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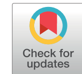
Research Article

Microtubule dynamics modulate sensing during cold acclimation in grapevine suspension cells



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ABSTRACT

Cold acclimation is of practical relevance, since it can avoid cold-induced damage in various crops. To efficiently activate cold acclimation requires that the chilling stress is perceived and processed efficiently. In the current work, we use a transgenic cell line of V. rupestris expressing a GFP-labelled tubulin to follow the effect of cold acclimation and the relation between microtubules and the expression of the transcription factor *Cold Box Factor 4* (*CBF4*) as molecular readout for adaptive responses to cold stress. We find that chilling induced cold tolerance correlated with increased *CBF4* expression. We show that cold acclimation can be achieved through stabilisation of microtubules by taxol, as well as through transient elimination of microtubules by pronamide in the absence of cold stress. Furthermore, results from inhibitor studies indicate that transcriptional activation of *CBF4* appears to be under control of calcium influx. We screened a population of the ancestor of *V. sylvestris* and could identify different clades with strong induction of *CBF4*, indicative of genetic variation in cold adaptability that can be used for breeding. We summarize our findings into a working model where microtubule dynamics controls the sensitivity of cold induced calcium influx mediating the induction of *CBF4* culminating in cold hardening.

1. Introduction

Cold stress, as one of the major abiotic stresses, restricts the spatial distribution of many plant species [1], and often acts in concert with other side effect stresses, such as the cold-induced osmotic and oxidative stresses [2,3]. While freezing injury is attributed to membrane damage caused by ice crystals [4], in many crop plants even chilling (temperatures below 10 °C, but above the freezing point) can cause irreversibly impact on morphology, growth, and development of plants [5] and freezing injury (below 0 °C).

Grapevine is not only belonging to the first fruits domesticated by humans, but also represents one of the economically most important crops. Since it is widely grown and distributed in temperate regions, differences in cold tolerance are of high agronomical relevance [6]. Some wild grapes, such as the Siberian species *V. amurensis*, are well adapted to winter temperatures as low as -40 °C [7–9]. However, domesticated grapevine (*Vitis vinifera*) is mostly sensitive, especially in buds, flowers and leaves, and often severely damaged by extensive winters with low temperatures, and also, more importantly, by sudden temperature drops taking place during mid spring, when young leaves already have emerged. Especially such spring cold episodes result in major economic losses [10–12]. For example, a sudden freezing event happening in January in the Finger Lakes region caused the almost half of the *V. vinifera* yielding lost [13], or a short period with severe frost (-20 °C) lasting for only three days in 2007 reduced the annual grape production of Iran by 20% [6]. Thus, improving the cold tolerance of grapevine has been a primary goal for grapevine breeders.

However, breeding is not the only possible approach to reduce or avoid cold induced damage - alternatively, cold tolerance can be stimulated by physiological manipulation [14]. For example, priming of adaptive signaling by either pretreatment with specific compounds or moderate stress can improve adaptation to various environmental challenges [15]. A classic example of priming is so called cold acclimation or cold hardening: Exposure to non-freezing temperature prior

Abbreviation: CBF4, cold box factor 4; CBF/DREB1, C-repeat binding factor/dehydration responsive element-binding factor 1; COR, cold responsive genes; AP2/ERF, apetala2/ethylene response factor; DMSO, dimethyl sulfoxide; BA, benzyl alcohol; MT, microtubules; EGTA, ethylene glycol-bis (-aminomethyl ether)-*N*,*N*,*N*',*N*'-tetra acetic acid; GdCl3, gadolinium chloride; LatB, latrunculin B; CRPK1, cold responsive protein kinase 1

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to freezing temperature can effectively improve cold tolerance of otherwise cold-sensitive plants [16]. Cold acclimation was also demonstrated in grapevine, where the temperature drop in late autumn can boost freezing tolerance of dormant buds during the subsequent winter [17]. When the chilling period in late autumn is long enough to allow for complete cold acclimation, many *V. vinifera* cultivars can endure freezing stress down to −15 °C in midwinter without lethal damage [7,18]. Cold acclimation is linked with various physiological and biochemical changes, such as cessation of plant growth, reduction of water content, accumulation of abscisic acid (ABA), modifications of cell membranes, and the synthesis of sugars or cryoprotective proteins, allowing to circumvent freezing damage [19,20].

To efficiently activate cold acclimation requires that the chilling stress is perceived and processed efficiently, shifting the focus on the early events of signaling. While it has become clear that the plasma membrane is the primary site, where low temperature is perceived, which is accompanied by changes of membrane proteins, calcium influx, and activation of protein kinases [3,21], the details of cold sensing and transduction have remained far from thoroughly understood. In our previous work, we have, therefore, used a pharmacological strategy in a grapevine cell line to dissect early cold signaling upstream of cold-induced disassembly of microtubules [22]. The fast response to cold stress resulting in the disassembly of MTs in involves the influx of calcium, activation of a membrane-located NADPH oxidase, activation of a G protein, phospholipase D, and the synthesis of jasmonic acid.

This early response of microtubules seems to be relevant for efficient activation of cold acclimation. For instance, taxol, a drug stabilizing microtubules, has been shown to interfere with cold acclimation in spinach [23] and rye [24]. This was surprising, because generally, stabilization of microtubules, for instance by abscisic acid rendered microtubules cold stable [25,26], and microtubules of fully acclimated cells are cold stable as well [27]. This apparent discrepancy can be resolved, when the timing of events is considered as worked out in a study on cold acclimation in winter wheat [28]: Here, the development of freezing tolerance in different cultivars of winter wheat correlated with a transient disassembly of microtubules, while microtubules recovered with progressive acclimation and then acquired resistance to a freezing shock. When such a transient disassembly was evoked artificially (in the absence of chilling) using a pulse-chase treatment with pronamide, a mild inhibitor of tubulin assembly, it was possible to induce freezing tolerance in a cultivar that otherwise was freezing sensitive [28]. Thus, the relationship between microtubule integrity and cold tolerance depends on the time point, when this relationship is probed [29].

The signaling evoked by or linked with this early and transient disassembly of microtubules must be translated into activation of gene expression and synthesis of new proteins that are required for cold tolerance. A family of transcription factors, named CBF/DREB1 proteins, has been identified in *Arabidopsis* as key factors for cold acclimation. The members of this gene family induced in response to cold stress, and activate the expression of a set of COR genes that enhance cold tolerance [3,30]. The CBF proteins belong to the AP2/ERF transcription factor family and bind to a specific CRT/DRE motif present in the promoters of many COR genes [31]. After their discovery in *Arabidopsis*, CBFs have been studied in numerous species, including wheat, strawberry, barley, rice, maize and also grapevine. While all three members of the *Arabidopsis* CBF family, are rapidly induced after cold treatment [32], only *CBF1* and *CBF3* could be shown to be required for freezing tolerance using mutant approaches, indicative of additional functions of these transcription factors [33]. Nevertheless, overexpression of CBFs generally can enhance freezing tolerance as shown for rapeseed (*Brassica napus* L., [34]), tomato (*Solanum lycopersicum* L., [35]) and poplar (*Populus balsamifera* subsp. *Trichocarpa*, [36]). It should be mentioned, however, that some plants, such as strawberry [37] harbour a fourth member of this family, *CBF4* that is induced more slowly, but seems to be tightly correlated with cold acclimation. Also in grapevine, four members of the CBF family have been isolated and were found to confer cold tolerance, if expressed in *Arabidopsis thaliana* as host [38]. Although they are all induced by low temperature, there seems to be also functional specificities: While *CBF1-3* are induced not only by low temperature, but also by osmotic stress [39], *CBF4* is not responsive to osmotic stress [40]. Likewise, *CBF2* has been shown to be responsive to pathogen attack and also to confer, upon overexpression in *Arabidopsis*, elevated immunity [41]. *CBF4* differs from the other members by a much higher stability of induction in response to cold [40]. This temporal pattern would be consistent with a role of *CBF4* in cold acclimation. In fact, when *CBF4* was overexpressed in grapevine, freezing tolerance was found to be increased [18].

In our previous work we have dissected the events of cold signaling upstream of microtubule disassembly. Now, we investigate two events that act downstream of microtubules - one is cold acclimation, the other is the activation of *CBF4*. We ask specifically, how the cold-induced disassembly of microtubules is linked with cold acclimation, whether the cold activation of *CBF4* is linked with microtubule integrity, and how membrane fluidity and calcium influx as initial factors for the signalling leading to microtubule disassembly act on the expression of *CBF4*. Finally, we test, whether the European Wild Grape as wild ancestor of cultivated grapevine (*V. vinifera*) still harbours genetic diversity with respect to the activation of *CBF4*.

2. Materials and methods

2.1. Plant material

The transgenic cell line of *V. rupestris* expressing GFP in fusion with β-tubulin *AtTUB6* [42], was used in this study and cultivated in suspension as described in Wang & Nick [22].

Different genotypes of *V. sylvestris* (including 70 genotypes) and the wild species *V. amurensis* and *V. coignetiae* were used, collected and preserved in Botanical Garden of the Karlsruhe Institute of Technology. This collection has been genotyped as described in *Duan et al.* [43] using a set of 79 informative SSR markers: VV- [44], VVMD- [45,46] VVZAG- [47], GF- [48]; VMC- [49], UDV- [50], and VCHR- [51]. Pairwise genetic distances were calculated using the GenAlex 6.5 plugin for Excel (http://biology-assets.anu.edu.au/GenAlEx/), and a phylogenetic tree was inferred from this distance matrix by the UPGMA algorithm using the software MEGA7 [52].

2.2. Cold stress treatment in suspension cells

Cold shock (0 °C) and chilling (8 °C) treatments were performed at day four after subcultivation, by incubating the entire Erlenmeyer flasks either in ice water (0 °C), or in a cold room (8 °C), respectively, for a period of 72 h (sampling at 0, 24, 48, 72 h after the onset of the treatment) while shaking in the dark on an orbital shaker (KS260, IKA Labortechnik, http://www.ika.de) at 150 rpm. To investigate cold acclimation, cells were pretreated in cold room (8 °C) for 72 h and then transferred into ice water (0 °C) sampling aliquots every 24 h for the analysis of microtubule integrity, as well as the assay for viability. In parallel, cells cultivated at room temperature (27 °C) throughout were collected and used as negative control.

2.3. Cold stress treatments in grapevine leaves

For the cold treatment on fully expanded grapevine leaves, the fourth or fifth leaf counted from the apex was used for all the genotypes. Leaves were transferred into a big petri dish on moist filter paper individually, and then transferred into a 4 °C chamber. The leaves were harvested at different time points (0, 1, and 3 h) after the onset of the cold treatment, immediately frozen in liquid nitrogen, and stored at -80 °C for RNA analysis.

2.4. Chemical treatments in suspension cells

To manipulate the MTs under cold shock treatment, cells were pretreated with the microtubule-stabilising compound taxol and oryzalin, a compound, which inhibits microtubule polymerisation (Sigma-Aldrich, Germany). From preparatory experiments, where we had tested (1–10 μM) of both drugs over different time intervals, we defined 10 μM for both taxol (applied for one hour) and oryzalin (applied for half an hour) as optimal pretreatment to stabilize or eliminate microtubules, respectively. After the drug pretreatment, the cells were transferred to cold stress (0 °C), and sampled every 24 h for viability and the observation of microtubules.

In addition, to test whether a transient depolymerization of microtubules could initiate cold hardiness, the mild and reversible microtubule-assembly inhibitor pronamide (Fluka, Germany) was used at different concentrations (0, 25 and 50 μM) for 2 h at 27 °C to transiently eliminate microtubules and thus, to mimic the effect of chilling at 8 °C. In next step, pronamide was washed out, by filtering cells through a nylon mesh, and resuspension in fresh MS medium without pronamide. Subsequently, cells were allowed to recover the microtubules for 3 h at 27 °C, before challenging them with ice water (0 °C) for another 72 h. During the different steps of this procedure, cells were collected at the end of each specific step as well as every 24 h after the onset of the cold treatment to score viability, and analyze cortical microtubules. For all experiments, solvent controls with DMSO (used to dissolve the different compounds) were conducted as references, whereby the concentration of DMSO was equal to that in the drug sample.

