Cold signal into nucleus

A lesson from CBF4 and Kinesin 14

Zur Erlangung des akademischen Grades eines

DOKTORS DER NATURWISSENSCHAFTEN

(Dr. rer. nat.)

von der KIT-Fakultät für Chemie und Biowissenschaften
des Karlsruher Instituts für Technologie (KIT)

genehmigte

DISSERTATION

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Tag der mündlichen Prüfung: 25. Oktober, 2022

Die vorliegende Dissertation wurde am Botanischen Institut des Karlsruher Institutsfür Technologie (KIT), Botanisches Institut, Lehrstuhl 1 für Molekulare Zellbiologe,von Oktober 2018 bis Oktober 2022 angefertigt.

Hiermit erkläre ich, dass ich die vorliegende Dissertation, abgesehen von der Benutzung der angegebenen Hilfsmittel, selbständig verfasst habe.

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Zudem erkläre ich, dass ich mich beim Anfertigen dieser Arbeit an die Regeln zur Sicherung guter wissenschaftlicher Praxis des KIT gehalten habe, einschließlich der Abgabe und Archivierung der Primärdaten und dass die elektronische Version mit der schriftlichen übereinstimmt.

Karlsruhe, August 2022

Wenjing Shi

Acknowledgement

According to many legends, getting a degree is a one-way process of gradually and furtively losing your hair. Fortunately, I was spared this nightmare. Woo-hoo, they're satisfied with me, and getting more pals.

Looking back, I understand it was more than a stroke of luck. Prof. Nick, my supervisor, had offered me this fantastic opportunity to travel 7872 kilometers and spend four years in Germany. He has always been professional in his work and nice to me throughout the last four years. The moments in regular meetings motivated and pushed me to pursue my PhD. Thank you, boss, for making my academic year more bearable.

I'm also proud of the progress I've made in the kitchen. Delicacies mean everything to me. There are no problems or troubles that a good meal can’t solve. I even believe I am capable of graduating from Le Cordon Bleu Culinary Arts Institute as a result of my constant efforts. Taking excellent care of myself is extremely beneficial.

Of course, I'd be remiss if I didn't mention my lovely coworkers. First and foremost, Dr. Riemann. I can't express my thanks in words. You're the real Baymax, friendly, kind, and laid-back, unlike the other drab scientists. There are also numerous coworkers, such as Sabine, Ernst, Gabi, and the ever-young Azubis, who are passionate about their profession and contribute significantly to our institute. Thank you for your time and work. The last but not the least, here comes my dear Chris, Nathaniel, Noemi, Kai and so on. You guys are super cool. I say it not only because I admire that you can drink a lot of beers, but also because of you, life is not boring at all. Wait, still sounds like I like you guys because of your alcoholic ability. So be it.

Finally, my feelings are directed toward my family. I admire their unwavering support and will always stand by them.

Until now, I've begun to recognize that my PhD career is coming to an end. It's time to bid farewell. Life goes on; don't be concerned; be content. And, y'all stay sexy.

Wenjing Shi

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Abbreviations

CBF: CRT/DRE binding factor

PM: plasma membrane

MTs: microtubules

CaM: calmodulin

MAPK: mitogen-activated protein kinase

MAP3Ks/MAPKKKs/MEKKs: MAP kinase kinase kinases

MAP2Ks/MAPKKs/MKKs/MEKs: MAP kinase kinases

MAPKs/MPKs: MAP kinases

MAPs: microtubule-associated proteins

CMLs: calmodulin-like proteins

ROS: reactive oxygen species

$^{\bullet}$OH: hydroxyl radicals

$O_2^{\bullet -}$: superoxide anions

H_2O_2: hydrogen peroxide

SOD: superoxide dismutase

COR genes: cold-responsive genes

ICE1: inducer of CBF expression 1

HOS1: osmotically responsive gene 1

OST1: open stomata 1

KIFs: kinesin superfamily proteins

KLC: kinesin light chain

KCBP: kinesin-like calmodulin-binding protein

JA: jasmonic acid

DLK: Dual localization kinesin

D-N: *VvDLK* localizing in nucleus

D-M: *VvDLK* decorating microtubules and localizing in nucleus

DMSO: dimethylsulfoxide

CHX: Cycloheximide

AMD: Actinomycin D

SDS: sodium dodecyl sulphate

Zusammenfassung

Kältestress, einschließlich Abkühlung (0-20℃) und Frost (0℃), wirkt sich nachteilig auf den Pflanzenstoffwechsel und das Transkriptom aus. Die Pflanze erwirbt jedoch eine erhöhte Kältetoleranz durch eine frühere Exposition gegenüber leichtem Kältestress, der eine angemessene Kältereaktion in der Pflanzenzelle auslöst, aber keine irreversiblen Schäden verursacht. Die Kälteakklimatisierung ist von besonderer Bedeutung für die Toleranz gegenüber Spätfrösten im Frühjahr, da diese als Folge der verschwommenen Saisonalität im Kontext des globalen Klimawandels immer häufiger auftreten. C-Repeat-Bindungsfaktoren (CBFs) sind entscheidende Transkriptionsaktivatoren bei der Reaktion von Pflanzen auf niedrige Temperaturen. CBF4 unterscheidet sich durch eine langsamere, aber beständigere Regulation und seine Rolle bei der Kälteakklimatisierung.

In der aktuellen Studie untersuchen wir die Funktionen des CBF4 der Weinrebe, VvCBF4. Die Überexpression von VvCBF4, das mit GFP fusioniert ist, in BY-2-Tabakzellen verleiht Kältetoleranz. Darüber hinaus wandert dieses Protein als Reaktion auf Kältestress vom Zytoplasma in den Zellkern, was mit der Anhäufung von Transkripten für andere CBFs und das auf Kälte reagierende (COR) Gen ERD10d einhergeht. Diese Reaktion ist bei Kälte anders als bei Frost und wird durch vorgelagerte Signale wie oxidativen Burst, Proteasom-Aktivität und Jasmonat-Synthese unterschiedlich reguliert. Dieser Unterschied zwischen Kühlen und Gefrieren zeigt sich auch in der Regulierung der CBF4- und ERD10d-Transkripte in Blättern verschiedener Rebsorten, die sich in ihrer Kältetoleranz unterscheiden. Wir schlagen ein Modell vor, bei dem die Qualität des Kältestresses durch verschiedene vorgelagerte Signale vermittelt wird, die den Kernimport und damit die transkriptionelle Aktivierung von CBF4 bei Weinreben regulieren.

Darüber hinaus wurde in früheren Arbeiten unseres Labors gezeigt, dass sich ein Minus-End-gesteuertes Klasse-XIV-Kinesin aus Reis OsDLK (Dual localization kinesin) von den kortikalen Mikrotubuli in den Zellkern bewegt und zwar spezifisch bei Kältestress. Die Überexpression von OsDLK-GFP führte zu einer unterdrückten Expression der tabakspezifischen Homologen von CBF4, *Avr9/Cf9*, was auf eine mögliche Verbindung zwischen DLK und CBF4 bei der Kältesignalisierung hindeutet (Xu *et al.*, 2022). Um die molekulare Funktion von DLK bei der Kältereaktion zu verstehen, überexprimierten wir ein DLK aus der Weinrebe (*VvDLK*), die am N-Terminus mit GFP fusioniert war, in BY-2-Tabakzellen. In unserer Studie wurden zwei Subpopulationen beobachtet. Bei der einen erscheint das GFP-Signal im Zellkern (D-N) mit hohen DLK mRNA-Spiegel. Die andere ist, dass VvDLK nicht nur im Zellkern lokalisiert ist, sondern auch Mikrotubuli dekoriert (D-M) und eine mysteriöse schwere Bande (90kD) aufweist, die fest an Mikrotubuli bindet. Angesichts der differenten Zellsterblichkeit der

beiden Subpopulationen als Reaktion auf Kälte und Gefrieren wird D-N als kälteresistenter Phänotyp angesehen, während D-M kälteempfindlich ist. Um diesen Unterschied zu erklären, fanden wir Transkriptunterschiede von *Avr9/Cf9*, COR-Genen, die möglicherweise eine Rolle bei der Kältetoleranz spielen und den Unterschied zwischen den beiden Populationen bei der Reaktion auf Froststress erklären. Dieser Unterschied von *VvDLK* zwischen Kühlen und Gefrieren ist auch in Blättern verschiedener Rebsorten zu beobachten, die sich in ihrer Kältetoleranz unterscheiden, was auf einen positiven Zusammenhang zwischen VvDLK und Kältetoleranz hinweist, der mit der CBF4-Aktivität zusammenhängen könnte.

Abstract

Cold stress including chilling (0-20℃) and freezing (0℃) adversely affects plant metabolism and transcriptomes. However, plant acquires increased cold tolerance by perior exposure to mildly chilling stress, which adequately trigger cold response in plant cell but don't cause irreversible injury. cold acclimation is of accentuated relevance for the tolerance to late spring frosts as they become progressively common as consequence of blurred seasonality in the context of global climate change. C-repeat binding factors (CBFs) are crucial transcriptional activators in plant responses to low temperature. CBF4 differs by a slower, but more persistent regulation and its role in cold acclimation.

In the current study, we explore the functions of CBF4 from grapevine, VvCBF4. Overexpression of VvCBF4 fused to GFP in tobacco BY-2 cells confers cold tolerance. Furthermore, this protein shuttles from the cytoplasm to the nucleus in response to cold stress, associated with accumulation of transcripts for other CBFs and the cold responsive (COR) gene ERD10d. This response differs for chilling as compared to freezing and is regulated differently by upstream signalling involving oxidative burst, proteasome activity and jasmonate synthesis. This difference between chilling and freezing is also seen in the regulation of CBF4 and ERD10d transcripts in leaves from different grapevines differing in their cold tolerance. We propose a model, where the quality of cold stress is transduced by different upstream signals regulating nuclear import and, thus, transcriptional activation of grapevine CBF4.

Moreover, perior work in host lab discovered a rice minus-end-directed class-XIV kinesin OsDLK (Dual localization kinesin) moves from cortical microtubules into the nucleus and specifically in presence of cold stress. Overexpressing OsDLK-GFP harbored a suppressed expression of the tobacco homologues of CBF4, *Avr9/Cf9*, suggesting a possible link between DLK and CBF4 in cold signalling (Xu *et al.*, 2022). To understand the molecular function of DLK in cold response, we cloned and transformed overexpression of DLK from grapevine (VvDLK) fused GFP at N-terminus into tobacco BY-2 cells. There are two subpopulations observed in our study. One is GFP signal appears in nucleus (D-N) with extremely high *DLK* mRNA levels. The other one is VvDLK not only localizes in the nucleus but also decorates microtubles (D-M), haboring a mysterious heavy band (90kD) tightly binding to microtubules. Besides, given by the cell mortality of two subpopulations in response to chilling and freezing, D-N is considered as cold-resistent phenotype, whereas D-M is cold- susceptible. To explain this difference, we found transcript differences of *Avr9/Cf9*, COR genes may play a role in cold tolerance and attribute to the difference of two populations in response to freezing stress. This difference of *VvDLK* between chilling and freezing is also seen in leaves from different grapevines differing

in their cold tolerance, indicating a positive association between *VvDLK* and cold tolerance, which may link with CBF4 activity.

1. Introduction

Global warming, one of the most detrimental aspects of climate change affects all stages of plant growth and development primarily during their vegetative phase as well as flowering and reproductive development, including seed development, germination, and photosynthesis (Lippmann *et al.*, 2019). The elevated temperature in past decades has caused about 5% reproductive loss of major staple crops such as wheat, maize or rice per degree centigrade (Zhao *et al.*, 2017a). Besides, since 1736, flowering has advanced on average 4–7 days with each °C of increase, leading to precocious bud break driven by elevated temperature, from a study on 500 plant taxa in Massachusetts (USA) and 400 British plant species (Fitter and Fitter, 2002; Miller-Rushing and Primack, 2008). Additionally, extreme weather phenomena like early autumn frost, late frost in spring, as well as cold and hot waves during the early summer occur more often, leading to an aggravating negative impact on plants. For viticulture, a correlative analysis of harvest times and climatic conditions in France and Switzerland over the past four centuries showed that harvests have shifted to earlier time points, such that maturation is progressively uncoupled from the dry autumn weather required for high-quality wine (Cook and Wolkovich, 2016). The freezing tolerance of grapevine drops rapidly after bud break (Fuller and Telli, 1999). The progressively blurred onset of spring with unusually warm weather in March and late freeze episodes in April, therefore, has especially devastating consequences for viticulture. Thus, understanding the molecular mechanisms underlying cold stress and cold adaptation has turned into a very relevant research topic.

1.1 Cold stress

The quality of cold stress is strongly dependent on the temperature. Traditionally, two versions of cold stress are discriminated (for classical reviews see (Lyons, 1973; Guy, 1990)): Chilling stress is defined as above the freezing point (0-20℃), affecting the productivity and quality of plants inhabiting tropical or subtropical climates (Zhang *et al.*, 2020). However, many of these plants can also acquire cold tolerance in response to chilling, which is called cold acclimation. Unlike chilling, the injuries caused by freezing stress (< 0℃) to plants are irreversible and can be lethal due to the formation of intracellular ice crystals. Whether a given plant is tolerant to chilling or freezing, respectively, is strongly dependent on its genotype. In the case of the grapevine, *V. amurensis* can survive at –40 °C, while severe cane damage of *Vitis vinifera* starts already from +2.5℃ for Riesling and from -5℃ for Pinot Noir, according to the USDA extension service (http://wine.wsu.edu/extension/weather/cold-hardiness/ visited April 14, 2021). The

ability to respond adequately to different temperatures obviously requires efficient and specific signalling.

1.2 Cold signalling pathway

1.2.1 Cold signal perception through membrane rigidification

The plasma membrane is the phospholipid bilayer, together with membrane proteins (integral proteins spanning the membrane as membrane transporters) and peripheral proteins loosely attaching to the outer membrane as enzymes, facilitating the interaction with the environment (Barkla and Pantoja, 2011). It not only separates and shields the cell from its environment but also serves as a sensor, interpreting environmental stimuli and transmitting signals into cells, leading to direct and rapid responses to external changes (Takahashi *et al.*, 2013). During freezing conditions, the formation of disulfide bonds between two glutathione molecules or sulfhydryl groups on adjacent proteins has been confirmed to cause freezing injury and electrolyte leakage, while cold acclimation can enhance the content of unsaturated fatty acid and phospholipids (Shewfelt, 1992). As a result, instant membrane rigidification perceived by membrane proteins has been designated as the initial response to cold stress. In *Synechocystis*, a mutation of *Hik33,* which encodes fatty acid desaturase has resulted in plasma membrane rigidification even at moderate temperature and enhanced expression of cold-inducible genes (Inaba *et al.*, 2003). Moreover, the application of pharmacological methods has further confirmed the role of the plasma membrane in cold perception. Under chilling stress, inhibiting membrane rigidification by benzyl alcohol (BA) reduced the expression of cold-inducible genes and the Ca^{2+} influx, while those responses were stimulated at room temperature in presence of dimethylsulfoxide (DMSO), the membrane rigidifier (Örvar *et al.*, 2000; Sangwan *et al.*, 2002; Wang *et al.*, 2018; Wang *et al.*, 2019).

The increase of permeability caused by membrane rigidification has been also found in more plants that undergo cold stress, resulting in increased generation of a proton gradient across the membrane by the H^+-ATPase, which transports a variety of solutes and ions, such as K^+, NO_3^-, nucleotides or nucleosides across the plasma membrane (Palmgren, 2001). A recent study about plasma membrane located 14-3-3 proteins, binding to PM H^+-ATPase shows the close link of plasma membrane localized H-$^+$-ATPase and 14-3-3 proteins as well as the activity of phospholipase D (PLD). In *Arabidopsis*, 6h exposure to 4 °C inhibited the H^+-ATPase and reduced its association with 14-3-3, while the interaction of H^+-ATPase and 14-3-3 was interrupted by phosphatidic acid (PA) released by cold activated phospholipase D (Muzi *et al.*, 2016). Moreover, overexpression of 14-3-3ω boosted the H^+-ATPase activity， indicating the essential function of plasma membrane localized proteins and ion channels on cold signalling (Visconti *et al.*, 2019).

