel can attenuate cold-induced gene repression. Conversely, the patterns observed in Cluster 2_3 point to a basal cold response that may still be marginally influenced by biogel. Such attenuation implies that the biogel could interfere with early cold perception or downstream transcriptional repression, potentially through mechanisms that help preserve membrane integrity and delay cellular dehydration [52]. Cluster 3, which contained the largest proportion of TAGs, comprised genes specifically modulated in NTF samples but not in BIOGEL-treated ones. The contrasting expression trends observed in the NTF vs. TNT and BIOGEL vs. NTF comparisons further support the hypothesis that biogel might dampen or prevent transcriptional responses typically triggered by cold stress. Cluster 4 (Figure 1E) revealed a large set of TAGs that were exclusively modulated by the biogel treatment, independent of cold stress, as no significant changes were detected between the two control conditions. The consistency of expression patterns and FC values in both BIOGEL vs. NTF and BIOGEL vs. TNT comparisons indicates a likely direct transcriptional effect of biogel itself. Moreover, the strong induction of genes in Cluster 4_2 may point to

the activation of preemptive defense or signaling pathways, such as those involved in cell wall remodeling, ROS scavenging, or secondary metabolite biosynthesis.

It is important to acknowledge that the very high FC values observed for these clusters may, at least in part, reflect technical artifacts, such as variability introduced by read counts in lowly expressed genes. However, the consistent enrichment of GO categories related to abiotic stress responses within these clusters suggests that these changes are not solely technical noise but may instead represent a genuine and biologically meaningful transcriptional reprogramming triggered by cold stress. The convergence of high FC estimates with functional enrichment analyses therefore supports the potential biological relevance of these transcriptional shifts.

4.2. Biogel Seems to Be Mainly Involved in Modulating Membrane-Related Gene Expression, Preventing Dehydration Under Severe Cold Stress

The GO enrichment analysis suggested that biogel may influence membrane-related processes and partially attenuate the transcriptional reprogramming typically induced by cold stress. While control tissues (NTF) exposed to prolonged frost conditions showed enrichment in stress- and transcription-related categories, biogel-treated samples were more frequently associated with genes linked to membrane integrity and structural functions, suggesting a possible protective role in maintaining cellular stability acting on lipid bilayer organization [51]. Notably, biogel appeared to reduce the repression of certain membrane-associated and stress-related genes (Cluster 2_2) while also eliciting specific transcriptional responses (Cluster 4) that were independent of severe cold exposure.

Even though the lack of biological replicates limited the conclusions of the RNA-Seq experiment of the present study, our results support the identification of candidate genes whose roles in cold stress response have been described in literature [35–47]. These genes (Table 3) have been analyzed using qRT-PCRs and showed consistent expression trends, corroborating the transcriptomic data.

Taken together, qRT-PCR results provide further insight into the potential mechanism of action of biogel in grapevine buds subjected to severe cold stress.

In particular, we look at several genes that were typically induced under freezing conditions in untreated vine buds (NTF) but were markedly repressed in biogel-treated samples (Cluster 2_1). These include the Chitinase encoding locus *Vitvi16g01978*, homolog of the *A. thaliana* Chitinase (*AtChi*), which contributes to stress response by degrading glycolipid- or carbohydrate-associated molecules, generating signaling compounds, and reinforcing cell walls [35]; Dirigent Protein 6 (*DIR6*), which guides lignin and phenolic compound formation and modulates stress-related hormone signaling [36]; Isoprene synthase (*IspS*), a key enzyme in isoprene biosynthesis [39]; Trehalose-6-phosphate phosphatase (*TPP*), which is involved in terpene accumulation, particularly isoprene [37,38]; Ethylene-responsive factor 80 (*ERF80*), a transcription factor regulating ethylene biosynthesis [40]; and Wall-associated kinases (*WAKs*), which participate in hormone signaling, tissue development, and directly connect the cell wall to the cytoplasm [41]. Collectively, these genes are associated with stress perception, defense activation, hormone signaling, and volatile compound synthesis, all processes typically triggered under severe stress conditions. Their downregulation in biogel-treated buds suggests that the coating may attenuate the perception of freezing stress, reducing the need for an emergency transcriptional response. This effect likely reflects the physical barrier properties of the biogel, which can buffer direct frost injury at the tissue surface, reducing water loss, thereby dampening the intensity of stress signals perceived by the cells.

In parallel, other genes that are typically repressed by frost in untreated buds were instead induced under severe cold stress in the presence of biogel (Cluster 3_1). These include the 4-coumarate-CoA ligase gene (*4CL*) [43,44], which promotes the accumulation

of metabolites such as coumarins, flavonoids, and lignin, and the AAA-type ATPase gene (*AAA*), a highly conserved eukaryotic gene involved in essential cellular processes, including proteolysis, proteasome function, vesicle-mediated secretion, membrane fusion, peroxisome biogenesis, and mitochondrial activity [45]. These genes contribute to cell wall reinforcement, energy metabolism, and stress recovery, and their activation in biogel-treated buds suggests a priming effect. In this way, it is likely that, rather than eliciting a generalized stress response, biogel-treated tissues appear to redirect transcriptional activity toward maintaining membrane integrity, sustaining metabolic functions. Similarly, Early Response to Dehydration Six-Like Transporter (*ERD6*) displayed a context-dependent induction, being activated only when cold stress coincided with biogel application. This suggests that biogel might fine-tune sugar transport and osmotic adjustments in a stress-specific manner, as *ERD6* mediates the transport of hexoses (i.e., glucose, fructose, galactose, mannose, and xylose) across the tonoplast [42].