To probe for the role of calcium on the cold response, cells were pretreated for 30 min at 27 °C either with the calcium chelator ethylene glycol-bis (-aminomethyl ether)-N,N,N',N'-tetra acetic acid (EGTA, Carl Roth, Karlsruhe, Germany), the Ca^{2+} ionophore A23187 (10 μM) (Sigma-Aldrich, Germany), or the calcium-influx inhibitor gadolinium chloride (80 μM, Sigma-Aldrich, Germany) before administering the cold treatment (0 °C). To increase membrane fluidity, 10 mM of benzyl alcohol (BA) were used, for a mild elimination of dynamic actin 200 nM Latrunculin B (Sigma-Aldrich, Germany) were employed. Again, appropriate negative controls were included, where the cells were treated with the same concentration of the respective solvent (DMSO or ethanol).

2.5. Cellular assays

Viability was quantified by the Evans Blue dye exclusion test [53] as described in Guan et al. [54]. At least 500 cells were counted for each independent treatment with at least four biological replications. Microtubules were visualised based on the GFP signal of the fusion protein using spinning-disc confocal microscopy as described in detail in Wang & Nick [22].

2.6. Quantification of microtubule density

The relative density of cortical microtubules was quantified from geometrical projections of confocal z-stacks using a strategy based on the image-analysis software ImageJ (https://imagej.nih.gov/)using the quantification protocol described in Tan et al. [55] using the freehand selection tool to select each individual cell before launching the "analyze particle" routine and setting the lower limit to 20 square pixels. Approximately 40 cells were calculated for each time point.

2.7. RNA extraction and cDNA synthesis

Total RNA from cells was extracted by Universal RNA Purification Kit (Roboklon, Germany), while the Spectrum™ Plant Total RNA Kit (Sigma, Germany) was used for RNA isolation from grapevine leaves. A slight modification of the protocol was performed to remove the genomic DNA contamination from the samples by digesting with DNase (Qiagen, Hilden, Germany). Quantity and quality of RNA were determined spectrophotometrically (Nano Drop (VWR, Radnor, USA), and by electrophoresis on a 0.8% agarose gel, respectively. The cDNA synthesis was performed by the M-MuLV cDNA Synthesis Kit (New England Biolabs; Frankfurt am Main, Germany) according to the protocol of the manufacturer protocol using 1 μg of RNA template.

2.8. Semi-quantitative PCR and real-time PCR

First, a semi-quantitative PCR was conducted as described previously [43] with 32 cycles of annealing at 58 °C for 25 s and 30 s synthesis at 68 °C, using *VvActin* as internal standard to quantify the steady-state transcript levels of C-repeated binding transcription factor (*VvCBF4 :* DQ497624.1). *VvActin* was amplified with the pair 5′-3' CTCTATATGCCAGTGGGCGTAC as sense primer and 5′-3' CTGAGGAG CTGCTCTTTGCAG as antisense primer, while *VvCBF4* was amplified with the pair 5′-3'CGAGGTAAGGGAGCCCAACAA as sense primer, and 5′-3' GCCACGCAGAGTCCGCAAAAT as antisense primer. The PCR was performed using *Taq* polymerase from New England Biolabs (NEB, Frankfurt, Germany).

Quantitative real-time PCR was performed using a CFX96™ real-time PCR cycler (Bio-RAD, USA) which contained 96-well plates for a reaction volume of 20 μl based on the manual provided by the manufacturer. Relative expression levels of *VvCBF4* was calculated with the $2^{-\Delta\Delta Ct}$ method [56] using *VvActin* as endogenous control for normalization.

3. Results

3.1. Chilling can induce cold tolerance, which is correlated with increased CBF4 expression

We first wanted to see, whether grapevine cells are able to develop cold tolerance in response to pretreatment with a mild cold stress (chilling). For this purpose, we had to calibrate the stringency of stress in a way that allowed us to see the effect of cold acclimation. To monitor this stringency, we used cell mortality as final consequence of cold stress. As shown in Fig. 1A, incubation of the suspension cells in ice water (establishing exactly 0 °C) produced a progressive increase in mortality, detectable from 24 h after the onset of cold treatment, and exceeding 40% after 72 h of treatment. Since this treatment corresponded to a roughly half-maximal effect, and involved a time interval that was appropriate for experimental manipulation, we selected these conditions as reference treatment, referred to as cold shock. In the next step, we incubated at lower stringency under a milder cold stress of 8 °C. Here, the cells remained viable, such that there was no significant increment in cell mortality (Fig. 1B). Therefore, this treatment (designated in the following as chilling) was found to be suited to test whether cold acclimation existed in these grapevine cells. We used a chilling pretreatment for 72 h to find out, whether this produced cold hardiness, which was then probed by a subsequent cold shock (Fig. 1C). During the acclimation phase, we did not observe any significant increment of cell mortality, which was consistent with the results from Fig. 1B. When these cold-acclimatized cells were subjected to 48 h of cold shock treatment (longer incubation was not feasible due to experimental constraints, because depletion of the medium becomes limiting), the mortality increased only very modestly (Fig. 1C). Thus, when compared to the increased mortality in naïve cells (Fig. 1D), the chilling pretreatment reduced mortality by 10%. Thus, chilling can induce a significant increase in cold hardiness to a subsequent cold shock.

Cold Box Factors (CBFs) are transcription factors known to play a critical role for the activation of adaptive genes under cold acclimation. In grapevine, *CBF4* has been shown to be the most responsive member of this gene family. Based on a study, where the four grapevine CBFs were expressed in Arabidopsis thaliana and shown to confer cold

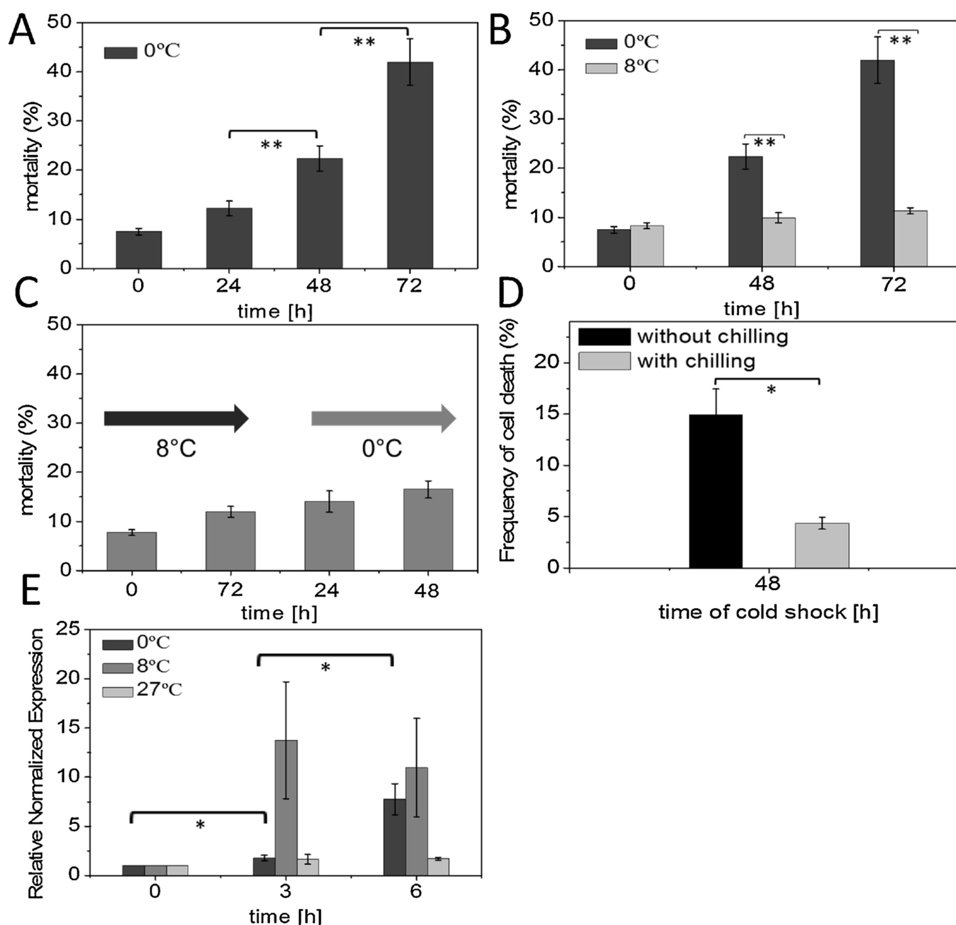

Fig. 1. Time course of cell mortality and the expression level of *CBF4* in response to cold stress in the *V. rupestris* microtubule marker line *GFP-TuB6*. Mortality was followed after treatment with ice water (cold shock, 0 °C, A), chilling at 8 °C (B), and cold acclimation treatment (firstly chilling treatment for 72 h, then cold shock treatment for another 48 h, C). At least 1500 cells were sampled for each data point. (D) Effect of cold shock over 48 h on mortality without (black bar) or with preceding (grey bar) cold acclimation by chilling for 72 h. (E) Steady-state transcript level of *CBF4* in response to cold stress (cold shock and chilling treatment), as compared to incubation at 27 °C as a negative control. * and ** indicate differences that are statistically significant at $P < 0.05$, and $P < 0.01$ level, respectively. Data represent mean values and standard errors from at least three independent experimental series are shown, error bars represent standard error.

tolerance [38], we first screened the expression of these transcription factors in *V. rupestris* leaves subjected to a stringent, but not lethal (4 °C) cold stress (Fig. S1A). While we did not detect any expression level of *CBFL* and *CBF2* over the entire 48 h of this experiment, *CBF4* and *ZFPL* were rapidly induced within 3 h after the onset of cold, and subsequently decreased from 6 h treatment. Since the rapid and transient accumulation of *CBF4* transcripts could be reproduced in the *V. rupestris* suspension cells in response to cold treatment (0 °C) treatment, we, therefore, decided to focus on this transcription factor, and studied the accumulation of *CBF4* transcripts under cold shock and chilling treatment in more detail (Fig. 1E). In response to cold shock, *CBF4* transcripts were increased significantly already at 3 h after the onset of a cold shock (~2-fold), and this induction kept increasing to nearly 8 fold at 6 h. For chilling, the induction of *CBF4* was strongly accelerated over that seen with cold shock. Here, around 12-fold induction at 3 h were observed, and the elevated levels of the transcript remained stable. In the absence of cold stress, when cells were kept at 27 °C, there was no significant change in the abundance of *CBF4* transcripts over the entire time interval 6 h of this experiment. Thus, the cold acclimation induced by chilling is preceded by a strong induction of *CBF4*.

3.2. Cold acclimation proceeds independently of microtubule integrity

Since microtubules disintegrate under cold shock, we were asking, whether cold acclimation induced by chilling would correlate with a recovery of microtubule integrity. Since the microtubules in our cell line were tagged by GFP-TuB6, we could follow the responses of cortical MTs to cold shock and chilling (Fig. 2A). To be able to integrate over the population, we quantified microtubule density by a quantitative imaging analysis strategy (Fig. 2B). We observed that nearly all cortical microtubules had been eliminated after 48 h of cold shock. After 48 h of chilling, the original dense cortical array seen in non-challenged controls had become considerably scarcer, but still numerous microtubules were clearly detectable. However, all these remaining cortical microtubules had totally disappeared after subsequent 48 h of cold shock, i.e. under a condition, where cold acclimation was clearly detectable (compare Figs. 1D and 2 A). In order to statistically verify these changes of cortical microtubules in response to cold stress, the density of cortical MTs was quantified (see the details in Material and Method). We measured a decrease in density by some 70% within the first day of cold shock (Fig. 2B). In contrast, chilling caused only as slight decrease by around 25% within 72 h of treatment. Despite this acclimation, these residual microtubules were eliminated by a subsequent cold shock treatment for 48 h to almost the same residual level as seen in the non-acclimatized cells (Fig. 2B). Thus, the development of cold acclimation was not accompanied by elevated cold hardiness of microtubules.