1.2.2 Microtubule-mediated cold perception

Microtubules are dynamically organized in diverse arrays (cortical MTs, preprophase band (PPB), mitotic spindle, and phragmoplast), participating in plant morphogenesis, including cell division, cell expansion, and cell differentiation (Chen *et al.*, 2016). Among those arrays, cortical MTs are quite dynamic through exhibiting polymerization-biased dynamic instability at one end and slow depolymerization at the other, therefore able to change their orientation in response to diverse stimuli (Wang *et al.*, 2020).

In addition, microtubules also play a role in signal transduction based on the ability to sequester and release molecules (Gundersen and Cook, 1999). Microtubules function as a ''thermometer'' and interact with Ca^{2+} to sense temperature fluctuations. The dynamic microtubules, upstream of Ca^{2+} activity modify plasma membrane-localized Ca^{2+} channel and thus triggers Ca^{2+} influx in signal transduction (Wang *et al.*, 2020). On the other hand, MTs act as downstream regulators of Ca^{2+}, for example, in the binding of Ca^{2+} to CaM (Calmodulin), phosphorylation of MAPs by MAPK resulting in MTs stability (Komis *et al.*, 2011). In plants, CaM can bind to cortical MTs and affect their dynamics in a calcium-dependent manner (J. CYR, 1991). Furthermore, ROS homeostasis can integrate with other molecules to affect the organization of MTs, which reveals the essential function of MTs in signal transduction (Wang *et al.*, 2021).

In *Arabidopsis*, the MAP65 family, which is involved in MTs bundling by concentrating at the plus end of MTs shows effects on microtubule nucleation and polymerization, thus strongly stabilising MTs and rendering the plant more resistant to cold treatment (Li *et al.*, 2009; Mao *et al.*, 2005; Tulin *et al.*, 2012; Van Damme *et al.*, 2004). The end-binding protein 1 (EB1) and CLIP-associated protein (CLASP) preferentially bind at the plus end of MTs to stabilize MT activity (Ambrose *et al.*, 2011; Ambrose *et al.*, 2007; Chan *et al.*, 2003). Moreover, WAVE-DAMPENED2-LIKE5 (WDL5), a microtubule-associated protein involved in MTs stabilization, is likewise specifically targeted and upregulated by EIN3, suggesting the interplay of MAP and other signal molecules in abiotic stress (Dou *et al.*, 2018; Sun *et al.*, 2015).

1.2.3 Ca^{2+}-mediated signalling

The calcium ion, as an essential second messenger contributes to the coordination of external stimuli and multifarious intracellular processes. Under unstimulated conditions, the level of free Ca^{2+} in the cytosol ($[Ca^{2+}]$cyt) of any organism is strictly limited to approximately 100nM, which is extremely lower than the extracellular concentration of 2mM. However, it soars to around 10-fold when exposed to ambient stimuli. The increase of $[Ca^{2+}]$cyt is either derived from extracellular Ca^{2+} crossing through the plasma membrane, or ion channels on the ER (Clapham, 1995).

Ca^{2+} sensing

Calcium sensor proteins are categorised into the three main families, including (i) the calmodulin (CaM) and calmodulin-like proteins (CMLs), (ii) the calcineurin-B-like proteins (CBLs) (iii) and the calcium-dependent protein kinases (CPKs) and calcium and calmodulin-dependent protein kinases (CCaMKs) (Ranty *et al.*, 2016). Among them, CaM is considered the ubiquitously expressed and well-established Ca^{2+} sensing protein. CaM is composed of two globular domains with 4 EF-hand motifs connected by a central helix. By binding to Ca^{2+} with various affinities, EF-hand domains change their conformation, exposing hydrophobic surfaces on each of the domains that are accessible to interact with their downstream target enzymes, triggering activation of their respective activities (e.g., CaMK and calcineurin) or regulation of the functions of target proteins in a Ca^{2+}-dependent manner (e.g., Orai) (Bagur and Hajnóczky, 2017). There are two distinct mechanisms of enzymes in Ca^{2+}-dependent activation, referring to the simultaneous or the sequential one. For example, the interaction and activation of CaMK with/by Ca^{2+} are only triggered by elevated [Ca^{2+}]cyt. On the other hand, part of Ca^{2+} binds with and activates CaM, while the other part interacts with other target proteins to form a low-affinity complex, providing the possibility for further interaction of Ca^{2+} with CaM EF-hands (Kincaid and Vaughan, 1986). Thus this successive binding mechanism allows a precise switch for regulating [Ca^{2+}]cyt.

Ca^{2+} signalling in animal

The immunological response of Ca^{2+} signalling in animal cells has been confined to a decrease in Ca^{2+} concentration in the ER, which triggers Ca^{2+} sensor-based activation of Ca^{2+} channels on the plasma membrane (Di Capite and Parekh, 2009). Generally, the dynamics of the Ca^{2+} concentration in the ER sensed by stromal interaction molecules (STIMs) trigger the formation of the pore-forming *Orai* proteins, generating the hexameric Ca^{2+} release-activated Ca^{2+} (CRAC) channel (Hou *et al.*, 2012; Rothberg *et al.*, 2013; Prakriya *et al.*, 2006). Activated CRAC leads to the continuous Ca^{2+} influx (Feske *et al.*, 2006). However, *Orai* genes are found in most gymnosperms, rather than being absent in angiosperms (Cai, 2007; Collins and Meyer, 2011; Edel *et al.*, 2017). Numerous studies have established that plants exhibit lower diversity of proteins responsible for Ca^{2+} influx, whereas animals have numerous protein families encoding for Ca^{2+} efflux (Wheeler and Brownlee, 2008; Edel and Kudla, 2015; Verret *et al.*, 2010).

Ca^{2+} influx in plant defence signalling

Intracellular homeostasis and modulation of Ca^{2+} under abiotic and biotic stress have remained interesting research topics to date. Thus, findings of the calcium channels that respond to environmental stimuli shed the light on this field. The large conductance elicitor-activated ion channel (LEAC), a Ca^{2+}-permeable channel in the plasma membrane, can be specifically activated by a peptide fragment named Pep-13, which is used as an inducer of plant defence, in a rapid and reversible way without significant alteration in membrane potential (Zimmermann *et al.*, 1997).

More voltage-dependent Ca^{2+}-permeable channels mediated and activated by depolarization of membrane were identified on the plant plasma membrane. A Ca^{2+}-permeable channel defined from the two-pore channel family (TPC1) was demonstrated to be involved in Ca^{2+} influx and stress responses in tobacco and rice coupled with interaction with mitogen-activated protein kinase (MAPK) signalling (Kadota *et al.*, 2004; Kurusu *et al.*, 2005). Cyclic nucleotide-gated ion channels (CNGC) are taken as other Ca^{2+}-permeable channels on the plant plasma membrane, consisting of six membrane-spanning helices and individual cyclic domains in the carboxy terminus for nucleotide-binding and calmodulin-binding (Sherman and Fromm, 2009). Heterologous expression of CNGC2 genes showed the accumulation of $[Ca^{2+}]$cyt, while a mutant of those was verified to lack a cyclic nucleotide-gated Ca^{2+} current through the plasma membrane or reduce ion influx (Leng *et al.*, 1999; Frietsch *et al.*, 2007; Ali *et al.*, 2007; Guo *et al.*, 2010). Moreover, glutamate receptor-like channels (GRLs), plant homologies of mammalian ionotropic glutamate receptors (iGluRs) were discovered in the *Arabidopsis* genome in 1998, which play a role in Na^+, K^+ as well as Ca^{2+} transportation by forming ligand-gated channels to varying degrees, depending on subunit composition form ligand-gated channels (Lam *et al.*, 1998; Lacombe *et al.*, 2001; Traynelis *et al.*, 2010).

Ca^{2+} efflux in plant signalling

As a signal molecule, $[Ca^{2+}]$cyt is regulated via a sophisticated efflux process, either to transduce the signal or maintain a relatively low basal level in preparation for the rapid influx by other stimuli. Existing research has classified two energized transport systems for Ca^{2+} efflux, e.g. (i) antiporters with high capacity but low affinity to exchange Ca^{2+} with a counter ion (e.g., H^+), (ii) pumps with low capacity but high affinity to suppress the magnitude and duration of Ca^{2+} increase in defence responses (Shigaki *et al.*, 2006; Spalding and Harper, 2011). On all major cellular membranes in *Arabidopsis*, 5 types of gene families have been identified as responsible for calcium efflux, including the mitochondrial calcium uniporter complex (MCUC), ER-type Ca^{2+}-ATPases (ECAs), autoinhibited Ca^{2+}-ATPases (ACAs), P1-ATPases (HMA1) and Ca^{2+} exchangers (CAX), leading to a rapid and efficient network to exclude Ca^{2+} from the cytosol (Bose *et al.*, 2011; Spalding and Harper, 2011; Pittman, 2011).

1.2.4 Cold signal transduction

1.2.4.1 Reactive oxygen species (ROS) formation in plant stress response

Reactive oxygen species (ROS) play a dual function in the plant life cycle. It is required in almost all biological processes *in vivo*, e.g., plant development and organogenesis, and it also serves as the signal molecule in plant responses to biotic or abiotic stress. On the other hand, oxidative stress caused by the production of ROS leads to irreversible DNA damage and even cell death. In plant

cells, ROS is classified into ionic and/or molecular forms, referring to hydroxyl radicals ($^{\bullet}OH$) and superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) respectively (Huang *et al.*, 2019). The mechanism of ROS regulation in intricate stress response is spatial-temporal, based on the type of ROS, cellular compartment of ROS generation as well as duration and intensity of ROS in cells (Castro *et al.*, 2021).

ROS compartmentalization

In the light, ROS are mainly derived from chloroplasts and peroxisomes in green plants, e.g., in chloroplasts, formation of singlet oxygen ($^1O_2$) at photosystem II (PSII), superoxide at PSI and PSII as byproducts, whereas the mitochondria exhibit the highest concentration of oxidized proteins (Asada, 2006; Pospíšil *et al.*, 2004; Bartoli *et al.*, 2004).

Hydroxyl radicals ($^{\bullet}OH$) formed by the activity of intrinsic peroxidases are the most reactive ROS, which can react with all biological molecules, including oxidization of the cell wall polysaccharides, thus resulting in cell wall loosening, and induction of DNA single-strand breakage (Kärkönen and Kuchitsu, 2015; HIRAMOTO *et al.*, 1996; Zepeda-Jazo *et al.*, 2011). Superoxide anions ($O_2^{\bullet-}$), the other ionic ROS, are active with a strong redox capacity, acting as the precursor of other ROS. $O_2^{\bullet}$ is mainly generated in illuminated chloroplasts by three common pathways, e.g., photosynthetic electron transport chains, membrane-dependent NADPH oxidase (RESPIRATORY BURST OXIDASE HOMOLOG proteins (RBOHs)) and mitochondrial respiratory electron transport chains. Among them, $O_2^{\bullet}$ can interact with superoxide dismutase (SOD) to produce hydrogen peroxide (H_2O_2) (Scandalios, 1993; Mhamdi and Van Breusegem, 2018). H_2O_2 generated from chloroplasts and mitochondria is considered a crucial redox molecule, due to its extreme stability as well as rapid and reversible oxidization ability in plant cells (Mittler, 2017; Mhamdi and Van Breusegem, 2018). It can operate as a retrograde signal and also transport cell membrane-localized aquaporins to cause long-distance oxidative damage and regulate cell signalling e.g., interacting with transcription factors, thus affecting nuclear gene expression (Wudick *et al.*, 2015; Ng *et al.*, 2013; Bienert and Chaumont, 2014; Möller *et al.*, 2019). Moreover, H_2O_2 also plays a role in signalling by integrating with hormones, e.g., abscisic acid (ABA) (Desikan *et al.*, 2004b; Desikan *et al.*, 2004a). Singlet oxygen ($^1O_2$) triggers chloroplast-to-nucleus retrograde signalling, causing regulation of nuclear gene expression, stress response and even programmed cell death (Suzuki *et al.*, 2012; Castro *et al.*, 2021).

ROS perception

To fulfil the function of the signal molecule, ROS are probably recognized by redox modifications, which lead to the alteration of protein structures and/or localisations, or interactions with target proteins. ROS in apoplast can be perceived in the extracellular space via diffusion through plasma membrane-localized aquaporins or anion channels (Möller *et al.*, 2019). The oxidative modification of cysteines, methionine and tyrosine have been well-established and studied as the main

mechanism for ROS sensing. For ROS-sensitive proteins, the cysteines tend to be oxidized in the higher pH environment, which means cysteines in the cytosol are more easily oxidatively changed under non-stimuli conditions. However, a transient alkalization triggered by a change of external stimuli in the apoplast can promote the oxidization of cysteines by ROS (Felle, 2001; Geilfus, 2017). Cysteine-rich receptor-like kinases (CRKs) and receptor-like kinases (RLK) are the most studied candidates for ROS sensors, being proven that cysteine residues are modified by extracellular ROS facilitating the activation of calcium channels (Bourdais *et al.*, 2015; Kimura *et al.*, 2020; Tian *et al.*, 2019).

ROS production in cold stress

Cold sensing by microtubule depolymerization triggered by extreme temperature, results in the alteration of the fluidity of plasma membranes, which also subsequently activates calcium channels on the plasma membrane that facilitate the influx of Ca^{2+} (Wang *et al.*, 2020; Wang *et al.*, 2018). Furthermore, calcium influx activates downstream signalling including the generation of ROS and NO as well as ABA. Meanwhile, cold stress can also induce the expression and activity of ROS scavengers (Heidarvand and Amiri, 2010; Janská *et al.*, 2010). On the other hand, stomatal closure caused by cold stress is taken as another explanation for ROS accumulation under cold stress because it disrupts the balance in the Calvin-Benson-Bassham cycle, increasing photosynthetic electron flux to O_2 (Logan *et al.*, 2006; Hu *et al.*, 2008).

1.2.4.2 Mitogen-activated protein kinase (MAPK) cascades in plant signalling

Composition and classification of MAPKs

Mitogen-activated protein kinase (MAPK) cascade is an evolutionarily conserved module, referring to three classical kinases, MAP kinase kinase kinases (MAP3Ks/MAPKKKs/MEKKs), MAP kinase kinases (MAP2Ks/MAPKKs/MKKs/MEKs), and MAP kinases (MAPKs/MPKs) through sequential phosphorylation to integrate external stimuli with signal transduction in all eukaryotes (de Zelicourt *et al.*, 2016). In the *Arabidopsis* genome, there are 80 MAP3Ks, 10 MAP2Ks and 20 MAPKs, as compared to 85 MAP3Ks, 5 MAP2Ks and 16 MAPKs in tomato, 120 MAP3Ks, 9 MAP2Ks and 26 MAPKs in apple, as well as 62 MA3PKs, 5 MAP2Ks, and 14 MAPKs in grapevine (*Vitis vinifera*), however, in which few have been investigated (Colcombet and Hirt, 2008; Wu *et al.*, 2014a; Sun *et al.*, 2017; ÇAKIR AYDEMİR and KILIÇKAYA, 2015).

Plant MAPKs are classified into two subtypes based on the amino acid sequence of T-X-Y (T: threonine, Y: tyrosine, and X: any amino acid) motifs, TEY and plant-specific TDY subtype (Zhang and Zhang, 2018). The TEY subtype consists of three groups: (i) group A (*Arabidopsis* MPK3 and MPK6), (ii) group B (*Arabidopsis* MPK4, MPK5, MPK11, MPK12, and MPK13), those from two groups have been proven to play roles in plant biotic and abiotic stress responses, as well as plant growth and development, (iii) group C (*Arabidopsis* MPK1, MPK2, MPK7, and MPK14), which are mostly

unknown (Xu and Zhang, 2015; Devendrakumar *et al.*, 2018). The plant-specific TDY subtype known as group D has 8 MAPKs (MPK8, MPK9, MPK15, MPK16, MPK17, MPK18, MPK19, and MPK20), being distinct from TEY MAPKs, because of auto-phosphorylating its own threonine and tyrosine residues and no proteins identified as the upstream for their activation (Nagy *et al.*, 2015).