Interestingly, canonical cold-response regulators showed only modest changes in expression across conditions (Cluster 5). These include Cold-regulated gene 78 (*COR78*), and Inducer of CBF expression 4 (*ICE4*), both involved in hormone-mediated signaling pathways that promote sugar accumulation in the cell membrane [46], as well as WRKY DNA-binding protein 33 (*WRKY33*), a transcription factor regulating ethylene synthesis, and calcium- and receptor kinase-mediated signaling [47]. This moderate regulation suggests that biogel probably does not suppress the basal function of the ICE-CBF-COR core pathway [50], which can maintain a minimal level of cold tolerance. Rather, the treatment appears to selectively dampen secondary, stress-amplifying transcriptional cascades while sustaining or enhancing structural and metabolic defenses.

These results support a model in which biogel may act through a dual mechanism. First, it seems to provide a physical barrier that mitigates cold-induced cellular damage, thereby reducing stress perception and limiting the need for extensive transcriptional reprogramming. Second, it might promote targeted reconfiguration of gene expression, restraining excessive defense activation while priming pathways associated with cellular integrity maintenance.

The proposed mechanism is further supported by the discovery, throughout GO analyses of the preliminary RNA-Seq data, of additional functional classes potentially involved in membrane stabilization in Clusters 2 and 4. For example, the Cluster 2_2 locus *BDA1* (Vitvi04g00139), repressed by cold stress in untreated samples (Supplementary File Table S1), encodes for a protein belonging to the ankyrin repeat-containing protein (ANK) class, which has been associated with plant immunity and environmental sensing, including temperature fluctuations [55]. Eleven ANKs were also present in Cluster 4 (three repressed, eight induced). Late Embryogenesis Abundant (LEA) proteins, well characterized in grapevine and other plants, protect membranes and proteins under dehydration and cold stress [56]. The LEA locus Vitvi16g01948 was induced by cold in control samples NTF (Cluster 2_3), whereas three LEA genes (Vitvi15g01083, Vitvi16g04392, Vitvi16g01159) were specifically induced by biogel treatment in Cluster 4. Similarly, two genes in Cluster 2_3 (Vitvi06g01222 and Vitvi16g01886), encoding *EIX1* (Ethylene-Inducing Xylanase 1) receptor-like proteins, were up-regulated by frost. Fifteen of such loci were identified in Cluster 4, showing mixed regulation (six repressed, nine induced). These receptors mediate extracellular stress perception and cold-induced signaling cascades, including MAP kinase pathways, contributing to cold tolerance [57,58]. At the structural level, CASP-like proteins (Critical Assessment of Structure Prediction-like proteins) were identified in both Clusters 2 and 4: Vitvi17g00053 (Cluster 2_1) was repressed by biogel treatment, while the two Cluster 4 CASP-like loci (Vitvi10g04312 and Vitvi01g02007) were respectively repressed and induced by the application of biogel. CASP-like proteins maintain cell wall-plasma

membrane contacts and Casparian strip integrity, and their modulation has been linked to enhanced cold tolerance by reducing mechanical stress under chilling [59,60].

Even at an early stage of investigation, these results suggest that biogel might mitigate the excessive cold-induced transcriptional reprogramming while enhancing membrane stability and limiting cellular dehydration [51], supporting the activation of defense-related functions and promoting a more balanced and resilient stress response in grapevine buds.

5. Conclusions

This study provides a first insight into the impact of a natural polysaccharide-based biogel on grapevine buds' genetic response to severe cold stress. The results, supported by qRT-PCR validation on candidate genes, suggest that this compound may attenuate the activation of stress-related transcriptional pathways, leading to a reduced perception of freezing and improved stabilization of cellular functions and membrane integrity. Although preliminary and focused on molecular changes under extreme frost conditions, these findings offer a valuable foundation for further research that should include experiments under milder cold stress to assess whether similar molecular effects result in measurable physiological protection. Moreover, the integration of complementary stress indicators and multi-season field trials will be essential to confirm the effectiveness of biogel and its practical applicability. Overall, however, this work highlights the potential of carob-based biogels as a sustainable and low-cost strategy to mitigate frost damage in vineyards under changing climatic conditions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture15212219/s1>, Table S1: Supplementary file S1. List of TAGs in each cluster (indicated in the name of Excel sheet) detected by Venn diagram. For each locus, gene_id, FC in each pair-wise comparison, functional description, GO ID with the corresponding e-Value, and GO descriptions are indicated. FCs of significantly down-regulated loci are in red, FCs of significantly up-regulated loci are in green; Figure S1: Supplementary file S2. Gene Ontology enrichment in clusters of co-expressed genes. In each bar graph, the top GO terms (x -axis) are plotted against the corresponding $\log_2$ of the reciprocal Fisher's p value. Bar plot colors point out the number of transcriptionally altered genes identified for each GO term, while MF, BP and CC indicate GO terms of Molecular Function, Cellular Component and Biological Process domains, respectively.

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