3.3. Microtubule stabilization by taxol is sufficient to induce cold acclimation

We had observed that microtubules completely disassemble under cold shock (Fig. 2A), which was followed by progressive cell death (Fig. 1A). Further, we have observed that, under chilling, microtubules disassemble only partially (Fig. 2A), correlated with a significant reduction of mortality in response to cold shock (Fig. 1D). This prompted us to ask, whether a stabilization of microtubules might mimic the chilling treatment with respect to cold acclimation.

We, therefore, used 10 μM taxol, a drug that inhibits microtubule disassembly to increase microtubule stability for 1 h, before challenging the cells by cold shock (Fig. 3A). In fact, this pretreatment could efficiently mitigate the mortality induced by cold shock. Even after 72 h at 0 °C, the taxol-pretreated cells showed only 50% of the mortality

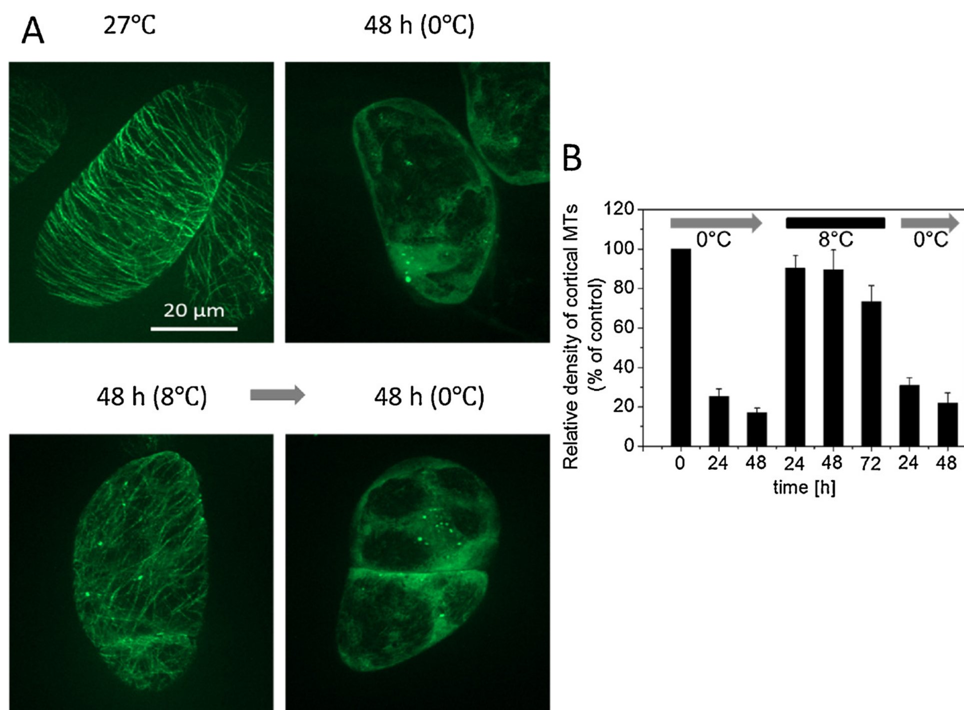

Fig. 2. Response of cortical MTs in the cell line *V. rupestris GFP-TuB6* to cold shock (0 °C) and chilling (8 °C) treatments as compared to cultivation at 27 °C. (A) Representative images showing the state of microtubules under the respective conditions. (B) Quantitative analysis of the relative density of cortical MTs representative of at least three independent experimental series with a population of at least 40 individual cells. Time course of microtubule density in response to cold shock (0 °C, left), chilling (8 °C, center), and cold shock following chilling (right) is shown.

observed in the control that was not pretreated. If compared to chilling (Fig. 1D, note that more than 48 h of chilling were not possible, due to medium depletion), this taxol pretreatment was fully equivalent in inducing cold hardiness. We confirmed by microscopy (Fig. 3C) and quantitative image analysis (Fig. 3B) that this taxol pretreatment rendered microtubules more resilient against cold shock. While in the non-pretreated control, microtubules had been mostly eliminated after 24 h of cold shock, in the taxol-pretreated condition the cortical array was still clearly detectable, although it has to be noted that the microtubules appeared thinner as compared to the situation prior to the cold shock (Fig. 3C). The quantification showed that the microtubule density was not significantly altered after 1 h of taxol treatment (Fig. 3B). After 24 h of cold shock, the density had decreased by only 20%, and even after 3 d of cold shock, still 60% of microtubules were present. In contrast, in non-pretreated cells (Fig. 1D, also integrated as black bars into Fig. 3B to allow the comparison), microtubule density had dropped to 25%

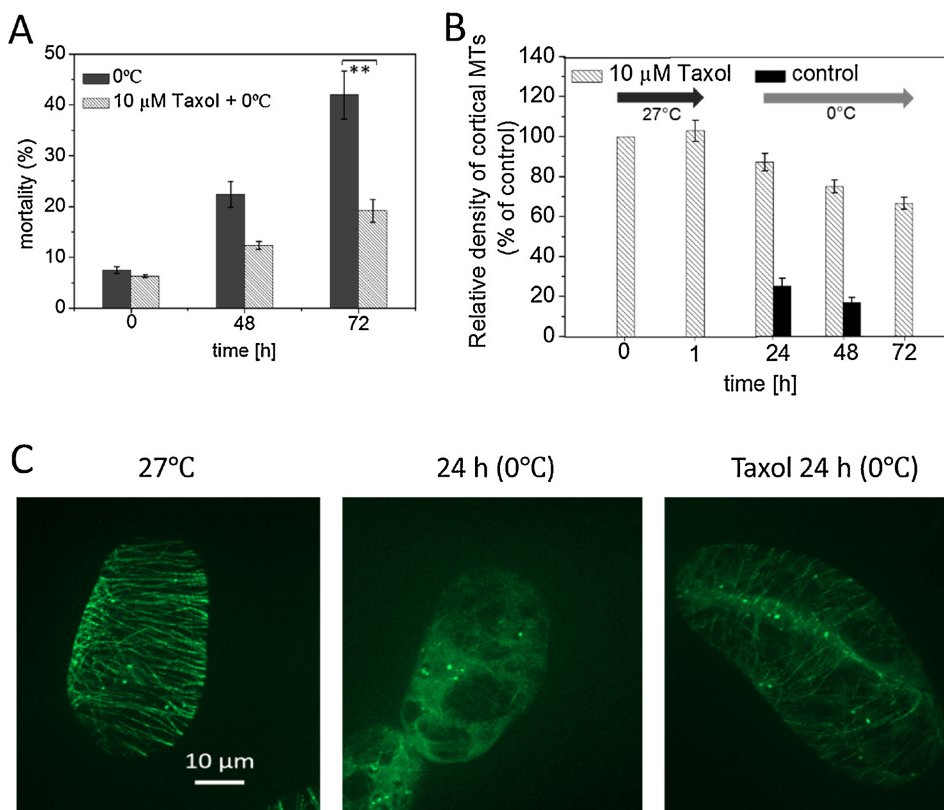

Fig. 3. Effect of taxol (10 µM, 1 h, 27 °C) on the cold response of mortality (A), and cortical microtubules (B, C) in the *V. rupestris* microtubule marker line *GFP-TuB6*. ** indicate differences that are statistically significant on the $P < 0.01$ level. Data represent mean values and standard errors sampling at least 1500 individual cells from at least three independent experimental series. (B) Quantitative analysis of the relative density of cortical MTs after pretreatment with 10 µM taxol for one hour at 27 °C to cold shock (0 °C) over up to 72 h (striped bars), the black bars show the response to cold shock without preceding taxol treatment. Data are representative of at least three independent experimental series with a population of 40 individual cells for each treatment. (C) Representative images showing the state of microtubules at 27 °C prior to the treatment and after 24 h without and with taxol pretreatment shows the situation under normal temperature.

already after 24 h of cold shock.

Thus, microtubule stabilization by taxol can fully replace chilling with respect to cold acclimation. Since chilling caused a rapid and stable accumulation of *CBF4* transcripts by one order of magnitude, we also tested the effect of taxol on the expression of *CBF4*, but we did not see any significant change of steady-state levels for these transcripts (Fig. S2). The cold hardening induced by taxol seems to develop independently of *CBF4* transcription.

3.4. Elimination of microtubules can induce cold tolerance, but only, when it is transient

Since stabilization of microtubules by taxol could phenocopy the effect of chilling with respect to induction of cold tolerance, we wondered, whether elimination of microtubules by oryzalin would have the opposite effect. Therefore, cells were pre-treated by oryzalin (for half an hour at 27 °C) and then treated by cold shock treatment. Microtubules were completely eliminated by this treatment and remained eliminated during the cold shock (Fig. S3). Against our expectation, the mortality of the oryzalin-treated cells was not increased over the cells treated by cold shock without pretreatment (Fig. S3). We also did not see any significant response of *CBF4* transcripts in response to oryzalin (Fig. S2).

Oryzalin did not increase mortality under cold shock, although it eliminates microtubules irreversibly, and is not easily washed away. This prompted us to ask, whether a transient, reversible elimination of microtubules might induce tolerance to a subsequent cold shock. This is experimentally possible using pronamide, an herbicide that can be easily washed out, which allows to obtain a transient elimination of microtubules [28]. Indeed, we were able to eliminate microtubules transiently: After 2 h of pronamide treatment (25 μM), microtubules were strongly affected, and their density was reduced to 40% of the control (Fig. 4A,B; condition 2). After pronamide was washed out, microtubules recovered – already 1 h later, a full cortical network had reappeared in a density that was not significantly different from the starting situation (Fig. 4A,B; condition 3). These reassembled microtubules were still cold-sensitive, though, as seen by their complete elimination in response to a subsequent cold shock (Fig. 4A,B; condition 4). We followed then the progress of mortality under cold shock for pretreatment with 25 μM or 50 μM of pronamide compared to cells that were not pretreated (Figs. 4C, S4): Over the first 48 h, there was no significant difference between the three sets. However, after 72 h of cold shock, mortality in the pronamide-treated samples was significantly, by 30%, reduced as compared to the non-treated control. Compared with the taxol pretreatment which reduced mortality at 72 h of cold shock by 50% (Fig. 3A), the pronamide treatment is, therefore, less efficient.

Since pronamide has to be dissolved in DMSO (which also acts as a membrane rigidifier), we also included a solvent control. This solvent control was behaving as the non-treated control, if scored at 72 h of cold shock. However, at 48 h, the DMSO treated samples exhibited an (by almost 50%) elevated mortality over the values seen in the untreated control and the two pronamide samples.

Thus, a transient elimination of microtubules can partially replace chilling with respect to the induction of cold tolerance. Similar to taxol, this cold hardening induced by pronamide occurs without a response of *CBF4* transcription (Fig. S2).