MAPKs cascade activation

MAP3Ks activated by stimulated plasma membrane receptors phosphorylate conserved serine/threonine in the S/T-X3-5-S/T motifs of MAP2Ks, as a consequence activated MAP2Ks phosphorylate both threonine and tyrosine residues of MAPKs on their T-X-Y motifs (Jonak *et al.*, 2002; Cristina *et al.*, 2010). At last, phosphorylated MAPKs interact with various downstream targets, e.g., kinases, transcription factors, enzymes and proteins to transmit and amplify environmental stimuli signals into cells (Zhang *et al.*, 2018).

Works on *Mekk1, mkk1/mkk2* mutants have confirmed the negative role of MEKK1-MKK1/2-MPK4 module in plant defence interplaying by enhanced salicylic acid level and ROS accumulation (Brodersen *et al.*, 2006; Kong *et al.*, 2012; Pitzschke *et al.*, 2009; Suarez-Rodriguez *et al.*, 2007). Furthermore, studies on the rapid and strong activation of MAPK3, 4 and 6 by flg22, together with the activation of MAPK4 and MAPK6 by harpin have supported their function in plant immunity (Suarez-Rodriguez *et al.*, 2007; Gao *et al.*, 2008; Takahashi *et al.*, 2007; Desikan *et al.*, 2001).

Abiotic stress has been proven to trigger at least two MAPK modules in *Arabidopsis*, including MEKK1-MEK1/MKK2-MPK4 and the other MAPK cascade with MPK6 and p44MAPK.21 (Ichimura *et al.*, 2000). In the salt or cold response, MEKK1 acts as the upstream of MKK2, which further activates downstream MPK4 and MPK6, while MKP1 interacting with MPK6 and MPK4 negatively regulates salt tolerance (Ulm *et al.*, 2002; Teige *et al.*, 2004). Besides, the role of MAPK in drought stress has been established by induction of MEKK1 and MPK3 in *Arabidopsis* and activation of OsMSRMK2 and OsMAPK5 in rice plants (Mizoguchi *et al.*, 1996; Agrawal *et al.*, 2002; Xiong and Yang, 2003). Under cold stress, phosphorylated MKK2 by MEKK1 activates downstream MPK4 and MPK6, playing a positive role in cold tolerance (Teige *et al.*, 2004; Yang *et al.*, 2010). Besides, in *Arabidopsis*, MPK3/MPK6 can phosphorylate and destabilize ICE1 activity in freezing stress with impaired freezing tolerance (Li *et al.*, 2017a).

ABA-activated MAPK signalling module

Recently, a new MAPK module MAP3K17/18-MKK3-MPK1/2/7/14 activated by abscisic acid (ABA) has received considerable attention. The abundant transcript and protein levels of MAP3K17 and MAP3K8 were only detected under ABA treatment with a slower response, which may be involved in the long-term stress response in plants (Danquah *et al.*, 2015). Besides, MPK9 and MPK12 are the positive redundant regulators of ABA signalling in guard cells (Salam *et al.*, 2012; Khokon *et al.*, 2015). The coherent evidence of the correlation between MAPK and ABA signalling in *Arabidopsis*

has been clarified as MAP3K17/18-MKK3-MPK1/2/7/14 Pathway. In this pathway, MAP3K17 and MAP3K18 upstream of MAP3Ks phosphorylate MKK3 and consequently active MPK1, MPK2, MPK7 and MPK14 in ABA-dependent responses under drought stress (Danquah *et al.*, 2015; Matsuoka *et al.*, 2015; Mitula *et al.*, 2015). Besides, PP2C phosphatase ABI1, as the core module in ABA signalling directly interacts with and stabilizes MAP3K18 (Mitula *et al.*, 2015).

1.2.4.3 CBF-dependent signalling pathway

CBF (C-repeat binding factor) transcript factors, also known as dehydration-responsive element-binding protein 1s (DREB1s), belonging to APETALA2/ethylene response element-binding family (AP2/EREBP) are critical in cold signalling by recognizing and binding to CCGAC cis-element of downstream cold responsive (COR) genes to confer cold tolerance in plants (Shi *et al.*, 2018). The activation of COR genes has been proven by ectopic expression of CBFs even at moderate temperatures, resulting in enhanced cold acclimation (Liu *et al.*, 1998; Stockinger *et al.*, 1997). In *Arabidopsis*, the functions of three members (CBF1, CBF2, and CBF3 (known as DREB1A, DREB1B, and DREB1C), tandemly arranged in an 8.7-kb region of chromosome 4 have been addressed by constructing mutant or overexpression of those proteins (Shi *et al.*, 2018). Triple-mutant *Arabidopsis* seedlings *cbf1,2,3* (*cbfs*) were conferred to be more freezing susceptible than single-mutants *cbf2*, *cbf3*, and double-mutant *cbf1,3*, indicating the essential role of CBFs in cold tolerance (Zhao *et al.*, 2016; Jia *et al.*, 2016). Moreover, CBFs driven by the cauliflower mosaic virus (CaMV) 35S promoter exhibited higher induction of stress target gens and stronger cold tolerance (Jaglo-Ottosen *et al.*, 1998; Hsieh *et al.*, 2002; Gilmour *et al.*, 2000; Oh *et al.*, 2005). Except for the regulation of downstream COR genes, CBFs are also involved in the regulation of other CBF members. For example, in *Arabidopsis* CBF2 can negatively regulate CBF1 and CBF3 to fine-tune the accurate induction of COR genes for cold hardiness and plant development under low temperatures (Novillo *et al.*, 2004; Novillo *et al.*, 2007). Bioinformatic analysis has shown the overlapping and distinct target genes for each CBF protein, indicating the functional diversity of CBF proteins (Zhao *et al.*, 2016; Jia *et al.*, 2016; Shi *et al.*, 2017).

There are up to six CBF paralogs in *Arabidopsis*, executing different functions via specificity of expression in tissue, response time and regulation pattern in response to various abiotic stresses (Shi *et al.*, 2017). Differing from CBF1-3, expression of CBF4 has been found to be induced by drought rather than cold stress, and overexpressing CBF4 in *Arabidopsis* activated downstream COR genes in both cold and drought stress (Haake *et al.*, 2002). On the other hand, stronger and more persistent induction of CBF4 was observed in grape plants in response to chilling stress but neither drought nor salt stress, as compared to the rapid induction of other CBF genes (Xiao *et al.*, 2008). Furthermore, overexpression of a *Populus euphratica* CBF4 gene in poplar has induced strong induction of COR and higher levels of proline and sugars for stress tolerance (Tian *et al.*, 2017). Ectopic expression of *Solanum tuberosum* CBF1(StCBF1) and CBF4 in *Arabidopsis* exhibits cold tolerance with activation of COR genes, while the CBF4 line is more tolerant than the CBF1

line under cold stress (Sun *et al.*, 2021).

The expression of CBF genes has been verified under sophisticated control by a MYC-like basic helix–loop–helix (bHLH) transcription factor ICE1 (inducer of CBF expression 1), which can bind to the MYC-binding sites (CANNTG) in CBF1-3 promoters, facilitating their expression in cold stress (Chinnusamy *et al.*, 2003). Given the impaired freezing tolerance and induction of CBF genes in *ice1* mutant coupling with elevated CBFs expression in overexpressing ICE1 line, the positive role of ICE1 in regulating CBF genes has been well studied (Chinnusamy *et al.*, 2003; Ding *et al.*, 2015a). In moderate temperatures, ICE1 is constitutively synthesized and also continuously degraded by osmotically responsive gene1 (HOS1) in the 26S proteasome-mediated manner (Dong *et al.*, 2006). Under low temperature, SIZ1 stabilizes ICE1 by sumoylation of it at Lys393 and reduces its poly-ubiquitination by HOS1 (Miura *et al.*, 2007). OST1, a Ser/Thr protein kinase can phosphorylate ICE1 at Ser278, preventing the degradation mediated by HOS1, thus enhancing freezing tolerance (Ding *et al.*, 2015a). Besides, the work about MAPK-involved cold response shows the negative regulation of CBF genes caused by MPK3/6-mediated phosphorylation of ICE1 at conserved Ser/Thr residues, while MPK4 negatively regulates MPK3/6 resulting in stabilized ICE1 transcriptional activity, thus enhances CBFs expression and cold tolerance (Li *et al.*, 2017a; Zhao *et al.*, 2017b).

Other than the regulation by ICE1, there are some other proteins independently regulating CBFs expression in *Arabidopsis*. For example, CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 3 (CAMTA3) rapidly induced the expression of CBFs and increased cold tolerance, while MYB15, EIN3 and PHYTOCHROME-INTERACTING FACTOR 3 (PIF3) have been shown negatively regulate CBF genes by binding to their promoters to repress their expression (Kim *et al.*, 2013; Kim *et al.*, 2017; Shi *et al.*, 2012; Lin *et al.*, 2018).

1.2.4.4 CBF-independent signalling pathway

Microarray analysis has shown that only 12% of COR genes in the *Arabidopsis* genome are CBF regulons, which means a large range of COR genes may be involved in the CBF-independent signalling pathway (An *et al.*, 2017). In *Arabidopsis*, the *hos9* mutant (HOS9, a homeodomain protein) exhibited lower cold tolerance coupling with the same induction of CBF genes when compared to wild type and distinct cold-activated downstream COR from CBF regulons (Zhu *et al.*, 2004). HOS10 (an R2R3-type MYB), may positively regulate cold response in the ABA-mediated manner (Zhu *et al.*, 2005). Ectopic overexpression of MYB4 (a R2R3-typeMYB), a cold-inducible transcription factor lead to enhanced expression of COR genes, proline levels as well as freezing tolerance (Vannini *et al.*, 2004; Agarwal *et al.*, 2020). Additionally, in rice, the cold-induced MYB3R-2 (an R1R2R3 MYB) was found to positively regulate cold tolerance in a CBF-independent pathway (Dai *et al.*, 2007). An AP2 family transcription factor RELATED TO ABI3/VP1 (RAV1) has been recently found to play a positive role in cold tolerance by improving membrane stability and regulating the expression of genes protecting the composition of the cell wall in plants (Ren *et al.*,

2021). In soybean, a C2H2-type zinc finger protein SCOF1 induced by cold stress was characterized to regulate the expression of COR genes, probably by enhancing the DNA binding activity of G-Box-binding factor 1 (Kim *et al.*, 2001).

To sophisticatedly trigger appropriate downstream molecular responses, the extracellular temperature drop, as physical stimulus has to be discriminated and sensed accurately, which requires a sufficient susception step (Wang *et al.*, 2020). Except for formation of a asymmetric mechanic force by membrane rigification, microtubules are one of potential structure for cold susception because of their structure, which allows cold signal to be integrated and transmitted as compression forces, resulting in changes in associated proteins such as microtuble-associated proteins (MAPs), kinesins, thus triggering downstream signaling (Nick, 2012).

1.3 Microtubules and kinesins

1.3.1 Microtubule structure and dynamic

In addition to actin filaments, one of the key constituents of the cytoskeleton in eukaryotic cells is the microtubule (MT), which is organized with α- and β-tubulin heterodimers by head-to-tail to form filaments (Wade and Hyman, 1997). α- and β-tubulins are highly homologous proteins with about 450 amino acids each (about 50 kDa), binding a guanine nucleotide, which is nonexchangeable in α-tubulin (N-site) and exchangeable in β-tubulin (E-site) (Downing and Nogales, 1998). In a protofilament, one end has an exposed α-subunit designated as the minus (-) end, while the other end is the plus (+) end with an exposed β-subunit (Wade and Hyman, 1997). In the end, 13 protofilaments parallel to one another with the same polarity to form a hollow tube.

Microtubules execute continuously rapid cycles of assembly and disassembly by GTP hydrolysis at the E-site of β-tubulin monomer to the addition of a tubulin dimer to the microtubule end, where it forms a stabilizing cap (Mandelkow and Mandelkow, 1994). When the stabilizing cap is lost, the microtubule rapidly shrinks, which is known as a catastrophe. When the rapid shrinking of MTs is rescued, the MTs turn back to growth. This nature of the microtubules is known as MTs dynamic instability (Heald and Nogales, 2002). The plus end is more dynamic with quick growth and shrinkage, while the minus end is protected from depolymerization (Ambrose and Wasteneys, 2014; Goodwin and Vale, 2010).

MTs stability is determined by various elements, such as microtubule-associated proteins (MAPs). Among them, the classical MAPs like MAP2 and Tua. They can bind to and probably neutralize the repulsive negative charge on the MTs surface (Heald and Nogales, 2002). Some MAPs such as the highly conserved XMAP215/Stu2p/TOG family, are thought to be enriched on a subset of the

spindle but not astral MTs (Gadde and Heald, 2004). CLIP-170 and EB1 known as microtubule-end binding MAPs, are involved in the following events: (1) copolymerization with new tubulin subunits; (2) selectively binding to the specific form of the microtubule ends; (3) interaction with other proteins like CLASPs and APC to serve as the attachments for growing or shortening microtubules to cellular membranes or kinetochores; (4) releasing microtubules from the centrosome (McNally, 2000; Gadde and Heald, 2004). In addition to the role of conventional MAPs in MTs stability, the lissencephaly protein LIS1 has been found that bind to the dynein-dynactin motor complex to microtubule ends involved in MTs stability (Huang *et al.*, 2012; Li *et al.*, 2005; Smith *et al.*, 2000).

Besides, microtubules as rigid polar polymers also serves as the track for the motors, the dynein and kinesin superfamilies, which drive cargo transport along microtubules using the energy from ATP hydrolysis (Ross *et al.*, 2008). The numerous motors move along the complex cytoskeleton with network of microtubule and actin filament intersections, maneuvering various cellular processed.

1.3.2 Cytoskeletal motor proteins

The cellular cytoskeleton is widely accepted as a highly dynamic and complex network, consisting of diverse cross-linked biopolymers, which fulfil multiple essential intercellular processes, such as serving as the scaffold for motor proteins, adjusting cell shape and spatial organization (Hafner *et al.*, 2016). Actin filaments together with microtubules (MTs) contribute to the formation of the dynamic tracks for intracellular transport driven by molecular motor proteins including kinesins, dyneins and myosins (Appert-Rolland *et al.*, 2015). Myosins are a superfamily of actin motor proteins moving towards the plus or minus direction of actin filaments, while kinesins are mostly plus-end motors as compared to minus-directed dynein motors (Hafner *et al.*, 2016).