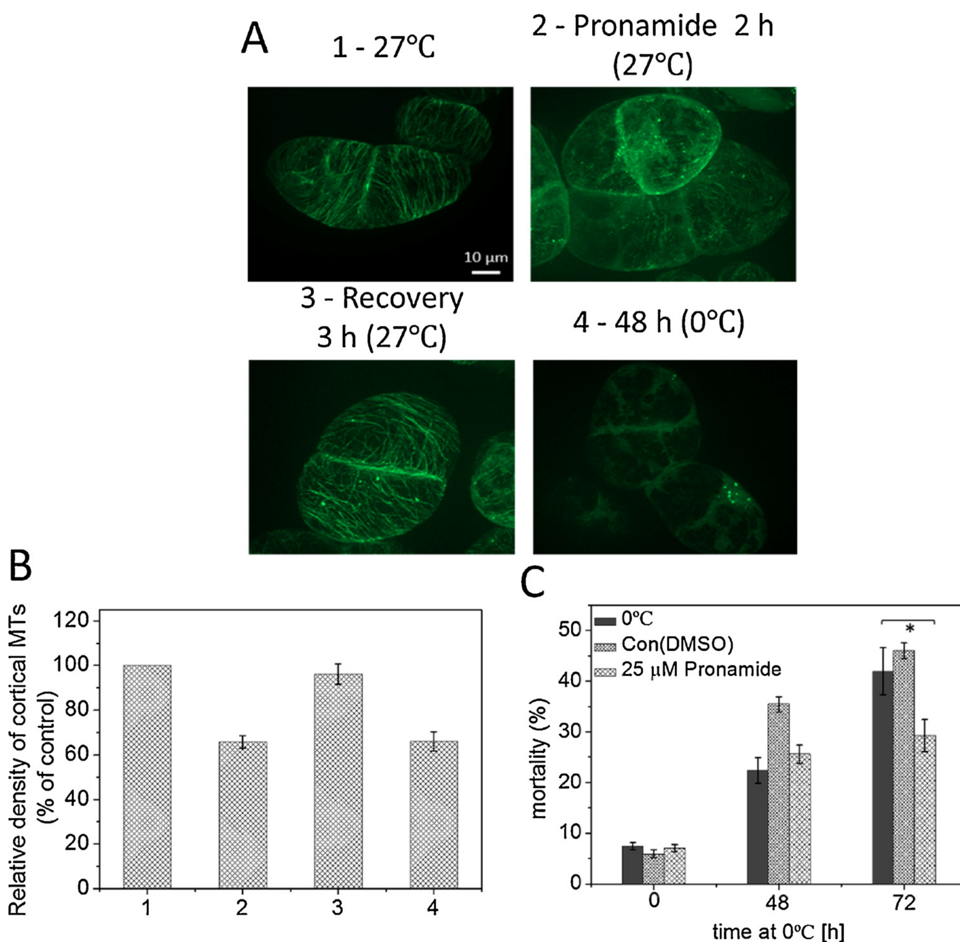

Fig. 4. Effect of transient pronamide treatment (25 μM, 2 h, 27 °C, followed by washout) on the cold response of mortality (C), and cortical microtubules (A, B) in the *V. rupestris* microtubule marker line *GFP-TuB6*. (A) Representative images showing the state of microtubules at 27 °C prior to the treatment (condition); after pre-treatment with 25 μM pronamide for 2 h at 27 °C (condition 2); after subsequent washout of pronamide and recovery for another 3 h at 27 °C (condition 3); and after cold shock (0 °C) for another 48 h following this washout (condition 4). (B) Quantitative analysis of the density of cortical MTs for the four conditions described in A. Observations are representative of at least three independent experimental series with a population of 30 individual cells for each treatment. (C) Mortality after transient treatment with 25 μM pronamide (as described in A), as compared to the solvent control (DMSO, used to dissolve pronamide), or without any pre-treatment under cold shock treatment. * indicate differences that are statistically significant on the $P < 0.05$ level. Data represent mean values from at least four independent experimental series, error bars represent standard errors.

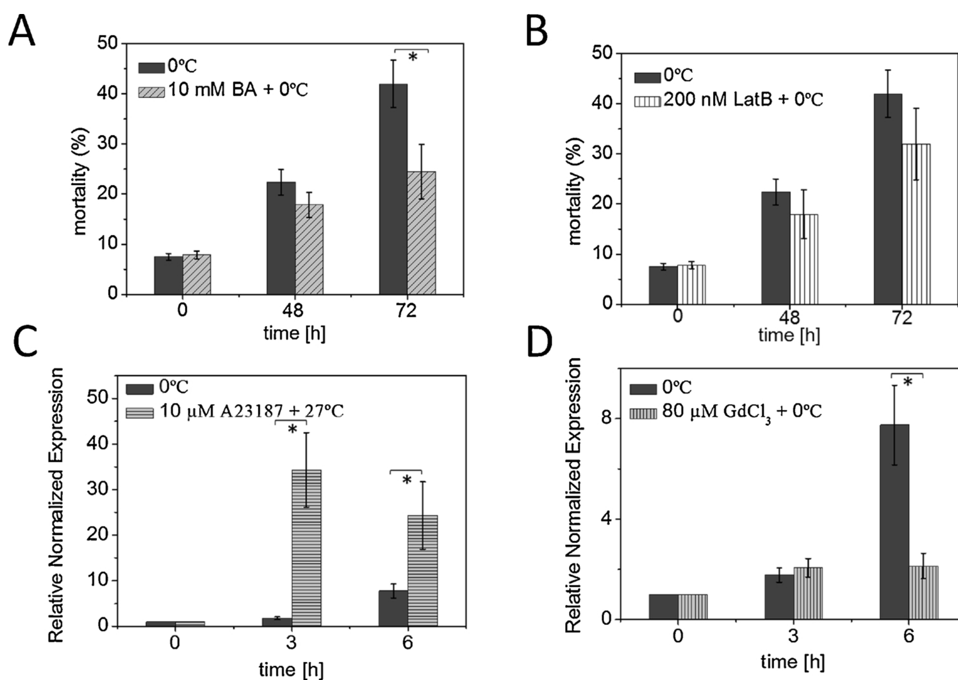

Fig. 5. Time course for the cold response (0 °C) in the *V. rupestris* microtubule marker line *GFP-TuB6* for cell mortality (A, B) and the expression level of *CBF4* (C, D) after pretreatment for 30 min with benzyl alcohol (BA, 10 mM), Latrunculin B (LatB, 200 nM), the calcium ionophore A23187 (10 μM) and the calcium-channel blocker $GdCl_3$ (80 μM). Mortality is shown in (A, B), steady-state transcript levels for *CBF4* in (C, D). * indicate differences that are statistically significant on the $P < 0.05$ level. Data represent mean values from at least four independent experimental series sampling 1500 cells per data point (A, B), and from three independent experimental series with three technical replicates per data point (C, D), respectively.

3.5. Benzyl alcohol can increase cold tolerance

The status of plasma membrane is imperative for plant survival during cold stress. A pre-treatment with 10 mM benzyl alcohol (BA), a compound known to promote the fluidity of plasma membranes and stabilizing microtubules against cold shock [22] is therefore expected to mitigate cold-induced mortality. In fact, as shown in Fig. 5A, this BA pre-treatment reduced mortality under cold shock by around 50% when scored at 72 h. However, at 48 h, this effect was still minute and below the threshold for significance. Again, the transcript levels of CBF4 did not show any significant response to BA (Fig. S5A). The fluidity of the membrane is also controlled by the highly dynamic, cortical actin [57,58], such that a mild elimination of actin should increase fluidity. We employed a low concentration of Latrunculin B to sequester G-actin and remove actin filaments based on their innate turnover. This approach caused only a weak reduction of mortality by around 20% (Fig. 5B) which remained under the threshold for significance. These results demonstrate that the increase of membrane fluidity can decrease the cell mortality in response to cold stress. This effect seems to be at least partially independent of the disassembly of actin, and is not accompanied by any induction in the expression of *CBF4*.

3.6. Calcium influx is necessary and sufficient for the expression of CBF4

The cold-induced reduction in the fluidity of plasma membranes is known as important input for cold signalling and has been shown, in our experimental system, to control the behavior of cortical microtubules under cold stress [22]. A second important input is the transient influx of calcium into the cytoplasm, resulting in the disassembly of MTs as early responses to cold stress. Our previous results indicate that the activation of cold tolerance by chilling correlates with a rapid and strong activation of *CBF4*. On the other hand, none of the microtubule-modifying compounds, nor the *bona-fide* membrane fluidizer benzyl alcohol caused any significant response in the expression of *CBF4* (Fig. S5A) indicative of a branched signalling. We, therefore, asked for the link between calcium influx and expression of *CBF4*. First, we tested whether an influx of Ca^{2+} is sufficient for the induction of *CBF4*, using the calcium ionophore A23187 which allows calcium to enter the cytoplasm in the absence of an external stimulus (Fig. 5C). We observed that treatment with A23187 at 27 °C induced *CBF4* very efficiently (around 35-fold at 3 h) and thus even was superior to chilling (compare Fig. 1E). Unfortunately, to test a potential increase of cold tolerance by ionophore pre-treatment was not possible, due to the long-term cytotoxicity of A23187. In the next step, we asked, whether calcium influx is necessary for the induction of *CBF4*. This was tested using the calcium-channel blocker $GdCl_3$ [22] prior to a cold shock. In fact, this treatment almost completely suppressed the induction of *CBF4* in response to cold. While steady-state transcript levels were increased 8-fold after 6 h of cold shock in the non pre-treated cells (Fig. 5D), it had reached only doubled in the cells treated with $GdCl_3$. Similar to the ionophore experiment, it was not possible to test whether $GdCl_3$ would increase the mortality under chilling, because this compound is already cytotoxic by itself, if administered over a longer period. However, we were able to address this issue using the calcium chelator EGTA (Fig. S5B). Since EGTA did not cause any increase in cold-induced mortality, extracellular calcium seems to be dispensable for cold acclimation. In contrast, the influx of calcium is shown by our experiments to be necessary and sufficient for cold activation of *CBF4*.

3.7. Cold induction of CBF4 in planta is elevated in a cold tolerant species

Our previous experiments showed a correlation between cold acclimation and the induction of *CBF4* (Fig. 1D, E). On the other hand, pharmacological manipulation of cortical microtubules (Figs. 3 and 4) or membrane fluidity (Fig. 5), while effective in the induction of cold hardiness, did not induce *CBF4* (Figs. S1, S5A). Conversely, expression of *CBF4* is under strict control of calcium influx (Fig. 5), while treatment with the calcium chelator EGTA did not render the cells more sensitive to cold shock (Fig. S5B). We, therefore, wanted to test the link between cold tolerance and induction of *CBF4 in planta*. As test system, we used two closely related species of *Vitis* that are adapted to different latitudes: While *Vitis amurensis* settles in the harsh continental climate of Siberia and Manchuria and is therefore cold tolerant [8], *Vitis coignetia* inhabits deciduous forests in the four islands of Japan as well as the Korean peninsula and is therefore cold sensitive [59]. When leaf discs excised from the two genotypes were subjected to a chilling treatment (4 °C), *CBF4* transcripts were found to accumulate rapidly by around 6-fold in *V. amurensis*, remaining elevated (at around 4-fold) even one day later (Fig. 6A). In contrast, *CBF4* did not show any induction in *V. coignetia*, and after 24 h was even downregulated. Thus,

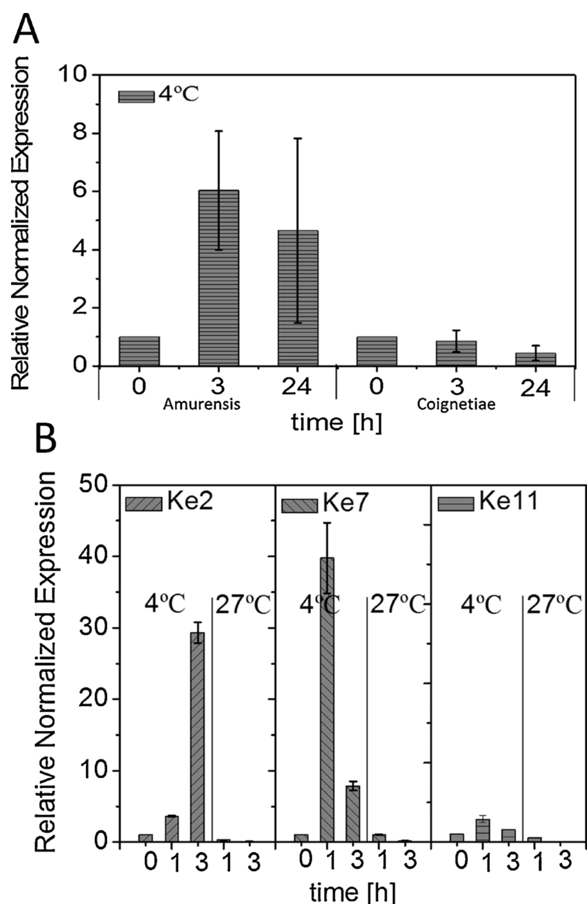

Fig. 6. Temporal expression pattern for *CBF4* in leaf discs of different *Vitis* genotypes in response to cold stress (4 °C). (A) Comparison of the cold tolerant *V. amurensis* and the cold susceptible *V. coignetiae*. Mean values and standard errors from three independent experimental series and three technical replicates per data point are shown. (B) Representative examples for different temporal patterns in different genotypes of *V. sylvestris* under cold stress (4 °C) as compared to control conditions (27 °C). Mean values and standard errors come here from a single experimental series with three technical replicas per data point performed in 2016. Left: Stable induction (type I) as seen in genotype Ke2, center: transient induction (type II) as seen in genotype Ke7, right: no induction (type III) as seen in Ke11.

the inducibility of *CBF4* is high in cold-tolerant species, but absent in cold-sensitive species of grapevine.