1.3.2.1 Myosin-actin motors

To date, the myosin motors have been categorized into 35 classes by phylogenetic analysis of their heavy chains (Odronitz and Kollmar, 2007). Myosin-I consisting of a motor domain, the light chainbinding domains (LCBD), and a tail domain, is the second-largest myosin family in humans with eight isoforms, widely expressed in vertebrates, playing crucial roles in many cellular processes, such as regulation of actin dynamics, tension sensing, membrane morphology and trafficking as well as nuclear transcription (Greenberg and Ostap, 2013). Myosin II is responsible for the generation of cytoskeletal tension (Fernandez-Gonzalez *et al.*, 2009). Besides, myosin V is the best-understood myosin family for the cargo-transporting mechanism (Figure. 1). The ADP releasing from the trailing head of myosin leads to this head subsequently binding of ATP and rapid dissociating, while the other head forms a power stroke, positioning the new leading head, which can rapidly release Pi and generate a strong binding conformation. Therefore changing myosin V back to the previous state while moving forward by 36 nm (Hammer and Sellers, 2012). Notably,

The class VI myosins is a unique myosin motor family from all other myosin families, moving vesicles out towards the cell periphery, whereas myosin VI more likely brings materials or

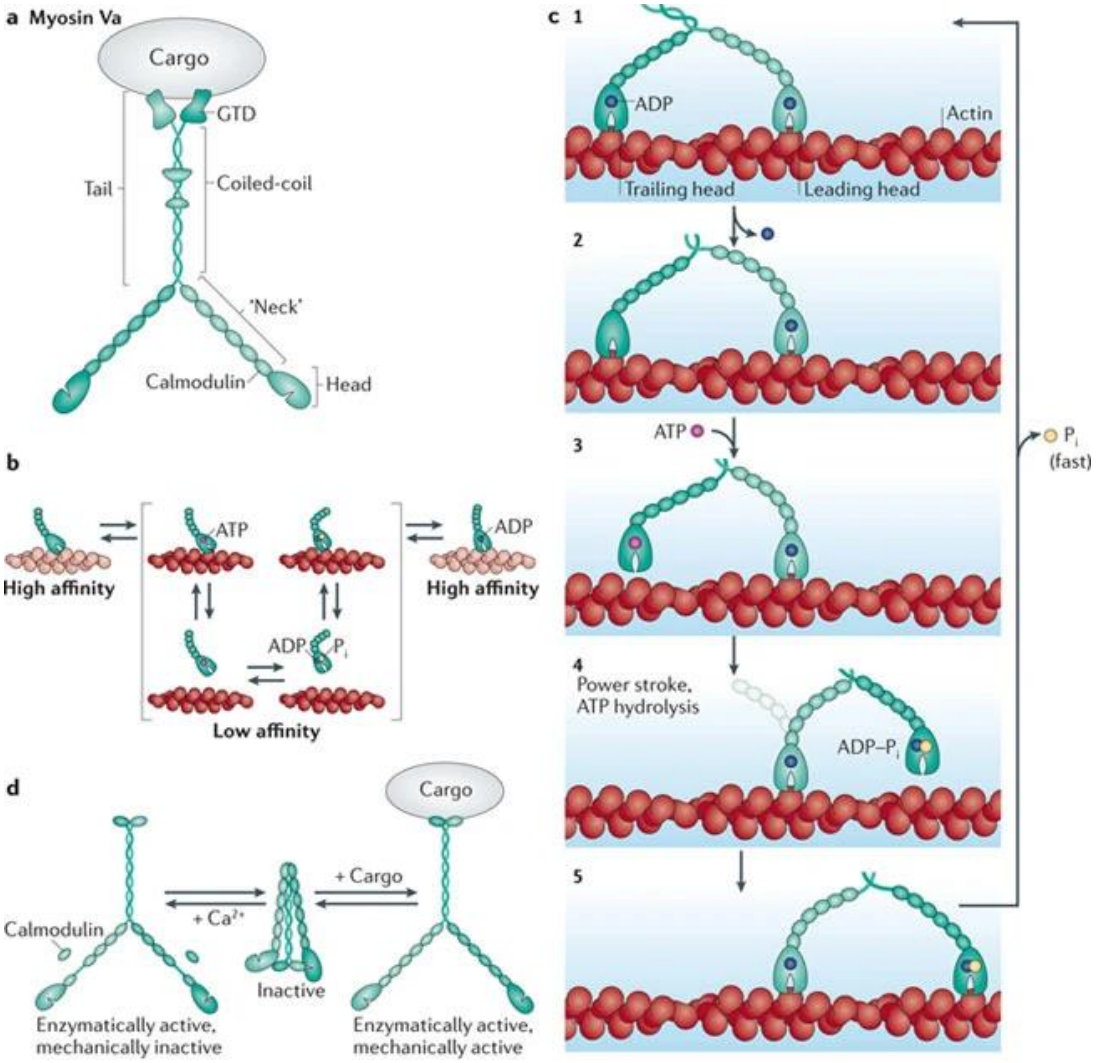

Figure. 1 Principle of myosin V moving along microtubules and transporting cargoes. Image source: Hammer, J., Sellers, J. Walking to work: roles for class V myosins as cargo transporters. Nat Rev Mol Cell Biol 13, 13–26 (2012). https://doi.org/10.1038/nrm3248

membranes into the cell. Numerous cellular functions require a subset of myosin superfamily members to traffic in the reverse direction (Titus, 2000; Sweeney and Houdusse, 2010).

1.3.2.2 Microtubule-based motors

Dynein-missing in the plant kingdom

Dynein is a robust microtubular minus-end-directed motor, walking rapidly and generating forces which are comparable to plus-end-directed kinesin towards the cell centre (Canty and Yildiz, 2020). The heavy chain of dynein is comprised of two identical motor domains together with a hexameric AAA ring, consisting of the stalk, buttress (or the strut), and linker (Bhabha *et al.*, 2016). The binding of ATP triggers a series of conformational changes around the entire ring, and rearranges all of the

AAA domains into a closed ring conformation (Roberts *et al.*, 2012). However, on its own, dynein remains in an inactive conformation, motility is activated when the complexes with dynactin and a cargo adaptor are formed (Lu and Gelfand, 2017). Compared to myosin V and kinesin, one head of dynein takes a step forward to a new tubulin-binding site, while the other trailing head remains bound in place (Figure. 2) (Bhabha *et al.*, 2016). In addition to transporting cargoes into cells, dyneins have been found to have a diversity of intracellular functions, referring to tethering and stabilizing microtubules plus ends, facilitating nuclear envelop breakdown and mitochondrial re-localization (Hendricks *et al.*, 2012; Salina *et al.*, 2002; Sánchez-Madrid and Serrador, 2007).

Kinesin

Kinesin superfamily proteins（KIFs）are involved in mitosis and directional transport by recognizing and binding to multiple cargos (e,g,. vesicles, organelles, protein complexes and mRNAs and membrane-associated complexes) (Hirokawa and Noda, 2008). There are 45 genes encoding KIFs in the mouse and human genome, which may be facilitated by two isoforms being generated by alternative splicing (Miki *et al.*, 2001). According to the phylogenetic analyses, those KIFs constitute 15 distinct families, from kinesin 1 to 14B (Figure. 2a), categorized as three classes

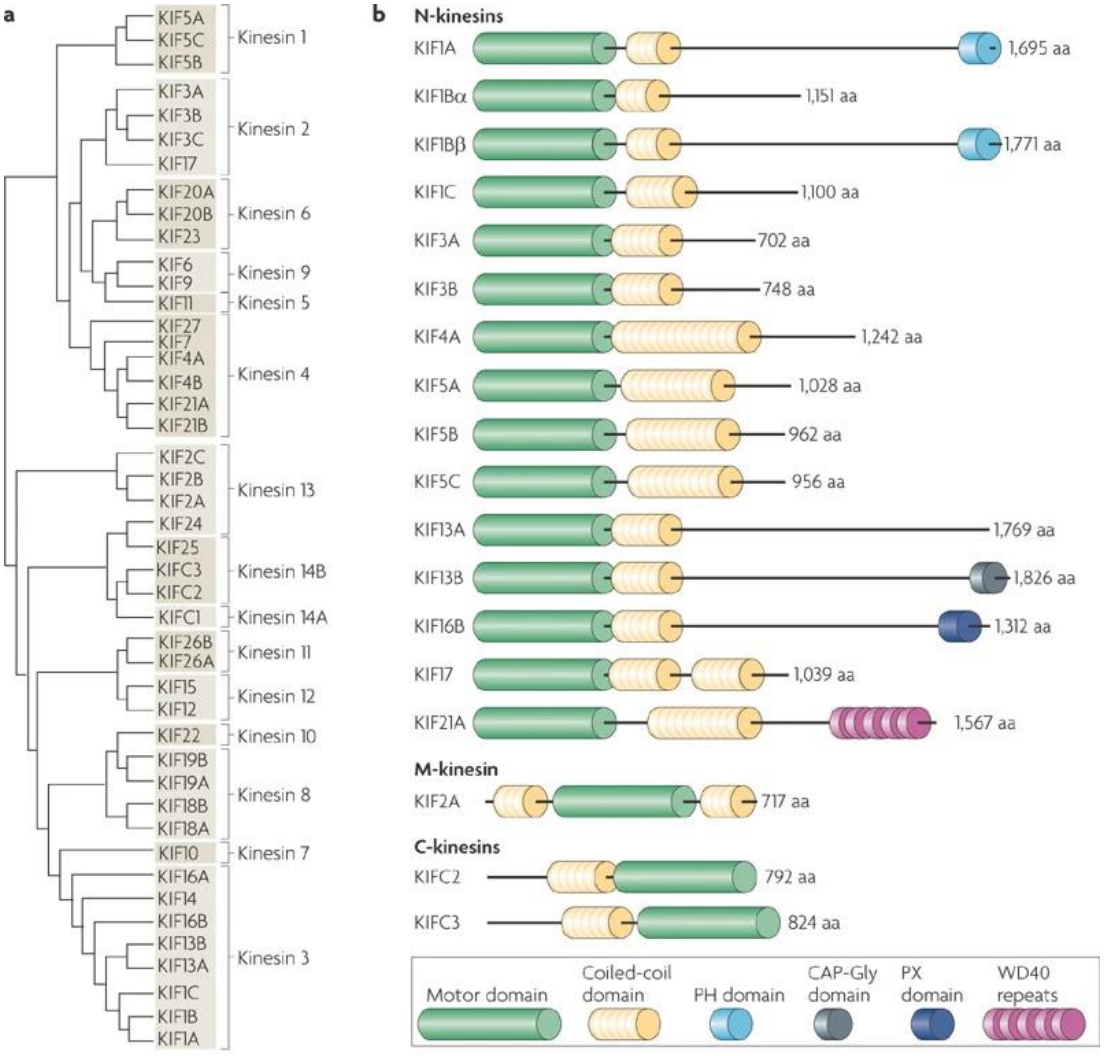

Figure. 2 The phylogenetic analysis and structure of major kinesins in mice. Image source: Hirokawa, N., Noda, Y., Tanaka, Y. et al. Kinesin superfamily motor proteins and intracellular transport. Nat Rev Mol Cell Biol 10, 682–696 (2009). https://doi.org/10.1038/nrm2774.

by the position of the motor domain in the molecule (Figure. 2b): (1) N-terminal kinesins show a motor domain in the amino-terminal region, moving towards to plus end of the MTs, (2) C-terminal kinesins have a motor domain in the carboxy-terminal region, moving to minus end, (3) M-kinesins owning a motor domain in the middle, depolymerizing the MTs (Hirokawa *et al.*, 2009). Among the 45 kinesins, the human genome has only three C-terminal and three M kinesins, the rest are N-terminal kinesins (Miki *et al.*, 2001).

Conventional kinesin is comprised of two monomers, which include an N-terminal motor head, a neck linker, a coiled-coil region and a globular tail domain in each monomer (Kozielski *et al.*, 1997). The coiled-coil regions of two monomers interwind with each other, forming a common stalk (Bloom *et al.*, 1988). The neck linkers are the mechanical element, which can execute nucleotide-dependent conformational changes, generating a power stroke and determining the directionality of kinesin movement (Rice *et al.*, 1999; Sablin *et al.*, 1998; Endow and Waligora, 1998; Case *et al.*, 1997). The motor domains play a role in binding to the MTs and nucleotides (Scholey *et al.*, 1989; Bloom *et al.*, 1988). The kinesin light chain (KLC) in the tail region modulates the affinity of kinesin and cargoes (Bloom *et al.*, 1988). In the inactive state, the C-terminal region of KHC interacts with the neck region, inhibiting its ATPase activity (Woźniak and Allan, 2006). Previous studies on kinesin 1 have indicated the role of KLC in inhibiting the function of the inactive KHC (Verhey *et al.*, 1998). The loss of the KLC function has been shown to impair axonal transport, cause severe neuron defects and even death (Rahman *et al.*, 1999; Stenoien and Brady, 1997).

1.3.3 Plant kinesins

The genes encoding kinesins in flowering plants have been expanded, for example, *Arabidopsis* has 61 compared to 41 in rice, while several families are missing in higher plants (Reddy and Day, 2001; Richardson *et al.*, 2006). In the plant kingdom, except for plant kinesin-5 and kinesin-14 subfamilies, most kinesins in the plant kingdom evolutionarily differ from animal kinesins (Li *et al.*, 2012).

The kinesin family 1 is the first reported kinesin, involved in organelle transport and nuclear movement (Holzinger and Lütz-Meindl, 2002; Pilling *et al.*, 2006). Compared to animal KHCs, kinesin family 1 from plants and fungi lacks the light-chain-binding domains (LCBDs), but contains the domains compromising the function of cargo binding such as a transmembrane domain or ARM-repeat domain (Miki *et al.*, 2005). The kinesin-1 family has been thought to deputize the function of the absent Kinesin-2 family in higher plants. Besides, the kinesin-3, kinesin-6, kinesin-9 and kinesin-11 families are missing in higher plants, perhaps because in plants their functions

somehow have been taken over by the actin-based motor proteins myosin or other motors (Ding *et al.*, 2019). The kinesin-4 family exhibits more family members in higher vertebrates than in plants while plant harbours much more members of the kinesin-5, -7 and -14B families rather than animals (Miki *et al.*, 2005). The kinesin-5 family is the most conserved, functioning as the formation of a bipolar spindle in mitosis (Mann and Wadsworth, 2019). The members of kinesin-7 (15 in *Arabidopsis* and 6 in rice) with family-specific long necks localize to the cytokinetic apparatus and mitochondria in plants, differing from animal kinesin-7 members that capture spindle microtubules at kinetochore sites (Nishihama *et al.*, 2002; Strompen *et al.*, 2002; Miki *et al.*, 2005). Kinesin-8 motors participate in controlling spindle length and regulating chromosomal movements in the length-dependent microtubule depolymerization manner (Varga *et al.*, 2009; Locke *et al.*, 2017). The kinesin-10 family is predicted to directly bind to chromosomes, mediating chromosomal movements in cell division (Tokai *et al.*, 1996). In addition to traditional function, member of kinesin-11 Kif11 interacts with ribosomes to enhance translation, while Smy1 doesn't bind to MTs (Lwin *et al.*, 2016; Pei *et al.*, 2017). In plants, kinesin-12 members are involved in various developmental processes, such as the male gametophyte, development of the embryo, seedling- and seed development, in contrast to the crucial role in the spindle assembly in animals (Müller and Livanos, 2019). Unlike most other kinesins translocating along the microtubule lattice, kinesin-13 family concentrates at microtubule ends and induces MTs depolymerization *in vitro* (Mennella *et al.*, 2005). It has been shown to localize to entire Golgi stacks in cotton and to be involved in Golgi-associated vesicles in *Arabidopsis* (Lu *et al.*, 2005; Wei *et al.*, 2009). Besides, six kinesin families (Kinesin-1, Kinesin-3, Kinesin-5, Kinesin-8, Kinesin-11, Kinesin-13) in mammals have been proven to interplay with the MAPK pathway (Liang and Yang, 2019).

1.3.4 The minus-end kinesin-14 motors in plants

Most kinesins are microtubule-based plus-end motors, whereas kinesin-14 members are the reverse of typical N-terminal KIFs, conferring reverse motility, while they could substitute the function of dynein in minus end-directed membrane trafficking in animal cells (Vale, 2003). Certain members of the plant kinesin-14 family harbour domains, which are absent in non-plant counterparts, indicating the plant-specific function of this family (Richardson *et al.*, 2006). There are 21 predicted kinesin-14 members in *Arabidopsis*, only a few of which are minus end-directed motors including Kinesin-like calmodulin-binding protein (KCBP), ATK1 (KatA), ATK5, calponin-homology domain 1 (KCH), while motor domains in the bulk of kinesin-14 are either near the N-terminus or in the middle of the molecule (Reddy and Day, 2001; Zhu and Dixit, 2012).

A plant-specific kinesin-14 member Kinesin-like calmodulin-binding protein (KCBP) possesses a second MT binding site in the N-terminal tail domain, which is ATP-independent, contributing to the stabilization of cortical MTs (Kao *et al.*, 2000). Besides, KCBP also guides the phragmoplast to

the cortical division site (Buschmann *et al.*, 2015). Loss of ATK5 leads to impaired formation of early, indicating the role in promoting spindle organization and integrity (Ambrose and Cyr, 2007). Moreover, ATK5 may not play a critical role in cortical MTs organization proven by normal cortical MTs organization in atk5 mutant (Ambrose *et al.*, 2005). ATK1 is a non-processive motor, participating in force generation in the spindle during meiosis (Marcus *et al.*, 2002). OsKCH1 interplays with cortical microtubules and actin microfilaments during nuclear positioning (Frey *et al.*, 2010). Of other members of the kinesin-14 family, AtKP1 involves itself in mitochondrial movement, and KCA1 and KCA2 are responsible for chloroplast movement (Zhu and Dixit, 2012).