When differences in the inducibility of *CBF4* are readouts for cold acclimation, it would be feasible to screen wild *Vitis* germplasm for differential regulation of *CBF4* for subsequent breeding of varieties with elevated cold tolerance. The European Wild Grape, *Vitis vinifera* ssp. *sylvestris* as ancestor of cultivated Grapevine, *Vitis vinifera* ssp. *vinifera*, is of special interest. The largest viable population in Germany of this endangered plant has been genetically copied in the Botanical Garden of the Karlsruhe Institute of Technology. We tested the expression pattern of *CBF4* in a set of genotypes from this collection to detect potential differences in the responses to cold. In fact, we were able to detect three response patterns, exemplarily shown in Fig. 6B: TypeI-showed continuous induction of *CBF4* transcripts over 3 h of cold treatment. This group included 30 genotypes; In TypeII, the expression of *CBF4* was maximal after 1 h cold treatment, but dropped subsequently. This group with transient induction included 26 genotypes; Type III, did not show any induction of *CBF4*, in some cases even a downregulation of this transcript. This group comprised 14 of the tested genotypes (Table. S1).

We mapped these patterns of gene regulation onto the phylogenetic relationship of these genotypes as analyzed based on microsatellite markers [60]. This projection shows that the three response patterns are not equally distributed over the population, but concentrated in certain clades, for instance between two sister lines (Fig. 7, red and blue arrows, A–E) indicative of genetic factors that are shared within these clades and are correlated with the cold inducibility of *CBF4*.

4. Discussion

In our previous work, we have examined the early cellular signalling activated by cold stress focussing on potential factors upstream of microtubule disassembly, including calcium influx, membrane rigidification, activation of a NADPH oxidase and G-protein, in concert with a phospholipase D [22]. Since the transient disassembly of microtubules has been shown to be necessary and sufficient to activate cold adaptation, consistent with results from other plant models [28,29], we addressed in our current work the relation between microtubules and downstream events, such as cold acclimation and the expression of the transcription factor *Cold Box Factor 4* (*CBF4*) as molecular readout for adaptive responses to cold stress. We also asked the question, whether early signals such as membrane rigidification of calcium influx, can modulate the expression of *CBF4*, and whether this modulation is dependent on microtubule integrity. With consideration of a potential application on breeding of tolerance, we probed for, and confirmed a genetic variation in the expression of *CBF4* in a germplasm of *Vitis sylvestris*, the ancestor of cultivated grapevine.

These data allow to discuss three aspects of cold acclimation, which is more straightforward beginning with the downstream events and moving upstream: (1) What is the role of *CBF4*? (2) What is the role of calcium influx? (3) What is the role of microtubules?

4.1. Induction of CBF4 correlates with cold acclimation

The transcription factor family CBF/DREB1 are generally considered as central factor regulating gene expression in response to cold [3]. These responses might be linked with cold-related damage. However, some of these responses might also be part of long-term adaptation to low temperature. When we probed the temporal pattern of the four known CBFs that, upon ectopic expression, induced cold tolerance in *Arabidopsis* [38], we observed that only two of them, *ZFPL* and *CBF4*, were expressed in cold-treated leaves of *V. rupestris*. Since *CBF4* was also cold-induced in our *V. rupestris* suspension cells, further work focussed on this factor. Moreover, stable overexpression of grapevine *CBF4* had been shown to lead to elevated freezing tolerance [18].

We selected therefore *CBF4* as *bona-fide* marker for adaptive gene expression and found that this marker was strongly upregulated under chilling at 8 °C, a treatment that was also efficiently mitigating mortality induced by a subsequent cold shock (Fig. 1). In naïve cells, the cold shock could also induce the expression of *CBF4*, however, to a lower extent, correlated with a mortality elevated over that seen in adapted cells. It should be emphasised that transcripts for a trigger of cold acclimation have to be translated into protein (leading to a decrease of steady-state levels), the protein has to activate genes involved in cold acclimation, transcripts have to be made from those genes, translated into proteins, and these proteins have to exert their effect, before cold hardiness can become manifest. Thus, the rapid and transient accumulation of CBF4 transcripts that precedes the expression of cold hardiness (which occurred much later), is a condition sine qua non for the concept that *CBF4* is a trigger of cold-acclimation.

A second piece of evidence comes from the comparison of the cold-tolerant species *V. amurensis* with the cold-sensitive *V. coignetia* (Fig. 6). Again, the induction of *CBF4* was truly matching with the degree of cold tolerance.

Since the expression of the transcription factor, *CBF4* can be used as a marker for cold tolerance, we employed the activation of *CBF4* to screen a population of *V. sylvestris*. In fact, this population, representing the entire genetic diversity for this species still present in German,

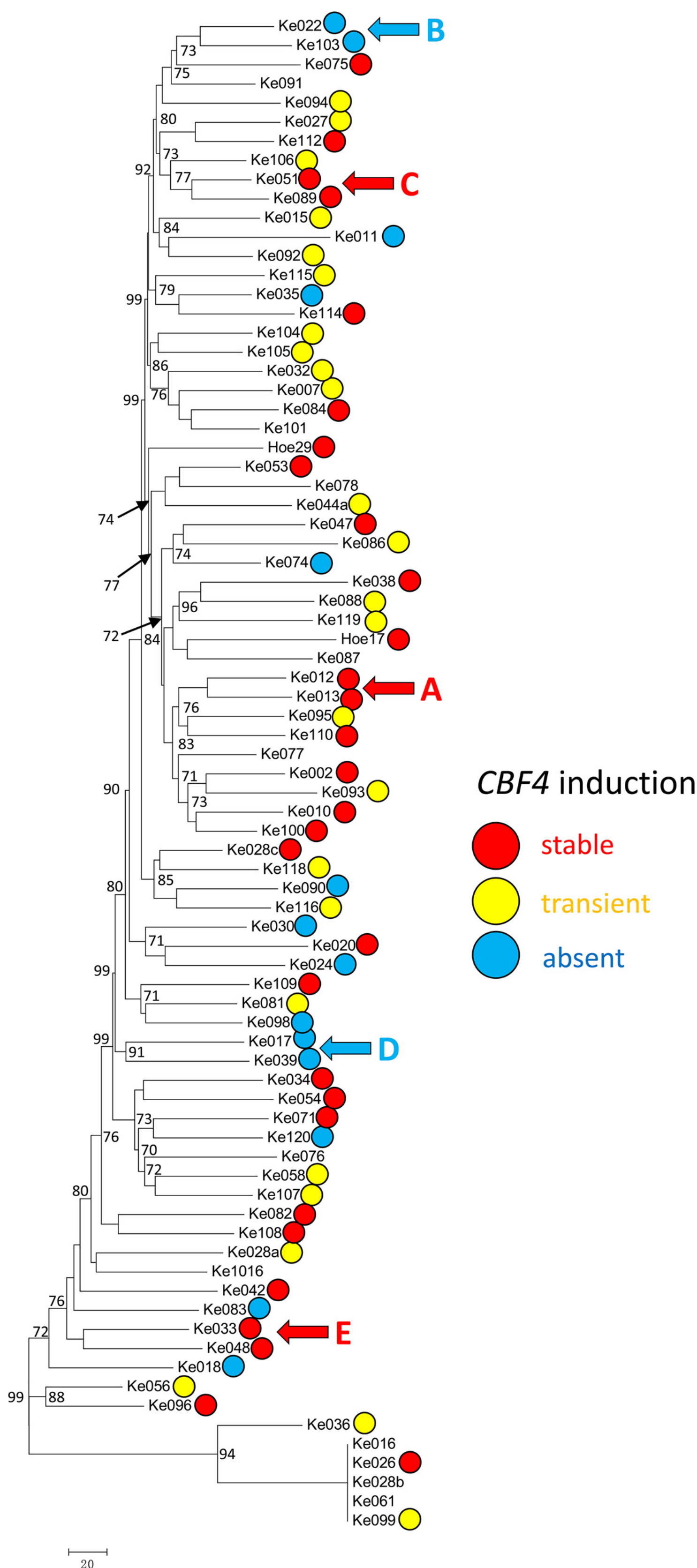

Fig. 7. Mapping of the regulation patterns for *CBF4* on phylogenetic relationships in the Ketsch population of *V. sylvestris* inferred from microsatellite markers using the UPGMA algorithm. Red circles: stable induction of *CBF4* (Type I), Yellow circle: transient induction of *CBF4* (Type II), Blue circle: no induction of *CBF4* (Type III). A–E represented pairs of closely related *V. sylvestris* genotypes that share the *CBF4* response pattern indicative of overlapping genetic regulators. The tree was tested using 1000 burn-ins, bootstrap values > 70% are indicated on the nodes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

displayed different patterns that in some cases clustered in specific phylogenetic clades (Fig. 7). The molecular mechanisms responsible for the different response patterns have to be elucidated, of course, but irrespective of the mechanism, this genetic variability supports the feasibility of using these differences as source for breeding new varieties with elevated cold hardiness. A further prediction of this hypothesis is that fast *CBF4* inducers also excel in terms of cold acclimation, will be validated in future experiments.

4.2. Cold induction of CBF4 is caused by calcium influx

The induction of *CBF4* occurs in the nucleus. However, the sensing of low temperature occurs in the plasma membrane [61]. This means that the signal has to be conveyed by a second messenger. The rapid influx of calcium into the cytoplasm will lead to accumulation of calcium in the cytosol, which culminates in activation of the ICE-CBF-COR cascade pathway followed by improved tolerance [3,62]. The missing link between elevated cytosolic calcium levels and transcriptional activation might be calcium dependent protein kinases, as concluded from experiment, where overexpression of such kinases conferred cold tolerance in rice [63]. However, there must be some mechanisms existing how the signal is transduced from cold sensing at the membrane to the calcium influx and finally to the nucleus. Recently, one typical research finds a plasma membrane protein kinase, named CRPK1, which could phosphorylate 14-3-3 proteins, triggering its nuclear shuttling under cold stress to negatively regulate the cold tolerance in *Arabidopsis* [64]. We have therefore tested, whether modulation of cytosolic calcium levels would modify the induction of *CBF4*. While treatment with the calcium ionophore A23187 induced *CBF4* in the absence of cold stress (Fig. 5C), treatment with $GdCl_3$ treatment suppressed the cold response of transcription compared to that with only cold treatment (Fig. 5D). This demonstrates that calcium influx is necessary and sufficient for the induction of *CBF4*. This finding is congruent with previous reports that the cold activation of the *Brassica napus* BN115 promoter could be induced by the calcium ionophore A23187 in the absence of cold treatment and inhibited by gadolinium ions under low temperature [65].