Interestingly, recent work on the rice homologues of *Arabidopsis* ATK1 and ATK5 (OsDLK)exhibit a conventional cortical MTs localization, however, shifts into the nucleus during the interphase of mitosis (Xu *et al.*, 2018a). The nuclear localization of OsDLK was also proven in various systems, including the rice leaf sheath, *Arabidopsis thaliana* protoplasts and *Nicotiana benthamiana* leaves (Xu *et al.*, 2018a). Besides, the OsDLK was shifted into nucleaus in response to chilling and accumulated by prolonged cold, which specifically modulates tobacco CBF4 expression by probably binding to its promoter, indicating the link between this kinesin-14 protein and cold signaling (Xu *et al.*, 2022).

1.4 The scope of this study

Although it has been a while to classify the cold stress into two signalling, freezing and chilling, the distinction of those has been mainly compared in two typical models, rice, which is susceptible to mild cold stress for chilling study, and *Arabidopsis* as chilling resistant species to investigate freezing response. And exstincting researches have recognised the critical role played by CBF1-3 in the rapid response of plants to either chilling or/and freezing stress, however, some plants possess CBF proteins, such as up to six CBF paralogs in *Arabidopsis*. Previous work from our lab found a correlation between the expression of grapevine CBF4 and the acclimation to cold stress (Wang *et al.*, 2019). To test, whether this correlation derives from a causal relationship, we generated transgenic lines in the cellular model tobacco BY-2 that overexpressed CBF4 from the grapevine variety Pinot Noir in fusion with GFP to test, whether these cells show constitutive cold tolerance. Moreover, the difference of chilling and freezing response has been compared in tobacco background, including tobacco endogenous CBFs, downstream Cold-reponsive (COR) genes (the chaperone Early Responsive to Dehydration 10) as well as CBF4-dependent MAPK pathway, ROS and calcium influx. This direct comparison of chilling and freezing in the same model gives evidence that the mechanisms of cold signalling differ depending on the physiological context of the respective stress condition.

In addition, a mysterious rice Kinesin-14 *OsDLK* with minus-end direction exhibited two subcellular

locations during the interphase and correlated with the expression of the tobacco homologue of CBF4, indicating the link between this kinesin and regulation of cold-induced genes. To further confirm the authenticity of these phenomena, the heterologous system was generated by stably overexpressed *Vitis* homologue of *OsDLK*, *F6HRV2*, fused with GFP at the N-terminal and transformed into the tobacco BY-2 cell line. The subcellular localization of this kinesin was observed, unexpectedly showing two sub-populations with one being in the nucleus and the other in the nucleus as well as the MTs. Thus, this kinesin was named *vitis* the dual localization kinesin (*VvDLK*), and D-N for nuclear subpopulation, D-M for localization with nucleus and MTs. To understand the reasons for these two different localizations, the mRNA levels and protein expression were addressed in both sub-populations. Moreover, the opposite cold response was observed in the two sub-populations, D-N as the cold-tolerant phenotype and the cold-sensitive D-M. The difference of cold-induced CBF4 and COR was found between D-N and D-M in order to uncover the relationship between this kinesin and cold signalling.

2. Materials and methods

2.1 Establishment of transgenic cell lines

2.1.1 Overexpression of *VvCBF4* fused with GFP via GATEWAY CLONING

Leaves of grapevine (*Vitis vinifera* L. cv. Pinot Noir, clone PN40024, used for the reference genome (Jaillon et al., 2007)) were collected after exposure to 4℃ for 6 h. Total RNA was extracted using a commercial RNA isolation kit (Sigma Aldrich, Deisenhofen, Germany), and reversely transcribed into cDNA from 1 µg of mRNA template using the M-MuLV cDNA Synthesis Kit (New England Biolabs, Frankfurt am Main, Germany) according to the protocol of the manufacturer. Full-length *VvCBF4* (GenBank KF582403.1) was amplified with the modified oligonucleotide primers listed in **Supplementary Table S1** for subsequent integration into the GATEWAY pDONR/Zeo vector system (Invitrogen) using initial denaturation for 3 min at 98°C, and 30 cycles of denaturation for 30s at 98°C, annealing for 30s at 55°C, and elongation for 1 min at 72°C, with a terminal elongation step for 4 min at 72°C employing a proofreading Q5 polymerase (New England Biolabs, Germany). After verification of the amplicon sequence, the insert was then transferred into the binary vector pK7WGF2.0 making use of the recombinase function of the Gateway system (BP clonase II mix, Invitrogen Corporation, Paisley, UK). This construct allows for the expression of *VvCBF4* as a fusion with the green fluorescent protein (GFP) at the C-terminus driven by the CaMV 35S promoter. The fusion construct was introduced into suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells using a strategy based on *Agrobacterium tumefaciens* (strain LBA4404) according to (Klotz and Nick, 2012). After co-cultivation for 3 d, droplets of the cell suspension were transferred on selective plates with 100 mg/L cefotaxime and 100 mg/L kanamycin. After four weeks of selection in the dark at 25°C, cells from a single growing callus were transferred into a liquid BY-2 medium (see below) and then replated and re-picked on selective plates three times before being used for the experiments.

2.2.1 Overexpression of *VvDLK* fused with GFP via GATEWAY CLONING

Total RNA from leaves of grapevine (*Vitis vinifera* L. cv. Pinot Noir, clone PN40024, used for the reference genome (Jaillon et al., 2007)) was extracted using a commercial RNA isolation kit (Sigma Aldrich, Deisenhofen, Germany), and reversely transcribed into cDNA from 1 µg of mRNA template using the M-MuLV cDNA Synthesis Kit (New England Biolabs, Frankfurt am Main, Germany)

according to the protocol of the manufacturer. Full-length *VvDLK* (UniProtKB/Swiss-Prot accession no. F6HRV2) was amplified with the modified oligonucleotide primers listed in Supplementary Table S1 for subsequent integration into the GATEWAY pDONR/Zeo vector system (Invitrogen) using denaturation for 3 min at 98°C, and 30 cycles of denaturation for 30 s at 98°C, annealing for 30 s at 55°C, and elongation for 1 min at 72°C, with a terminal elongation step for 4 min at 72°C employing a proofreading Q5 polymerase (New England Biolabs, Germany). After verification of the amplicon sequence, the insert was then transferred into the binary vector pK7WGF2.0 making use of the recombinase function of the Gateway system (BP clonase II mix, Invitrogen Corporation, Paisley, UK). This construct allows for the expression of *VvDLK* as a fusion with the green fluorescent protein (GFP) at the N-terminus driven by the CaMV 35S promoter. The fusion construct was introduced into suspension-cultured tobacco (Nicotiana tabacum) BY-2 cells using a strategy based on Agrobacterium tumefaciens (strain LBA4404) according to (Klotz and Nick, 2012). After co-cultivation for 3 d, droplets of the cell suspension were transferred onto selective plates with 100 mg/L cefotaxime and 100 mg/L kanamycin. After four weeks of selection in the dark at 25°C, cells from a single growing callus were transferred into a liquid BY-2 medium (see below) and then replated and re-picked on selective plates three times before being used for the experiments.

2.2 Cell culture

The suspension cells were cultivated weekly by transferring 1.5 ml to 100ml Erlenmeyer flask with a liquid medium containing 4.3 g/L Murashige and Skoog salts (Duchefa, Haarlem, the Netherlands), 30 g/L sucrose, 200 mg/L KH_2PO_4, 100 mg/L inositol, 1 mg/L thiamine, and 0.2mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 50 mg/L kanamycin at pH 5.8. Wildtype BY-2 cells were cultivated in the medium without kanamycin. Cells were shaken on an orbital shaker (KS260, IKA Labortechnik, Germany) at 150 rpm in the dark at 27 °C. The subpopulation D-M was subcultured every two weeks in the same MS medium with 50 mg/L kanamycin under the same condition.

2.3 Cold treatments

Freezing treatment (0℃) was administered on day 5 after sub-cultivation by placing the entire Erlenmeyer flask containing the cell suspension in an ice-water mixture on an orbital shaker (KS260, IKA Labortechnik, Germany) at 150 rpm in the dark. To administer a chilling treatment (4℃), cells were kept in a cold room, again on an orbital shaker. If not stated otherwise, the cells remained exposed to the cold treatment for 24 h.

2.4 Evans blue assay

Cells under freezing and chilling treatment were collected every 24h to continuously monitor cell mortality. 200µl cells were transferred into a mesh-like fixed with Polyamide fabric membrane (PA-41/31, Franz Eckert GmbH, Germany) and put on the tissue paper to remove the medium. Then the chambers were incubated in 2.5% (w/v) Evans Blue for 5 min followed by 5 times washing with distilled water. The dead cells were counted via microscopy by bright-field illumination. Data from each treatment at one time point were based on at least 4 independent experiments with more than 500 cells.

2.5 Measurement of cytosolic calcium

Extracellular alkalinization. The changes in extracellular pH were measured by a pH meter (pH 12, Schott Handylab) with a pH electrode (LoT 403-M8-S7/120, Mettler Toledo). The cell suspension (4 mL) was pre-equilibrated on a shaker at 25°C in the dark for 1 h, before administering cold stress.

Intracellular distribution of calcium via the fluorescent dye chloro-tetracycline. The cells were sampled after the respective treatment and then transferred into a mesh-like device for removing the medium. The filtered cells were fixed in 2.5% glutaraldehyde in 200 mM sodium phosphate buffer (pH 7.4) for 15 min. The fixative was washed out in three rounds (each 5 min) with staining buffer (50 mM Tris-HCl, pH 7.45), and excess liquid was carefully drained out by filter paper, before staining for 5 min with 100 µM chlorotetracycline. Unbound dye was washed out twice for 2 min, and cells were directly analysed by spinning-disc confocal microscopy. The green fluorescence was recorded by spinning disc confocal microscopy upon excitation with the 488-nm line of an Ar-Kr laser (Zeiss) and collecting the green emission. For evaluation, mean fluorescence intensity was quantified using ImageJ. Concerning, the GFP emission of the VvCBF4ox line, Data was calculated by the fluorescence intensity of each time point minus the mean of the initial fluorescence intensity from the untreated VvCBF4ox line.

2.6 Pharmacological treatments

Inhibition of transcription and translation. To test for the role of transcription in the regulation of CBF4, the inhibitor Actinomycin D (AMD, Sigma-Aldrich, Deisenhofen, Germany) was used, which intercalates into the DNA and, thus, preventing transcriptional elongation by polymerase I (for review see (Bensaude, 2011)). To address the role of translation, we used the protein synthesis inhibitor cycloheximide (CHX, Sigma-Aldrich, Deisenhofen, Germany). CHX inhibits eEF2-mediated translocation in eukaryotic ribosomes (Schneider-Poetsch et al., 2010). Both, *VvCBF4* overexpressor,

and non-transformed BY-2 wildtype cells were pre-treated for 2 h with either 100 µg/ml CHX or 50 µg/ml AMD under shaking at 150 rpm in the dark, before transfer to 0℃ and 4℃ for subsequent 24 h. As solvent control for AMD, cells were treated with 2.5% ethanol. Cells were sampled directly after the pre-treatment, and cells treated with cold only without inhibitor pre-treatment were included as additional controls.

Manipulation of cold signalling molecules. To address the role of the NADPH oxidase Respiratory burst oxidase Homologue (RboH) in cold signalling, cells were pre-treated with 10 µM of Diphenyleneiodonium (DPI), an inhibitor of the NADPH oxidase Respiratory burst oxidase Homologue (Eggenberger et al., 2017). The MAPK cascade was assessed by pre-treatment with 100 µM of the MAP kinase kinase blocker PD98059 (Zhang et al., 2006). The cold-induced transcription factor cascade is under the control of the regulator Inducer of CBF expression 1 (ICE1), a protein that is rapidly generated and broken down under room temperature. In contrast, this protein is accumulating in the cold, because its proteolysis in the proteasome is blocked. To address this, we used 100 µM of MG132, a specific inhibitor of the proteasome (Jang et al., 2005). All three inhibitors were purchased from (Sigma-Aldrich, Deisenhofen, Germany), and dissolved from stock solutions in DMSO. They were administered 30 min prior to the cold treatment, and the cells were incubated at 27℃ on an orbital shaker (KS260, IKA Labortechnik, Germany) at 150 rpm in the dark. After pre-treatment, cells were transferred to 0℃ and 4℃ for 24 h on the shaker in the dark at 150 rpm. As solvent control, cells were treated with 1% (v/v) DMSO, corresponding to the solvent concentration in the inhibitor treatments. Additionally, cells without pharmacological treatment under 0℃ and 4℃ for 24 h were added as references.

Inhibition of 14-3-3σ activity by BV02. To address the function of upstream translocating proteins 14-3-3 on CBF4 activation, BV02 was used to inhibit 14-3-3 protein-protein interactions between 14-3-3σ and c-AbI *in vivo*. The cells were pre-treated for 1h with 5 µM BV02 diluted in distilled water under shaking at 150 rpm in the dark before challenged with 0℃ or 4℃ for 24 h on the shaker in the dark at 150 rpm. Cells were sampled directly after the pre-treatment, and cells treated with cold only without inhibitor pre-treatment were included as additional controls.

Inhibition of nuclear import by GTP-γ-S. To support the molecular function of CBF4 in cold signalling achieved by translocating to the nucleus, the non-hydrolysable GTP analogue GTP-γ-S was used to block the nuclear import of the VvCBF4ox line. Cells after pre-treatment with 500 µM GTP-γ-S dissolved in distilled water for 30 minutes at room temperature were cultivated under cold conditions (0℃ or 4℃) for the following 24 h on the shaker in the dark at 150 rpm. And cells without pharmacological treatment under 0℃ and 4℃ for 24 h were taken as references.

Inhibition of Jasmonate biosynthesis by phenidone. Previous work has revealed several Jasmonate-ZIM domain (JAZ) proteins, the repressors of jasmonate signalling interact with ICE1 and suppress its transcriptional activity, thus negatively regulating cold tolerance (Hu et al., 2013).

To address the positive role of ICE1 in CBF4 expression, the 1-phenylpyrazolidinone (phenidone) was recruited to inhibit jasmonate biosynthesis. Cells were pre-treated with 2 mM phenidone at room temperature for 30 minutes on the shaker in the dark at 150 rpm, and transferred into cold conditions (0℃ or 4℃) for the following 24 h. As solvent control, cells were treated with the same volume of ethanol, corresponding to the solvent concentration in the inhibitor treatments. Additionally, cells without pharmacological treatment under 0℃ and 4℃ for 24 h were added as references.

2.7 Measurement of mitochondrial superoxide

MitoSOX™ Red (Thermo Fisher, M36008) is a novel fluorogenic dye, which provides a way to follow the accumulation of mitochondrial-derived superoxides in vivo. Cells were incubated with 5 μM MitoSOX™ reagent for 10 min in the dark, then gently washed three times with PBS buffer. The fluorescence intensity of cells exposed to 0℃ and 4℃ was recorded for a time-course by spinning disc confocal microscopy after excitation by the 561-nm emission line of an Ar-Kr laser (Zeiss). Images were processed, and the mean fluorescence intensity of the entire image was analyzed using ImageJ.

2.8 Quantification of CBF4, DLK transcripts in grapevine leaves

To get insight into the functional context of CBF4 induction, we used a series of grapevine species differing in their stress tolerance. These included the cold-tolerant *V. amurensis* (KIT-voucher 6540), the cold-sensitive *V. coignetiae* (KIT-voucher 6542), and *Vitis vinifera* L. cv. Pinot Noir (clone PN40024 that had been used for the reference genome (Jaillon et al., 2007)) from the germplasm collection established in the Botanical Garden of the Karlsruhe Institute of Technology. Plantlets were propagated clonally from wood cuttings and were used for this experiment at the age of 10 weeks. They were transferred either to a chilling treatment (in a cold room at 4℃) or subjected to a freezing chamber (-18℃) for specific time intervals (0, 1, 3, 6, 12 and 24 h). Then the leaves were immediately frozen in liquid nitrogen and the samples were stored at -80 °C for further investigation. The *Vvactin* gene was selected as the reference gene, while primers of the CBF4 gene for different genotypes were listed in **Supplementary Table S1**.