While the activation of *CBF4* seems to be under tight control of calcium influx, there have to be mechanisms to shut off transcription, once the adapted state has been reached. Here, calcium-independent signals might be important as well as shown by the recent finding in *Arabidopsis thaliana*, where the plasma membrane located protein kinase CRPK1, presumably depending on the fluidity of the membrane, phosphorylates 14-3-3 scaffold proteins that are then shuttling into the nucleus where they down modulate the expression of *CBFs* [64]. It remains to be elucidated, by which molecular mechanism the signal is conveyed to the nucleus in grapevine.

4.3. Microtubule stability modulates the sensitivity of cold signalling

Microtubules, in addition to their traditional function as structural component, are also involved in the signalling transduction, due to their specific character of complex and highly nonlinear dynamics [66]. Particularly, they can amplify signalling in response to mechanic stimuli, such as gravity and cold stress. The role of microtubules in cold tolerance has been reported to be positive, or negative, depending on the particular conditions of the respective experiment, such as species, organ, developmental state, pretreatment, or timing [29,66]. Unfortunately, generalisations based on comparisons of incomparable experiments have created considerable confusion. In a previous study conducted in winter wheat [28], a transient and initial microtubule disassembly was found to be necessary and sufficient (using a mild treatment with pronamide) to trigger cold acclimation. In the current study, both, the pre-treatment of pronamide, as well as a pre-chilling treatment triggered cold hardening linked with a partial disassembly of microtubules. Thus, the central conclusion from the study in winter wheat could be confirmed in grapevine cells: The initial disassembly of MTs is crucial for cold hardening. However, we further disclosed that treatment of taxol, a stabiliser of MTs, is also sufficient to induce cold acclimation. However, we also have seen differences between both systems: while in winter wheat, cold hardiness was accompanied by cold stability of microtubules, this was not seen in the grapevine suspension cells. Whether this difference in the long-term behaviour of microtubules is linked with the fact that suspension cells do not differentiate, while root cells do, or whether it is caused by the difference of species, is not clear at this time. What is clear, however, is that cold stability of microtubules is not required for cold acclimation, but might rather be the consequence of cold acclimation.

These findings lead to a paradox situation – how can stabilisation of microtubules and destabilisation of microtubules both lead to the same outcome, improved cold acclimation? One possibility to resolve the paradox would be to assume that stable and dynamic microtubules might involve different events in cold sensing. Interestingly, neither taxol, nor propyzamide, nor benzyl alcohol were able to mimic the effect of cold on the expression of *CBF4* (Fig. S1). Given the tight link between *CBF4* expression and calcium influx, one can infer that neither the manipulation of microtubules, nor the modulation of membrane fluidity can trigger calcium influx in the absence of cold stress. This leads to a model, where microtubules as well as benzyl alcohol do not convey the signal, but modulate by what sensitivity this signal is generated. In other words, it is the competence of the membrane to deploy cold signalling which is dependent on microtubules.

The primary stimulus for low-temperature sensing seems to be a drop in membrane fluidity [61], which will open calcium-influx channels. In other words; a physical stimulus (temperature dependent fluctuations of molecular mobility leading to electrostatic interactions of the charged head groups) is transduced into a chemical stimulus (increase of cytosolic calcium). While it is conceivable that mechanic force produced by molecular aggregation can cause conformational changes in a transmembrane channel such that it will open, there is an important biophysical argument that is often overlooked in molecular considerations of mechanic signalling: To activate a mechanosensitive channel, forces in the range of $1\ mN{\cdot}m^{-1}$ are required [67], which is close to the breakage point of plant membranes estimated to be around $4\ mN{\cdot}m^{-1}$ [68]. In other words: without efficient leverage, the cold-induced changes of membrane mechanics are orders of magnitude below activation of opening calcium channels to a degree that allows to discriminate a signal against thermal noise. There is evidence from both, plant and animal cells, that microtubules with their high Young modulus might provide such a leverage system able to focus and convey mechanic forces [29] generated by the drop of membrane fluidity to the calcium channels such that the threshold for opening is locally breached.

Modulation of calcium channels by microtubules has been repeatedly described for both plants [69] and animals [70]. In patch-clamp studies, their activity is usually stimulated after treatment with anti-microtubular compounds [71]. The situation in walled cells, where the cytoskeleton is tethered to the extracellular matrix, is quite different, but to monitor calcium influx in walled cells, is far from trivial. One approach is to measure the cotransport of protons with calcium leading to apoplastic alkalinisation, a common practice in analysis of plant defence [72]. In our previous work, we have used this strategy to test, whether elimination of microtubules by oryzalin would induce calcium influx in the same model, suspension cells of *V. rupestris*. We could show that oryzalin induced a significant alkalinisation response, while elimination of actin filaments by LatB did not ([73], Fig. S1). However, this response was minor (around 10%) of the response that could be evoked with bacterial elicitors. Also in the current work, the failure to induce *CBF4* as tight reporter for calcium influx shows that microtubule elimination *per se* is not sufficient to open up calcium channels in our system. However, it is the combination with a cold stimulus, where manipulation of microtubule becomes manifest. Microtubules are therefore a component that modulates the sensitivity of cold signalling. The term "sensitivity" is here used in *sensu strictus* of sensory physiology as competence of a system to deploy signalling in response to a stimulus, not in to colloquial meaning, where "sensitive" means just "affected by" (A thorough treatment of the concept of sensitivity is given in Trewavas [74].

Based on these considerations, our working model of a microtubule thermometer (Fig. 8A) assumes that calcium channels are connected by microtubules that are tethered to the membrane, such that they will collect mechanic force and transmit it as compression upon the

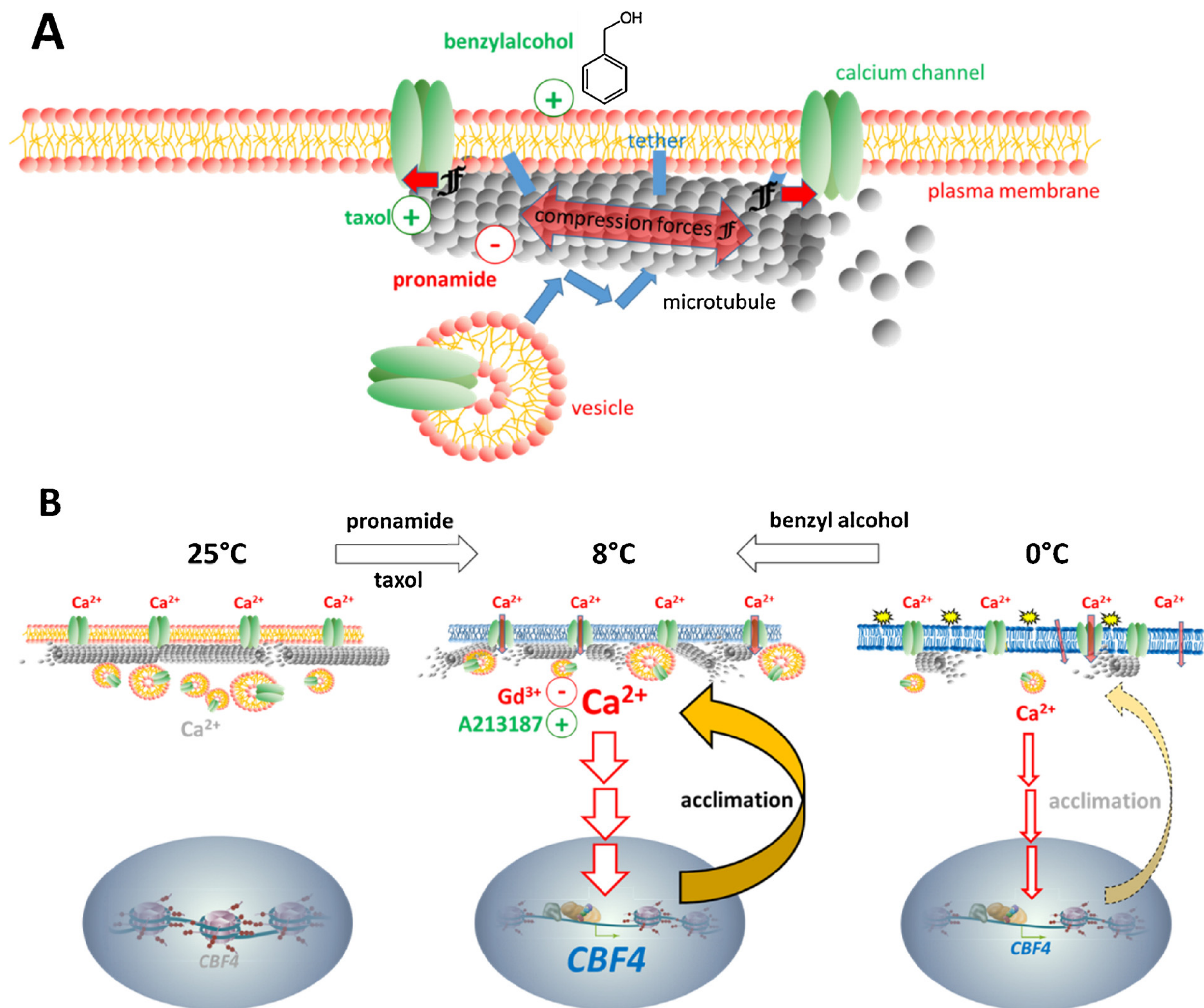

Fig. 8. Working model for the microtubule thermometer and its implications for modulation of cold acclimation. (A) The model assumes that stable microtubules transmit compression forces F collected from decreasing membrane fluidity via microtubule-membrane tethers (blue) on a mechanosensitive calcium influx channel (green). On the other hand, microtubules constrain the integration of recruitment of additional channels to the membrane. Taxol increases the efficiency for force transmission by stable microtubules, benzyl alcohol facilitate channel opening, and pronamide promotes recruitment of additional channels into the membrane. (B) Transduction of the signal generated by the microtubule-facilitated membrane sensor by increase of cytosolic calcium leading to transcriptional activation of *CBF4* culminating in cold hardening, representing the effects of the calcium ionophore A23187, the calcium-channel blocker Gd^{3+}, chilling at 8 °C and cold shock at 0 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

channels producing a mechanic leverage that will open the channels. Treatment with taxol will stabilise those microtubule levers and thus increase the sensitivity of the thermometer. However, microtubules convey a second function in addition: The plasma membrane of plant cells is highly dynamic. Measurements of turnover in coleoptile epidermal cells show that the entire membrane is recycled in as little as three hours [75]. In addition, the membrane is able to recruit additional vesicle material within seconds in response to osmotic challenges [58]. This recruitment depends on dynamic microtubules and is stimulated by sequestration of tubulin heterodimers. Therefore, our model predicts that a treatment with pronamide will stimulate the integration of additional membrane material (which means also the integration of additional calcium channels) and thus increase the sensitivity of the thermometer as well. Benzylaldehyde which disrupts the electrostatic interactions of charged head moieties of membrane lipids [76] is increasing the sensitivity of the sensor, because higher fluidity facilitates opening of the calcium channels due to reduced viscosity of the lipid environment, and which might further prolong cell survival during subsequent cold shock. Thus, microtubules, although important for cold hardening, are certainly not the only mechanism to acquire chilling tolerance. Also responses of membrane composition leading to higher fluidity have been recognised as central factors (reviewed in [61]). When, for instance, the abundance of desaturated lipids is increased by genetic engineering, this improves the chilling tolerance of *Arabidopsis* [77].

It should be noted that this model (Fig. 8A) defines microtubules as factors that modulate the sensitivity of signalling, not as factors of signalling itself. Sensitivity is used here in the strict physiological sense [74] as the competence to deploy a signal in response to a stimulus – in the absence of the stimulus, there is no signal.

There are important implications from this model (Fig. 8B) that are supported by our data: for instance, a mild cold stress (8 °C) should stimulate the integration of calcium channels into the membranes, and therefore lead to a distinct cytosolic calcium signal that will efficiently activate the expression of *CBF4*, while at severe cold stress the breakdown of the microtubule lever along with the failure to recruit additional calcium channels (because vesicle flow is slowed down and fusion is impeded by inhibited by membrane rigidity) will only yield a sluggish calcium signal, which in turn can activate *CBF4* only with some delay and at lower amplitude. The model can explain the effects calcium ionophore A23187 and the channel blocker $GdCl_3$, and it can explain, why both taxol and pronamide can improve survival under

0 °C. Neither benzyl alcohol, nor the microtubule drugs produce a response of *CBF4*, although they are efficient to prepare the cell to cope with severe cold stress, which might be taken as argument to call for a second microtubule-dependent pathway that does not rely on the expression of *CBF4*. However, it is possible to explain this phenomenon without the need to call for a second pathway: microtubule-disruption itself is not able to activate calcium influx (and, thus, also no response of *CBF4* expression), it can only amplify cold-triggered calcium influx, thus accelerating the signalling once the cold shock is administered. Thus, the drug effect upon microtubules will remain hidden (for instance, no induction of *CBF4* is observed), until the system is challenged by cold shock. Now, the drug effect becomes manifest as a more efficient activation of signalling and, thus, adaptation.

It should also be emphasized that the sensory role of microtubules addressed in the current work, have to be separated from the architectural role of microtubules in guiding cell expansion or in driving and aligning cell division. This architectural function of microtubules is a downstream response to the stimulus, not a part of stimulus perception itself. In cold acclimation of winter wheat roots [28] in a second step, a completely cold-tolerant array of cortical microtubules developed after several days. In the current experiments, such a stabilisation was not observed. This is possibly linked with the fact that proliferating cells of a suspension culture are endowed with more dynamic microtubules than cells in a tissue context that have left the meristematic zone. Moreover, since suspension cells die, if not timely subcultivated (leading to a new wave of mitotic activity), a potential long-term stability of microtubules does not have sufficient time to develop.

5. Outlook

Although calcium channels are involved in a plethora of plant stress responses, their molecular nature has remained elusive, with a few exceptions, such as the osmosensor OSCA1 [78]. Our model predicts that under chilling stress the abundance of the calcium channel responsible for cold sensing should increase. This would define a framework to identify potential molecular candidates, since these are expected to be upregulated under these conditions.

The mechanism, by which the signal is conveyed from the peripheral cytoplasm, where calcium levels become elevated into the nucleus, where *CBF4* is activated, represents a second target for future research. The finding that a non-conventional class-XIV kinesin from rice, dual localisation kinesin is imported into the nucleus in response to cold-induced disassembly of cortical microtubules, and that overexpression of this kinesin in tobacco cells amplifies the cold response of *CBF4* [79] indicates additional roles of microtubules in cold signalling, whose relation with CDPK-dependent signalling has to be clarified. In the current study, we did not address this. One approach might be to transiently express GFP-tagged versions of the grapevine 14-3-3 homologues and follow their potential nuclear import in response to cold shock.

The release of microtubule-regulated calcium-dependent cold signaling culminates in transcriptional activation of *CBF4*. One possible reason for the different activation patterns seen in the *sylvestris* population may be differences in recognition motives in the promoter regions of *CBF4*. This hypothesis could be tested using a dual-luciferase promoter-reporter assay [80–82] in the future work. The accumulation of *CBF4* proteins should in turn lead to activation of adaptive downstream genes depending on the presence of DRE/CRT motifs in their promoters. This would open a further target for breeding efforts, since even one amino acid difference in the CBF1 homologues from *V. vinifera* and the wild North American species *V. riparia* were found to lead to differential activation of target promoters [83]. We have launched a new set of experiments to test, whether the genetic variation in the expression patterns for *CBF4* in *Vitis sylvestris* correlates to corresponding differences in cold tolerance. A deeper understanding of adaptive cold signalling, therefore bears immediately on applications of agronomic relevance.

Conflicts of interest

The authors declare no conflict of interest.

Author contribution

LW and PN designed the research and wrote the paper. LW and ES performed the experiments, analyzed the data and wrote the paper. PN and MR revised the manuscript. All authors read and approved the final the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2018.11.008.

References

[1] T. Repo, K. Mononen, L. Alvila, T.T. Pakkanen, H. Hanninen, Cold acclimation of pedunculate oak (*Quercus robur* L.) at its northernmost distribution range, Environ. Exper. Bot. 63 (2008) 59–70.
[2] P.L. Steponkus, M. Uemura, M.S. Webb, Membrane destabilization during freeze-induced dehydration, Curr. Top. Plant Physiol. 10 (1993).
[3] V. Chinnusamy, J. Zhu, J.K. Zhu, Cold stress regulation of gene expression in plants, Trends Plant Sci. 12 (2007) 444–451.
[4] G.S. Sanghera, S.H. Wani, W. Hussain, N.B. Singh, Engineering cold stress tolerance in crop plants, Curr. Genomics 12 (2011) 30–43.
[5] J.M. Lyons, Chilling injury in plants, Annu. Rev. Plant Physiol. 24 (1973) 445–466.
[6] A. Ershadi, R. Karimi, K.N. Mahdei, Freezing tolerance and its relationship with soluble carbohydrates, proline and water content in 12 grapevine cultivars, Acta Physiol. Plant. 38 (1) (2016) 1–10.
[7] A. Fennell, Freezing tolerance and injury in grapevines, J. Crop. Improv. 10 (2004) 201–235.
[8] Y.Y. Ma, Y.L. Zhang, H. Shao, J. Lu, Differential physio-biochemical responses to cold stress of cold-tolerant and non-tolerant grapes (*Vitis L.*) from China, J. Agron. Crop. Sci. 196 (3) (2010) 212–219.
[9] W. Xu, R. Li, N. Zhang, F. Ma, Y. Jiao, Z. Wang, Transcriptome profiling of Vitis amurensis, an extremely cold-tolerant Chinese wild *Vitis* species, reveals candidate genes and events that potentially connected to cold stress, Plant Mol. Biol. 86 (4–5) (2014) 527–541.
[10] M.P. Fuller, G. Telli, An investigation of the frost hardiness of grapevine (*Vitis vinifera*) during bud break, Ann. Appl. Biol. 135 (1999) 589–595.
[11] H. Xin, W. Zhu, L. Wang, Y. Xiang, L. Fang, J. Li, et al., Genome wide transcriptional profile analysis of *Vitis amurensis* and *Vitis vinifera*in response to cold stress, PLoS One 8 (3) (2013) e58740.
[12] S.S.B. Rooy, G.H. Salekdeh, M. Ghabooli, M. Gholami, R. Karimi, Cold-induced physiological and biochemical responses of three grapevine cultivars differing in cold tolerance, Acta Physiol. Plant. 39 (12) (2017) 264.
[13] T.E. Martinson, G.B. White, Estimate of Crop and Wine Losses Due to Winter Injury in the Finger Lakes, (2004).
[14] A.A. Ghasemi, A. Ershadi, E. Fallahi, Evaluation of cold hardiness in seven Iranian commercial pomegranate (*Punica granatum* L.) cultivars, Hortscience 47 (2012) 1821–1825.
[15] G. Tanou, V. Fotopoulos, A. Molassiotis, Priming against environmental challenges and proteomics in plants: update and agricultural perspectives, Front. Plant Sci. 3 (2012) 216.
[16] C.L. Guy, Cold acclimation and freezing stress tolerance: role of protein metabolism, Annu. Rev. Plant Biol. 41 (41) (2003) 187–223.
[17] J. Levitt, Responses of plants to environmental stress, Chilling, Freezing, and High Temperature Stress vol. 1, New York Academic Press, 1980.
[18] R.L. Tillett, M.D. Wheatley, E.A.R. Tattersall, K.A. Schlauch, G.R. Cramer, J.C. Cushman, The *Vitis vinifera* C-repeat binding protein 4 (*VvCBF4*) transcriptional factor enhances freezing tolerance in wine grape, Plant Biotechnol. J. 10 (2012) 105–124.
[19] Z. Xin, J. Browse, Cold comfort farm: the acclimation of plants to freezing temperatures, Plant Cell Environ. 23 (9) (2000) 893–902.
[20] A. Janska, P. Marsik, S. Zelenkova, J. Ovesna, Cold stress and acclimation - what is important for metabolic adjustment? Plant Biol. 12 (2010) 395–405.
[21] D. Takahashi, B. Li, T. Nakayama, Y. Kawamura, M. Uemura, Plant plasma membrane proteomics for improving cold tolerance, Front. Plant Sci. 4 (2013) 90.
[22] L.X. Wang, P. Nick, Cold sensing in grapevine—which signals are upstream of the