2.9 RNA extraction and cDNA synthesis

Total RNA was extracted from BY-2 suspension cells using the innuPREP Plant RNA Kit (Analytik Jena, Jena, Germany). For extraction from leaves, the Spectrum TM Plant Total RNA Kit (Sigma, Germany) was used. DNase (Qiagen, Hilden, Germany) was utilized to remove DNA contamination from samples. The quality and quantity of RNA extracts were analyzed and measured by 1% agarose gel and Nanodrop device, respectively. Complementary DNA (cDNA) was synthesized from the 1 ug RNA template by using M-MuLV Reverse Transcriptase (New England Biolabs, Frankfurt, Germany).

2.10 Quantitative real-time PCR (RT-qPCR) analysis

The RT-qPCR was performed by using a CFX96TM real-time PCR cycler (Bio-RAD, USA) as described in previous work (Wang et al., 2019). The details including the sequence of the oligonucleotide primers are listed in Supplementary Table S1. Relative expression in cell cultures was calculated with the 2−ΔΔCt method based on L25 as the reference gene (Livak and Schmittgen, 2001) and normalized against the value of L25 in the non-transformed BY-2 WT prior to the cold treatment (0 h value). As for the CBF4 relative expression change, data was compared to the 0 h value in the OE-CBF4 line prior to the cold treatment. While CBF4 expression in grapevine leaves was shown as log2 (2−ΔΔCt), normalized to the expression level of actin in Pinot Noir under room temperature (0 h value). As for VvDLK relative expression change, data was compared to the 0 h value in the D-M subpopulation of the VvDLKox line prior to the cold treatment.

2.11 EPC affinity chromatography

Carboxy-ethyl-N-phenylcarbamate was synthetized and coupled to Sepharose 4B (Amersham-Pharmacia, Freiburg, Germany) according to (Mizuno et al., 1981). The soluble protein extract flowed through the column loaded with EPC Sepharose and glass wool as a filter filled in order from top to bottom. Then, the unbound protein bands were progressively washed by KCl (0 M, 0.05 M, 0.1 M, 0.15 M, 0.2 M, 0.25 M, 0.3 M, 0.35 M, 0.4 M, 0.45 M, 0.5 M, 1 M) and centrifuged at 15000g for 1 min in each KCl concentration. Then the fractions eluted in KCl buffer were mixed with 2% Na deoxycholate (DOC) for 30 min at 4℃ and precipitated by 100% tri-chloro-acetic acid (TCA) for 30 min at 4℃. The precipitation was centrifuged for 15 min at 4℃ and resuspended in SDS buffer with NaOH for following SDS-PAGE and Western blotting.

2.12 Protein extraction and Western blot analysis

Proteins were extracted from 5d tobacco BY-2 cells after sub-cultivation by the modified protocol

according to (Krtkova et al., 2012). Each sample contained 5 µL of a Protease Inhibitor Cocktail (Sigma-Aldrich, München, Germany) to avoid protein degradation. After denaturation of the soluble protein extracts at 98℃ for 10 min, 15µl of total protein was loaded onto 10% (w/v) polyacrylamide gels with a pre-stained protein ladder (P7704S, New England Biolabs). Tyrosinated and de-tyrosinated α-tubulin were immunoblotted by ATT and DM1A from monoclonal mouse antibodies (Sigma-Aldrich, Darmstadt, Germany), while β-tubulin was probed by DM1B (Sigma-Aldrich, Darmstadt, Germany). The relative protein unit was normalized to WT by mean values from 5 independent biological replicates by ImageJ.

3. Results

3.1 The role of CBF4 in cold stress

3.1.1 *VvCBF4*, a unique CBF gene from its family

The CBF4 proteins of *V. vinifera* are clearly differing from the other CBFs as manifested from a phylogenetic tree inferred for known sequences from different varieties of *V. vinifera* along with several wild species of Vitis (Figure. S1A). While CBF1-3 from the reference genome *V. vinifera* cv. Pinot Noir distribute separately with a high bootstrap support of >90%, the CBF4 proteins form a common cluster. Only the two North American species *V. riparia* and *V. cinerea* differ slightly forming a clade with a bootstrap value of just above 50%. A sequence alignment of the four CBF members from *V. vinifera* cv. Pinot Noir (Figure. S1B) reveals that AP2/ERF DNA-binding domain as well as predicted nuclear localisation signals are highly conserved between all members of the CBF family. In contrast, CBF4 differs from the other members by lacking a domain in the C-terminal half of the protein. This region, spanning around 20-25 amino acids in the other CBF members, is very rich in serines indicative of a potential regulation by kinases. As to obtain insight into the function of VvCBF4, we generated a C-terminal fusion with the Green Fluorescent Protein (GFP) and expressed this fusion construct in tobacco BY-2 cells under control of the constitutive CaMV 35S promoter. Upon transient expression following biolistic transformation and inspection by spinning-disc confocal microscopy, we observed a punctate signal in the perinuclear cytoplasm and the transvacuolar strands that emanate from the nucleus in these vacuolated cells (Figure. 1A, B). In contrast, the karyoplasm was void of any signal. Since *VvCBF4* (as well as the other members of the CBF family) harboured a bona-fide nuclear localisation signature (Figure. 1), we would have expected an intranuclear GFP signal. To address this further, we generated a stable transgenic line expressing the *VvCBF4* GFP fusion. When these cells were investigated at normal temperature (25°C), we observed again a cytoplasmic signal (Figure. 2C). When we zoomed into the GFP-channel and compared it with the overlay with the image obtained by Differential Interference Contrast (Figure. 2D), the GFP signal was located clearly outside of the nuclear envelope, while no signal was detectable in the karyoplasm, confirming the results from the transient expression. We wondered, whether the subcellular localisation might be conditional, and conducted an experiment, where the cells were subjected to severe cold stress (0°C for 4 h). This resulted in a generally reduced intensity of the signal (Figure. 2E), but now, the close-ups revealed that the protein was found in speckles localised inside of the nuclear envelope (Figure. 2F). Thus, in the absence of cold stress, the GFP fusion of *VvCBF4* is cytoplasmic, but seems to be

imported into the nucleus, when the cells experience cold stress.

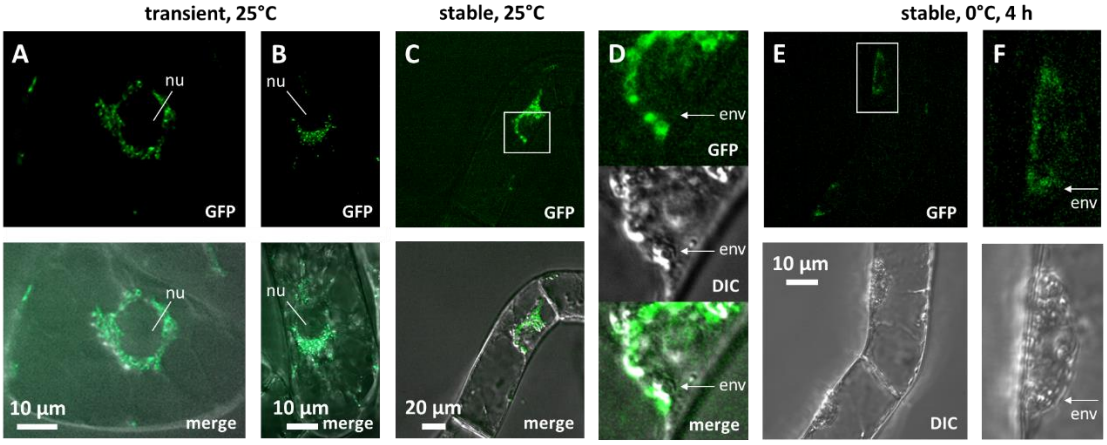

Figure 1. Subcellular localisation of *VvCBF4* in fusion with GFP, assessed in tobacco BY-2 cells as heterologous host. A, B transient transformation with a high-copy vector, cells incubated at 25°C. For two representative cells the GFP signals and the merge of the GFP signal with the differential interference contrast (DIC) are shown. Note that the GFP signal is localised outside of the nucleus (nu). C, D stable transformation with a binary vector, cells incubated at 25°C. D shows a zoom-in of the region marked by a white square in C. White arrows indicate the position of the nuclear envelope (env) separating karyoplasm and cytoplasm to show that the GFP signal is found outside of the nucleus. E, F stable transformation with a binary vector, cells incubated at 0°C for 4 h. F shows a zoom-in of the region marked by a white square in E. White arrows indicate the position of the nuclear envelope (env) separating karyoplasm and cytoplasm to show that this time the GFP signal is found inside of the nucleus.

3.1.2 *VvCBF4* mitigates mortality inflicted by cold stress

To address the role of *VvCBF4* for cold tolerance, we followed cell mortality under continuous cold stress either in the BY-2 cell line expressing *VvCBF4* in a stable manner or the non-transformed wild type (Figure. 2). When the wild type was kept at 0°C, mortality increased steeply from 24 h after induction, increasing by 3 to 4-fold over the resting level within 72 h. For the cells expressing *VvCBF4*, this cold-induced mortality was clearly mitigated by around 40% at 72 h, while the resting levels were comparable between both lines. For comparison, the mortality was observed under chilling stress (4°C). Here, a significant increase of mortality was observed much later (from 72 h of stress treatment) and to a lower amplitude (less than 2-fold over the resting level at 72 h of chilling). Again, the cells expressing *VvCBF4* seemed to be sturdier, but the difference was less pronounced (by around 20% at 72 h) and did not display a significant difference. Thus, expression of *VvCBF4* mitigates mortality inflicted by cold stress.

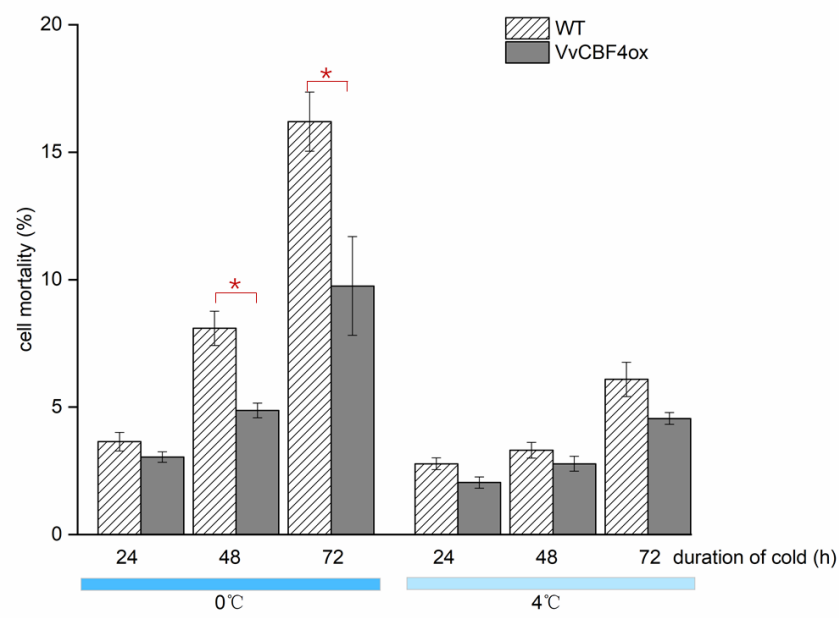

Figure 2. Cell mortality in response to cold treatment (4 ℃ and 0 ℃). Data are shown as means ± Standard Error from three independent experiments with 1500 cells each. Asterisk represents a statistically significant difference with Fisher's LSD test (P <0.05).

3.1.3 *VvCBF4* and its endogenous homologue show different regulation in tobacco BY-2

To understand why the transfected *VvCBF4* mitigated cold-inflicted mortality in the tobacco BY-2 recipient, we followed steady-state transcript levels of VvCBF4 along with transcripts of the tobacco CBF4 paralogue, *Avr9/Cf9*, over time in response to both chilling (4°C) and severe cold stress (0°C). The resting level of *Avr9/Cf9* was identical in non-transformed BY-2 cells and in cells overexpressing *VvCBF4* (Figure. 3A), indicating that the overexpression left the expression of the endogenous CBF4 paralogue unaltered. The resting level of the transfected *VvCBF4* was around 6.5 times higher as compared to the endogenous *Avr9/Cf9*, which is to be expected, since *VvCBF4* is driven by the constitutive Cauliflower Mosaic Virus 35S promoter. Interestingly, the steady-state level of *VvCBF4* transcripts increased significantly, when the overexpressor cells were exposed to cold stress (Figure. 3B). This induction was more pronounced for chilling as compared to severe cold stress. For chilling, the *VvCBF4* transcripts increased by 3.5-fold of the resting level (corresponding to around 25 times the resting level of *Avr9/Cf9*), but only 2-fold for severe cold stress (around 13 times the resting level of *Avr9/Cf9*).

In the next step, we investigated the regulatory pattern for the tobacco paralogue of CBF4,

Avr9/Cf9, under the same conditions. Irrespective of the absence (Figure. 3C) or the presence (Figure. 3D) of the transgene, there was a strong induction of *Avr9/Cf9* transcripts peaking at around 200-fold of the resting level at 48 h after the onset of severe cold stress (0°C). For chilling stress, only a relatively minute induction of less than 10-fold over the resting level resulted. Thus, the temperature dependency of the foreign *VvCBF4* (stronger induction at 4°C compared to 0°C) was inverted to the pattern seen for the endogenous *Avr9/Cf9* (weaker induction at 4°C compared to 0°C). The regulation of the endogenous *Avr9/Cf9* was not affected by the overexpression of *VvCBF4*.

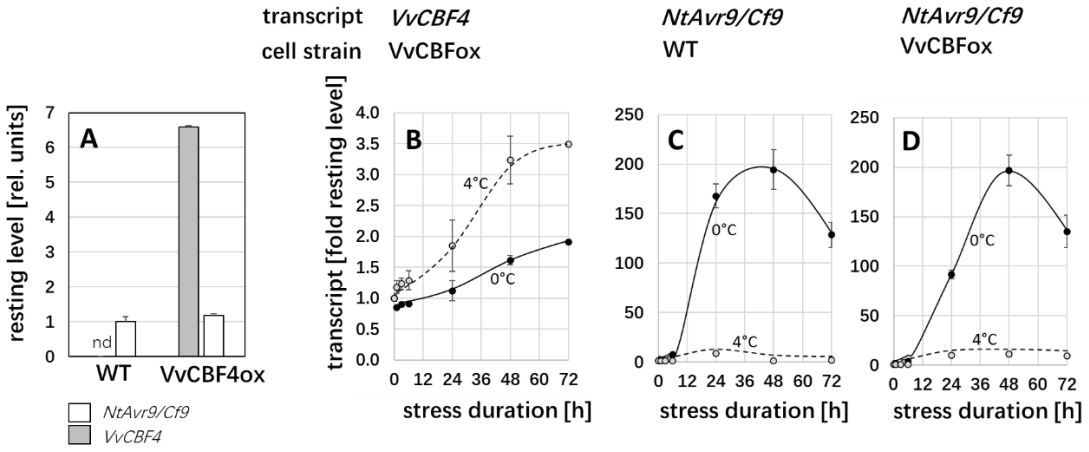

Figure 3. Regulation of transcripts for *VvCBF4* and its tobacco homologue *NtAvr9/Cf9* in response to chilling (4°C) and freezing (0°C) depending on cell strain. A resting levels for the transcripts of the two CBF4 homologues in the absence of cold stress in non-transformed BY-2 cells (WT) versus cells constitutively overexpressing *VvCBF4* in fusion with GFP (VvCBF4ox). nd non detectable. A-C Time course for steady-state transcript levels for VvCBF4 (B) and native *NtAvr9/Cf9* (C, D) during chilling and freezing in the background of the non-transformed WT (C), or cells overexpressing VvCBF4 (B, D). Data are expressed relative to the resting levels (given in A) and represent mean±SE from three independent experimental series, each in technical triplicates.