microtubular “thermometer”, Plant Cell Environ. 40 (11) (2017) 2844–2857.
[23] M.E. Bartolo, J.V. Carter, Effect of microtubule stabilization on the freezing tolerance of mesophyll cells of spinach, Plant Physiol. 97 (1991) 182–187.
[24] G.P. Kerr, J.V. Carter, Relationship between freezing tolerance of root-tip cells and cold stability of microtubules in rye (*Secale cereale* L. Cv Puma), Plant Physiol. 93 (1990) 77–82.
[25] M. Sakiyama, H. Shibaoka, Effects of abscisic-acid on the orientation and cold stability of cortical microtubules in epicotyl cells of the dwarf Pea, Protoplasma 157 (1990) 165–171.
[26] Q.Y. Wang, P. Nick, Cold acclimation can induce microtubular cold stability in a manner distinct from abscisic acid, Plant Cell Physiol. 42 (2001) 999–1005.
[27] K. Pihakaski-Maunsbach, T. Puhakainen, Effect of cold exposure on cortical microtubules of rye (*Secale cereale*) as observed by immunocytochemistry, Physiol. Plant. 93 (3) (1995) 563–571.
[28] A. Abdrakhamanova, Q.Y. Wang, L. Khokhlova, P. Nick, Is microtubule disassembly a trigger for cold acclimation? Plant Cell Physiol. 44 (2003) 676–686.
[29] P. Nick, Microtubules, and signaling in abiotic stress, Plant J. 75 (2013) 309–323.
[30] R. John, N.A. Anjum, S.K. Sopory, N.A. Akram, M. Ashraf, Some key physiological and molecular processes of cold acclimation, Biol. Plant. 60 (2016) 603–618.
[31] E.J. Stockinger, S.J. Gilmour, M.F. Thomashow, *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit, Proc. Natl. Acad. Sci. U. S. A. 94 (3) (1997) 1035–1040.
[32] J. Medina, R. Catala, J. Salinas, The CBFs: three *arabidopsis* transcription factors to cold acclimate, Plant Sci. 180 (2011) 3–11.
[33] F. Novillo, J. Medina, J. Salinas, *Arabidopsis* CBF1 and CBF3 have a different function than CBF2 in cold acclimation and define different gene classes in the CBF regulon, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 21002–21007.
[34] L.V. Savitch, G. Allard, M. Seki, L.S. Robert, N.A. Tinker, N.P. Huner, K. Shinozaki, J. Singh, The effect of overexpression of two Brassica CBF/DREB1-like transcription factors on photosynthetic capacity and freezing tolerance in Brassica napus, Plant Cell Physiol. 46 (2005) 1525–1539.
[35] T.H. Hsieh, J.T. Lee, Y.Y. Charng, M.T. Chan, Tomato plants ectopically expressing *Arabidopsis* CBF1 show enhanced resistance to water deficit stress, Plant Physiol. 130 (2) (2002) 618–626.
[36] C. Benedict, J.S. Skinner, R. Meng, Y. Chang, R. Bhalerao, N.P. Huner, C.E. Finn, T.H. Chen, V. Hurry, The CBF1-dependent low temperature signalling pathway, regulon and increase in freeze tolerance are conserved in *Populus* spp, Plant Cell Environ. 29 (2006) 1259–1272.
[37] G. Köhler, R.C. Wilson, J.V. Goodpaster, A. Sønsteby, X. Lai, F.A. Witzmann, M. Alsheikh, Proteomic study of low-temperature responses in strawberry cultivars (Fragaria× ananassa) that differ in cold tolerance, Plant Physiol. 159 (4) (2012) 1787–1805.
[38] Y. Takuhara, M. Kobayashi, S. Suzuki, Low-temperature-induced transcription factors in grapevine enhance cold tolerance in transgenic *arabidopsis* plants, J. Plant Physiol. 168 (2011) 967–975.
[39] H. Xiao, M. Siddiqua, S. Braybrook, A. Nassuth, Three grape *CBF/DREB1* genes respond to low temperature, drought and ABA, Plant Cell Environ. 29 (2006) 1410–1421.
[40] H. Xiao, E.A.R. Tattersall, M.K. Siddiqua, G.R. Cramer, A. Nassuth, *CBF4* is a unique member of the CBF transcription factor family of *Vitis vinifera* and *Vitis riparia*, Plant Cell Environ. 31 (2008) 1–10.
[41] J. Wu, K.M. Folta, Y.F. Xie, W.M. Jiang, J. Lu, Y.L. Zhang, Overexpression of Muscadinia rotundifolia CBF2 gene enhances biotic and abiotic stress tolerance in *Arabidopsis*, Protoplasma 254 (2017) 239–251.
[42] X. Guan, G. Buchholz, P. Nick, Tubulin marker line of grapevine suspension cells as a tool to follow early stress responses, J. Plant Physiol. 176 (2015) 118–128.
[43] D. Duan, D. Halter, R. Baltenweck, C. Tisch, V. Troster, A. Kortekamp, P. Hugueney, P. Nick, Genetic diversity of stilbene metabolism in Vitis sylvestris, J. Exp. Bot. 66 (2015) 3243–3257.
[44] P. This, A. Jung, P. Boccacci, J. Borrego, R. Botta, L. Costantini, et al., Development of a standard set of microsatellite reference alleles for identification of grape cultivars, Theor. Appl. Genet. 109 (2004) 1448–1458.
[45] J.E. Bowers, C.P. Meredith, Genetic similarities among wine grape cultivars revealed by restriction fragment length polymorphism (RFLP) analysis, J. Am. Soc. Hortic. Sci. 121 (1996) 620–624.
[46] J.E. Bowers, G.S. Dangl, C.P. Meredith, Development and characterization of additional microsatellite DNA markers for grape, Am. J. Enol. Vitic. 50 (1999) 243–246.
[47] K.M. Sefc, F. Regner, E. Tureschek, J. Glo, H. Steinkellner, Identification of microsatellite sequences in *Vitis riparia* and their application for genotyping of different *Vitis* species, Genome 42 (1999) 367–373.
[48] I. Fechter, L. Hausmann, E. Zyprian, M. Daum, D. Holtgräwe, B. Weisshaar, R. Töpfer, QTL analysis of flowering time and ripening traits suggests an impact of a genomic region on linkage group 1 in *Vitis*, Theor. Appl. Genet. 127 (2014) 1857–1872.
[49] R. Arroyo-García, J.M. Martínez-Zapater, Development and characterization of new microsatellite markers for grape, Vitis 43 (2004) 175–178.
[50] G. Di Gaspero, G. Cipriani, M.T. Marrazzo, D. Andreetta, M.J.P. Castro, E. Peterlunger, R. Testolin, Isolation of (AC)n-microsatellites in *Vitis vinifera* L. And analysis of genetic background in grapevines under marker assisted selection, Mol. Breed. 15 (2005) 11–20.
[51] G. Cipriani, M.T. Marrazzo, G. Di Gaspero, A. Pfeiffer, M. Morgante, R. Testolin, A set of microsatellite markers with long core repeat optimized for grape (*Vitis* spp.) genotyping, BMC Plant Biol. 8 (2008) 127.
[52] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (2016) 1870–1874.
[53] D.F. Gaff, O. Okong’o-Ogola, The use of non-permeating pigments for testing the survival of cells, J. Exp. Bot. 22 (1971) 756–758.
[54] X. Guan, G. Buchholz, P. Nick, The cytoskeleton is disrupted by the bacterial effector HrpZ, but not by the bacterial PAMP flg22 in tobacco BY-2 cells, J. Exp. Bot. 64 (2013) 1805–1816.
[55] S. Tan, C. Grün, X. Guan, Z. Zhou, U. Schepers, P. Nick, Gallic acid induces mitotic catastrophe and inhibits centrosomal clustering in HeLa cells, Toxicol. In Vitro 30 (2015) 506–513.
[56] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[57] P. Hohenberger, C. Eing, R. Straessner, S. Durst, W. Frey, P. Nick, Plant actin controls membrane permeability, Biochim. Biophys. Acta 1808 (2011) 2304–2312.
[58] Q. Liu, F. Qiao, A. Ismail, X. Chang, P. Nick, The plant cytoskeleton controls regulatory volume increase, Biochim. Biophys. Acta 1828 (2013) 2111–2120.
[59] K. Ip, M. Lam, A. Miller, Shading performance of a vertical deciduous climbing plant canopy, Build. Environ. 45 (1) (2010) 81–88.
[60] P. Nick, Schützen und nützen - von der Erhaltung zur Anwendung. Fallbeispiel Europäische Wildrebe, Hoppea Denkschr Regensb Bot Ges Sonderband (2014) 159–173.
[61] D.A. Los, N. Murata, Membrane fluidity and its roles in the perception of environmental signals, Biochim. Biophys. Acta 1666 (2004) 142–157.
[62] D.G. Zarka, J.T. Vogel, D. Cook, M.F. Thomashow, Cold induction of Arabidopsis CBF genes involves multiple ICE (inducer of CBF expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature, Plant Physiol. 133 (2003) 910–918.
[63] Y. Saijo, S. Hata, J. Kyozuka, K. Shimamoto, K. Izui, Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants, Plant J. 23 (2000) 319–327.
[64] Z.Y. Liu, Y.X. Jia, Y.L. Ding, Y.T. Shi, Z. Li, Y. Guo, Z.Z. Gong, S.H. Yang, Plasma membrane CRPK1-Mediated phosphorylation of 14-3-3 proteins induces their nuclear import to fine-tune CBF signaling during cold response, Mol. Cell 66 (2017) 117–128.
[65] V. Sangwan, I. Foulds, J. Singh, R.S. Dhindsa, Cold-activation of *Brassica napus* BN115 promoter is mediated by structural changes in membranes and cytoskeleton, and requires Ca^{2+} influx, Plant J. 27 (2001) 1–12.
[66] P. Nick, Microtubules as sensors for abiotic stimuli, in: P. Nick (Ed.), Plant Microtubules, 2008, pp. 175–203 Plant Cell Monographs.
[67] F. Sachs, C.E. Morris, Mechanosensitive ion channels in nonspecialized cells, Rev. Physiol. Bioch. P. 132 (1998) 1–77.
[68] A. Kell, R.W. Glaser, On the mechanical and dynamic properties of plant-cell membranes: their role in growth, direct gene transfer and protoplast fusion, J. Theor. Biol. 160 (1993) 41–62.
[69] J.P. Ding, B.G. Pickard, Mechanosensory calcium-selective cation channels in epidermal cells, Plant J. 3 (1993) 83–110.
[70] C. Savage, M. Hamelin, J.G. Culotti, A. Coulson, D.G. Albertson, M. Chalfie, *mec-7* is a beta-tubulin gene required for the production of 15-protofilament microtubules in *Caenorhabditis elegans*, Genes Dev. 3 (1989) 870–881.
[71] L. Thion, C. Mazars, P. Nacry, D. Bouchez, M. Moreau, R. Ranjeva, P. Thuleau, Plasma membrane depolarization-activated calcium channels, stimulated by microtubule-depolymerizing drugs in wild-type *Arabidopsis thaliana* protoplasts, display constitutively large activities and a longer half-life in *ton 2* mutant cells affected in the organization of cortical microtubules, Plant J. 13 (1998) 603–610.
[72] G. Felix, J.D. Duran, S. Volko, T. Boller, Plants have a sensitive perception system for the most conserved domain of bacterial flagellin, Plant J. 18 (1999) 265–276.
[73] X. Chang, P. Nick, Defence signalling triggered by Flg22 and Harpin is integrated into a different stilbene output in *Vitis* cells, PLoS One 7 (2012) e40446.
[74] A.J. Trewavas, Growth substance sensitivity: the limiting factor in plant development, Physiol. Plant. 55 (1982) 60–72.
[75] G.D. Phillips, C. Preshaw, M.W. Steer, Dictyosome vesicle production and plasma membrane turnover in auxin-stimulated outer epidermal cells of coleoptile segments from *Avena sativa* (L.), Protoplasma 145 (1988) 59–65.
[76] L.M. Gordon, R.D. Sauerheber, J.A. Esgate, I. Dipple, I. Robert, N. Marchmont, M.D. Houslay, The increase in bilayer fluidity of rat liver plasma MembranesAchieved by the local anesthetic benzyl alcohol affects the activity of intrinsic membrane enzymes, J. Biol. Chem. 255 (1980) 4519–4527.
[77] F.P. Wolter, R. Schmidt, E. Heinz, Chilling sensitivity of *Arabidopsis*thaliana with genetically engineered membrane lipids, EMBO J. 11 (13) (1992) 4685–4692.
[78] F. Yuan, H. Yang, Y. Xue, D. Kong, R. Ye, et al., Osca1 mediates osmotic-stress-evoked ca^{2+} increases vital for osmosensing in *arabidopsis*, Nature 514 (7522) (2015) 367–371.
[79] X. Xu, W. Walter, Q. Liu, I. Machens, P. Nick, A rice class-XIV kinesin enters the nucleus in response to cold, Sci. Rep. 8 (1) (2018) 3588, https://doi.org/10.1038/s41598-018-21816-w136.
[80] J. Höll, A. Vannozzi, S. Czemmel, C. D’Onofrio, A.R. Walker, et al., The R2R3-MYB transcription factors *MYB14* and *MYB15* regulate stilbene biosynthesis in *Vitis vinifera*, Plant Cell 25 (2013) 4135–4149.
[81] D. Duan, S. Fischer, P.R. Merz, J. Bogs, M. Riemann, P. Nick, An ancestral allele of grapevine transcription factor *MYB14* promotes plant defence, J. Exp. Bot. 67 (2016) 1795–1804.
[82] Y. Jiao, W. Xu, D. Duan, Y. Wang, P. Nick, A stilbene synthase allele from a Chinese wild grapevine recruits salicylic acid signalling for efficient defence, J. Exp. Bot. 67 (2016) 5841–5856.
[83] A. Nassuth, M. Siddiqua, H. Xiao, M.A. Moody, C.E. Carlow, Newly developed quantitative transactivation system shows difference in activation by *Vitis* CBF transcription factors on DRE/CRT elements, Plant Methods 10 (1) (2014) 32.