3.1.4 *VvCBF4* modulates expression of specific CBFs depending on temperature

The transcripts of the foreign *VvCBF4* were upregulated under cold stress (Figure. 3B), which was accompanied by a reduced mortality (Figure. 2). As to get insight into the potential functions of *VvCBF4* during the response to cold stress, we followed the expression of the endogenous CBF factors DREB1 and DREB3 (Figure. 4). The transcriptional change of *NtDREB1* was slight and distinct in wildtype and VvCBF4ox (Figure 4). For the freezing treatment, compared to the constant suppression of DREB1 in the VvCBF4ox line, the expression of DREB1 in WT was quite dynamic (Figure 4A). A drop of the DREB1 expression in the first 6 h was followed by the strong up-regulation at 24 h and down-regulation at 48 h of freezing (Figure

4A). Besides, the earlier induction of *NtDREB1* was shown at 3 h of chilling in wildtype than VvCBF4ox with a slight increase at 6 h, 48 h and 72 h of chilling (Figure 4A). Aside from the weak induction of NtDREB1 under cold stress, DREB3 abundance was notably and continuously accumulated under freezing stress in both cell lines, increasing from 6 h until peaking at 72 h, whereas enhanced expression of NtDREB3 in wildtype was observed from 24 h of chilling and stayed at the same level with extensive chilling treatment, as opposed to the perpetual suppression in VvCBF4ox (Figure 4B). Taken together, the data showed overexpressing CBF4 may suppress DREB1 expression with or without cold stress, and the response of DREB3 to chilling stress.

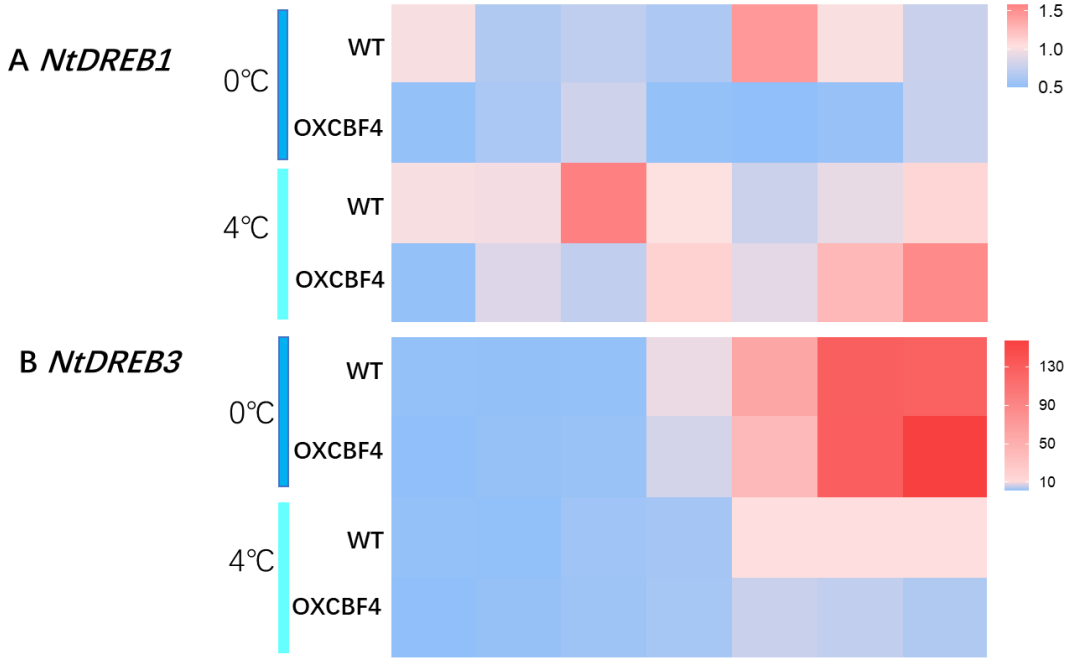

Figure 4. Relative expression level of endogenous CBFs in WT and VvCBF4ox line in response to chilling (4°C) and freezing (0°C). The colour code shows the fold changes in transcript levels normalized to the expression levels in non-transformed wildtype under room temperature. Data represent means of triplicate biological replicates.

3.1.5 Various regulations of downstream cold-responsive genes in response to different temperatures

Cold tolerance in plants largely relies on the regulation of COR gene expression activated by upstream transcription factors. For inspection of the regulating of downstream cold-responsive (COR) genes by CBF4 in cold signalling, the expression of 3 COR genes *NtERD10a*, *NtERD10c* and *NtERD10d* was observed. The mRNA level of *NtERD10a* in WT was rapidly induced for 1 h freezing and stayed at a higher level compared to a relatively low expression in VvCBF4ox (Figure 5). And the change of *NtERD10a* expression under chilling conditions was

negligible in both cell lines (Figure 5). *NtERD10c* and 10d have the same mRNA sequence harbouring different functions in cold signalling. *NtERD10c* was strongly induced in WT starting at 24 h chilling (6-fold) and freezing stress (11-fold), juxtaposing the late freezing response at 48 h and strongly suppressed induction under chilling stress in VvCBF4ox (Figure 5). Surprisingly, overexpression of *VvCBF4* strikingly induced the expression of *NtERD10d* (4-fold) without cold stress (Figure 5). On the other hand, the strong induction of *NtERD10d* was observed from 24 h (8-fold) under freezing in both cells with a stronger induction (5-fold more) in VvCBF4ox than WT after 48h freezing (Figure 5). However, under chilling stress, the induction of *NtERD10d* from 24 h was more effective in the WT (Figure 5). Thus, except *NtERD10a*, 10c and 10d are induced by cold, and responsible for long-term cold stress, especially the distinct induction of *NtERD10d* in different cold conditions. And 35S driven CBF4 expression restrains the expression of cold-induced genes during non-functional stages.

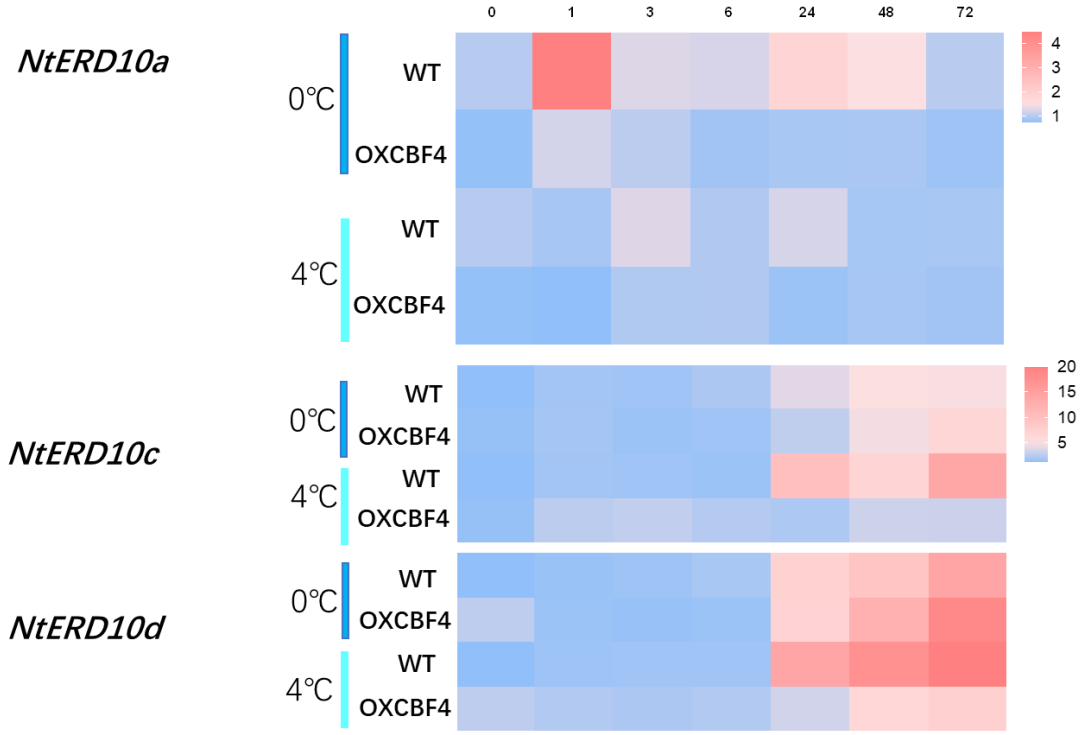

Figure 5. Relative expression level of cold-responsive (COR) genes in WT and VvCBF4ox line in response to chilling (4°C) and freezing (0°C). The colour code shows fold changes in transcript levels normalized to the expression levels in non-transformed wildtype under room temperature. Data represent means of triplicate biological replicates.

3.1.6 Nuclear import modulates the response of CBFs and COR to cold stress

As we had observed that VvCBF4 is translocated from the cytoplasm into the nucleus in response to cold stress (Figure. 1), and since ectopic overexpression of *VvCBF4* altered the response of endogenous CBFs and COR genes to cold stress (Figure. 4 and 5), we questioned,

whether translocation of *VvCBF4* into nucleus is required for this modulation of gene expression. To address this, we tested the effect of GTP-γ -S, which irreversibly blocks the GTPase function of Ran, and had been shown to block nuclear import also in plant cells (Merkle *et al.*, 1996). In the absence of cold stress, with exception of a slight inhibition of DREB1 transcripts, none of the tested transcripts displayed any change (Figure. 6). However, under cold stress, the expression of the tobacco CBF4 homologue *Avr9/Cf9* was modulated – the induction under freezing stress was reduced to ¼ after pre-treatment with GTP-γ-S indicating that nuclear import is essential for this induction by freezing stress. The relatively low induction of *Avr9/Cf9* transcript by chilling stress was not altered. In contrast, transcripts for *NtDREB1* that are downregulated around 5-fold by freezing stress, remain unaltered in presence of GTP-γ-S. Again, chilling caused only comparatively small effects that were reduced further by the inhibitor. The transcripts for the highly responsive *NtDREB3* were reduced from a 30-fold to a 16-fold induction by GTP-γ-S under freezing stress (under chilling stress, this transcript did not change significantly, neither for absence nor presence of the inhibitor). Thus, *NtDREB3* behaved parallel to *Avr9/Cf9*. For the COR gene *NtERD10a*, the inhibitor did not cause significant changes in the response to cold stress. However, *NtERD10c* and *NtERD10d* were altered clearly and with the same pattern by GTP-γ-S. Here, the induction by freezing stress was not changed, but the induction by chilling stress was boosted by around twofold. Thus, nuclear import supports the induction of the CBF4 orthologue *Avr9/Cf9* and the CBF *NtDREB3*, while it acts negatively upon the induction of the COR transcripts *NtERD10c* and *d*.

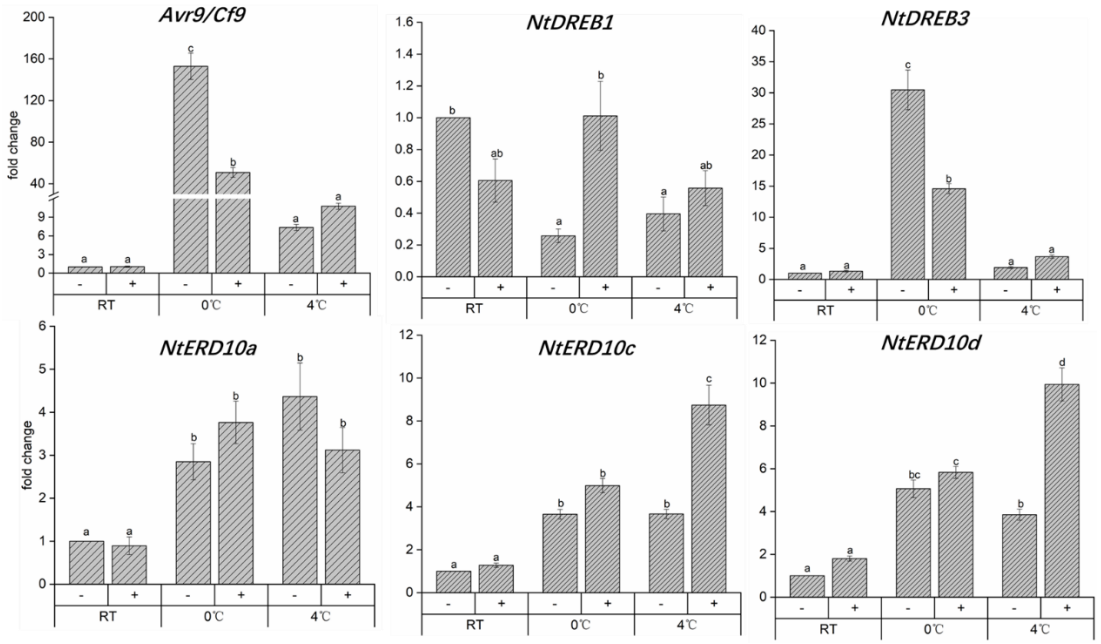

Figure 6. GTP-γ-S inhibition of quantitative real-time PCR analysis for endogenous CBF and COR genes in response to chilling (4°C) and freezing (0°C). Pre-treated cells with 500µM GTP-γ-S for 30 minutes in room temperature were cultivated under cold condition (0℃ or 4℃) for following 24h. - Represents cells under certain condition without GTP-γ-S, while + shows cells were also treated with GTP-γ-S. Different letters indicate the statistically significant difference with Fisher's LSD test ($P < 0.01$) by means ± SE from triplicate biological replicates.

3.1.7 The COR *ERD10d* transcript is negatively regulated by a protein

synthetised *de-novo*

To understand the mechanism behind the differential effect of chilling and freezing stress upon the induction of *ERD10d* transcripts (that derive by differential splicing from the same gene as *ERD10c*), we probed for the effect of Actinomycin D (AMD) as inhibitor of transcription, and Cycloheximide (CHX) as inhibitor of translation. To test, whether the treatment used in this experiment was sufficient, we first tested their effects on the transcripts of the foreign VvCBF4 that are driven by the constitutive CaMV 35S promoter (Figure. 6A). Indeed, inhibition of transcription by AMD significantly reduced *VvCBF4* transcripts to a residual level of around 35% of the resting level, irrespective of temperature. In contrast, inhibition of translation by CHX did not modulate the strong induction of *VvCBF4* transcripts (around 3-fold) in response to chilling stress, nor did it alter the weak induction of these transcripts in response to freezing stress. The pattern for *ERD10d* transcripts (Figure. 6B) differed fundamentally. While AMD eliminated the induction of these transcripts irrespectively of temperature, there was a clear difference with respect to the effect of CHX. In the absence of cold stress, *ERD10d* transcripts were induced 3-fold by CHX. Likewise, the around two-fold induction of *ERD10d* transcripts in response to chilling was further accentuated by CHX to around 4-fold of the resting level. In contrast, the strong induction of *ERD10d* transcripts in response to freezing remained mostly unchanged after pre-treatment with CHX. Thus, while transcription is necessary for the induction of *ERD10d*, there seems to be a negative regulator (both in the absence of cold stress and under chilling stress) that is synthetised as protein *de-novo*. However, this negative regulator is not needed for the response of this transcript to freezing stress.

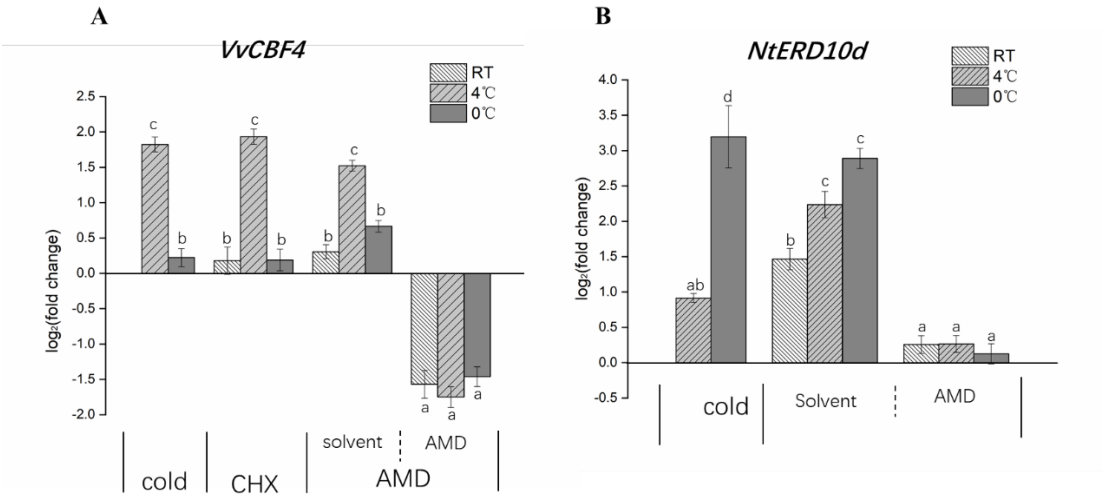

Figure 7. Role of transcription for expression of VvCBF4 and NtERD10d. Steady-state transcript levels for the foreign VvCBF4 (A) and the endogenous COR transcript NtERD10d (B) were measured after 24h incubation of VvCBF4 overexpressor cells and none-transformed BY-2 either at 25°C (RT), or chilling (4°C), or freezing (0°C) stress after pre-treatment for 2 h with either the transcription inhibitor Actinomycin D (AMD, 50 µg/ml) or the translation inhibitor Cycloheximide (CHX, 100 µg/ml). The solvent control of AMD was 2.5% EtOH, while CHX was diluted in an aequous stock. Data are normalised to the expression levels under room temperature without any of the inhibitors and represent means±SE of three independent experiments in technical triplicate. Statistically significant difference are represented as different letters with Fisher's LSD test ($P < 0.01$).

3.1.8 Temporal cold-induced expression of CBF4 correlates in freezing resistance

To get insight into a possible link between CBF4 expression and cold tolerance, we followed CBF4 transcripts in leaves from different Vitis genotypes differing in their cold tolerance, either under chilling (4°C) or severe freezing (-20°C). Under chilling stress (Figure. 6), the cold tolerant V. amurensis from Northern China responded by a rapid (within 3 h) and persistent (over 12 h) accumulation of CBF4 transcripts. In contrast, V. vinifera cv. Pinot Noir from Europe did not display any response of CBF4 to chilling. Interestingly, the cold sensitive V. coignetiae from Japan showed a significant elevation of CBF4 transcript levels from the first time point but did not produce any significant induction during subsequent chilling stress. The patterns for severe freezing stress (-20°C) were significantly different. Here, V. amurensis accumulated CBF4 transcripts slightly more sluggishly as compared to chilling stress, but again more persistently with a maximum at 12 h. In contrast, V.vinifera cv. Pinot Noir, which had not responded at all to chilling stress, produced a rapid, strong, but transient induction of CBF4 transcripts peaking at 6 h and complete breakdown afterwards. The cold susceptible V. coignetia was not responsive at all in the beginning, and accumulated CBF4 transcripts only from 12 h. Thus, the cold tolerance of V. amurensis correlated with a temporal pattern, where CBF4 transcripts were induced not very swiftly (no response after 1 h of cold stress), but persistently (over 12 h or beyond), irrespective of the type of cold stress (chilling versus severe freezing). The intermediate V. vinifera Pinot Noir was not responsive to chilling stress, indicative of a poor cold acclimation, while it was not able to sustain expression under freezing stress. The susceptible V. coignetia was already challenged by chilling stress responding by a precocious induction but failed to respond appropriately under freezing stress. In addition to these genotype-dependency, the response pattern differed with respect to stress stringency.

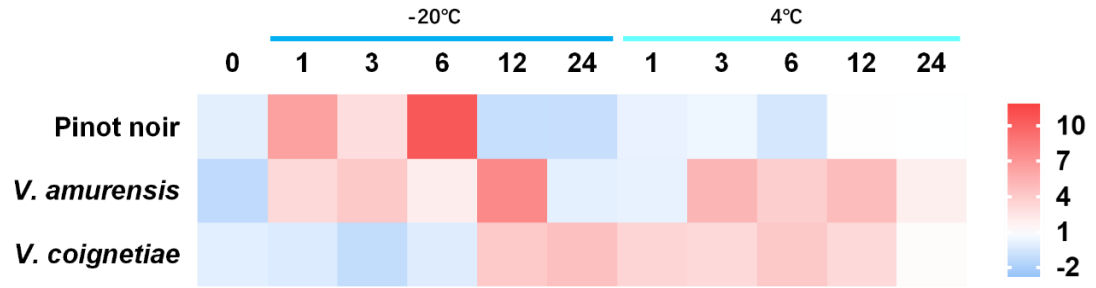

Figure 6. log2 fold changes in steady-state transcript levels of CBF4 in different Vitis genotypes in response to cold stress. Plants with the similar growth were challenged by chilling (4℃) and freezing (-20℃). The leaves were sampled at specific time points after cold stress. Color code represents log2 fold changes in transcript levels normalized expression level of Pinot Noir under room temperature. Data represent mean values from three biological replicates per genotype and time point.

3.2 CBF4-dependent cold signalling pathway

3.2.1 Overexpressing CBF4 alleviates calcium influx but aggravates intracellular calcium dynamic in cold signalling

Temporal and spatial dynamics of Ca^{2+} concentration represent a universal information code of cold signalling in plants. We first followed apoplastic alkalinisation during the 90-minute ice-water treatment because of proton coupling with calcium pumped into cells in response to cold stress. In both WT and transgenic line VvCBF4ox, the rapid and continuous increase of extracellular pH during the entire cold event was observed, followed by a gradual drop once cold treatment of the cells was stopped (Figure 1A). Notably, there was a remarkable difference between the two lines, VvCBF4ox and non-transformed tobacco BY-2. They showed a weaker pH change with a more rapid pH decline when cold stress was removed, suggesting CBF4 may indirectly or directly modulate intracellular Ca^{2+} in cold signalling.

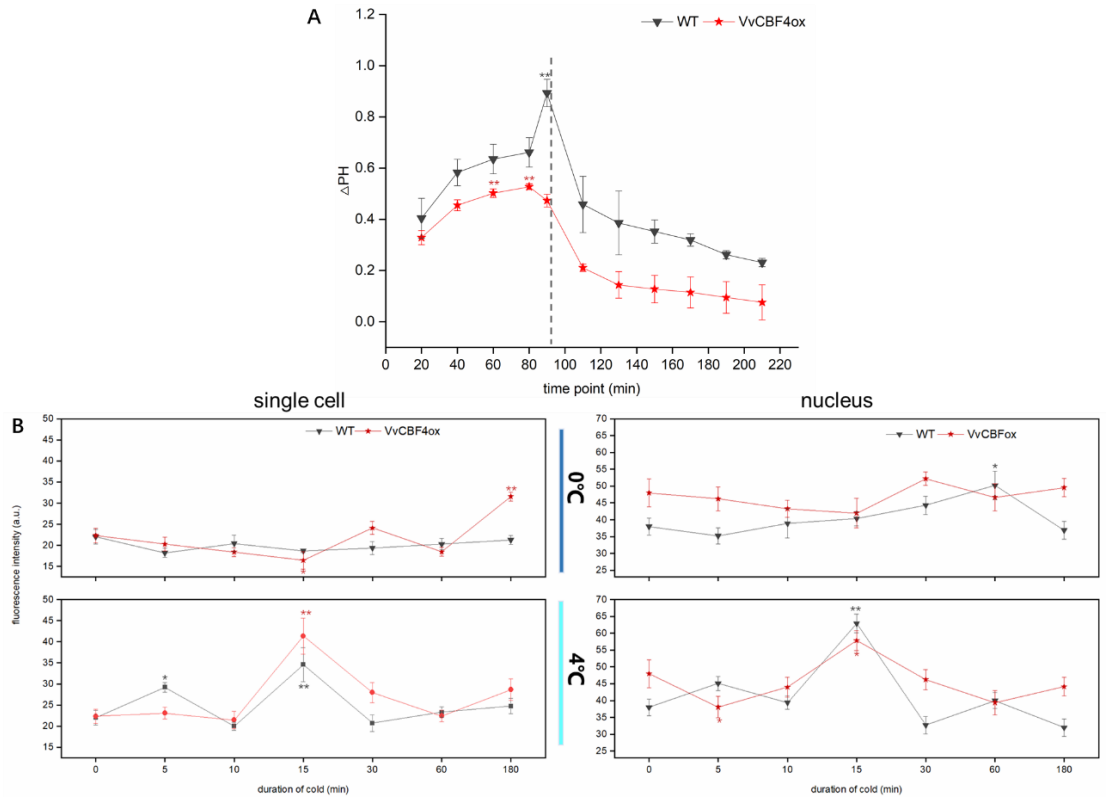

Figure 1. Calcium dynamics in response to cold stress. A. Time-course of the apoplastic alkalinisation dynamic in response to freezing (0℃). Data are shown as mean ΔpH± Standard Error from five independent experiments. Asterisk represents a statistically significant (LSD test, $P < 0.01$). B. Intracellular calcium concentrations differ in cold intensity. Data are shown as mean fluorescence intensity ± Standard Error from three independent experiments with at least 40 individual cells. Asterisk represents a statistically significant difference with Fisher's LSD test (*p <

0.05， ** p <0.01).

To have insight into the calcium distribution in cold signalling, the intracellular Ca^{2+} level was measured at low temperatures. Intracellular Ca^{2+} concentration in the VvCBF4ox line showed a gradual decline reaching the lowest point during the first 15 minutes from the onset of freezing (Figure 1B left). After the fluctuation of calcium levels from 15 min to 1 h of freezing exposure, a notable rise was observed after 1 h of freezing in the transgenic line (Figure 1B left). The Ca^{2+} signature in the wild type was quite steady after the freezing treatment (Figure 1B left). Unlike the indifferent response to freezing, the WT showed a more dynamic reaction to 4℃ (Figure 1B left). Rapid increases at 5- and 15-minute chilling were recorded up to its peak at 15 minutes (Figure 1B left). In contrast to the WT's response to chilling, the VvCBF4ox line had the same trend but a non-significant rise after 5 minutes of chilling treatment (Figure 1B left).

Furthermore, the signature of nuclear Ca^{2+} was employed to present the link between cold sensing and gene expression (Figure 1B right). There has been a slight drop at 5-min followed by a continuous increase up to a peak at 1 h of freezing in the nuclear Ca^{2+} level in WT (Figure 1B right). Conversely, a delayed rise at the point of 15 minutes followed by a gradual decrease of nuclear Ca^{2+} was shown during the first 30 minutes of freezing in the VvCBF4ox line (Figure 1B right). The tendency of these two lines after 1 h of treatment was opposite, the drop in the WT and a rise in the transgenic line (Figure 1B right). Same trend of Calcium dynamics as single cell, Ca^{2+} levels under chilling were more dynamic than in freezing conditions (Figure 1B right). After a few fluctuations, Ca^{2+} in the nucleus was accumulated to a peak at 15-minute of chilling in both cell lines with an extremely significant increase in the WT (Figure 1B right). What is striking was a significant drop of nuclear Ca^{2+} levels in the VvCBF4ox line during the first 5 minutes after the onset of chilling contrasting a rise at the same time in the WT (Figure 1B right). Overall, these results indicate that calcium ions may participate in the CBF4-dependent signalling pathway and play different roles in chilling and freezing signalling.

3.2.2 Different CBF4-dependent signalling in chilling and freezing stress

To get insight into the signalling underlying the regulation of cold-related transcripts, we determined transcripts for *VvCBF4* (in the tobacco BY-2 cell line VvCBF4ox) and *NtERD10d* (in both, VvCBF4ox and non-transformed BY-2 cells) after 24 h of either freezing (0°C) or chilling (4°C) stress after pre-treatment with different inhibitors (Figure. 2). The two stress treatments alone induced a different response of the two transcripts – *VvCBF4* was induced by chilling, but not by freezing stress, while *NtERD10d* was more induced by freezing stress and less by chilling stress (Figure. 2, mock). While the response of *VvCBF4* did not show obvious changes in presence of the solvent (1% DMSO), that of *NtERD10d* was clearly modulated. The induction by freezing stress was partially silenced by this solvent in the VvCBF4ox line, while it was

significantly enhanced in the non-transformed WT. For chilling stress, DMSO clearly enhanced the induction of *NtERD10d* in both cell lines. Thus, DMSO, which is not only a solvent, but also a membrane rigidifier, had a clear effect that was depending on the type of cold stress and the overexpression of *VvCBF4*. Pre-treatment with Diphenyliodonium (DPI), an inhibitor of the NADPH oxidase Respiratory burst oxidase Homologue, significantly quenched the induction of *VvCBF4* transcripts by chilling stress, while *NtERD10d* was not affected (neither in the overexpressor line or the non-transformed wild type). Inhibition of MAPK signalling by PD98059 suppressed the chilling response of *VvCBF4* transcripts even stronger. Here, we observed a mild (in the overexpressor line) or strong (in the WT) induction of *NtERD10d* transcripts. Again, the responses to freezing stress were modulated much less. The most salient changes were seen with MG132, a specific inhibitor of the proteasome. Here, both, *VvCBF4* and *NtERD10d*, were strongly induced under chilling stress (*NtERD10d* in both, overexpressor cells and WT). A comparable induction of *ERD10d* transcripts was seen for freezing stress, while *VvCBF4* transcripts did not exhibit this induction. Overall, the induction of *VvCBF4* transcript by chilling not only requires RboH and MAPK signalling but is also boosted by a factor that is swiftly degraded by the proteasome. This factor is not playing a role in freezing stress. This factor also boosts the accumulation of *ERD10d*, in contrast to *VvCBF4*, during freezing stress as well. The solvent DMSO is inducive for ERD10d, especially under freezing stress. This induction under freezing stress was silenced by the overexpression of *VvCBF4*. Instead, inhibition of MAPK signalling induced *ERD10d* under chilling, less so under freezing. This induction under chilling stress was silenced by the overexpression of *VvCBF4*. These data show that the response of the COR transcript depends on the presence of the foreign *VvCBF4* gene, and that the signalling differs qualitatively between chilling and freezing stress. The most striking trait is the strong dependence of the transcripts on the proteasome because its inhibition by MG132 was strongly enhancing the expression of both cold-related transcripts under chilling stress, in case of *ERD10d* also under freezing stress.

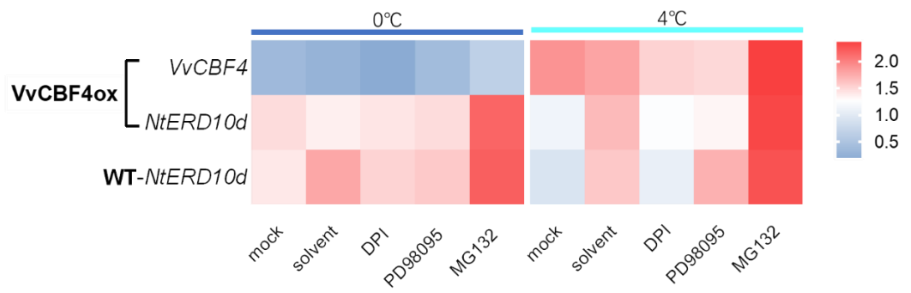

Figure 2. Heatmap of Log2 fold changes in transcript levels of *VvCBF4* and *NtERD10d* by inhibitors under cold stress. The colour code shows log2 fold changes in the steady-state transcript levels for the foreign VvCBF4 and the endogenous COR transcript NtERD10d in the VvCBF4 overexpressor as well as NtERD10d in non-transformed BY-2. Cells were incubated at either 24 h freezing (0°C) or chilling (4°C) after pre-treatment for 30 min with either 10 µM Diphenyleneiodonium (DPI), or 100 µM PD98059 or 100 µM MG132. As a solvent control, cells were pre-treated

with 1% (v/v) DMSO as same-volume treatments. Data are normalised to the expression levels under room temperature without any of the inhibitors and represent means ± SE of three independent experiments in technical triplicates.

3.2.3 The lipoxygenase inhibitor phenidone induces *VvCBF4* and VvCBF4-dependent *NtERD10d*

The strong effect of the proteasome inhibitor MG132 (Figure. 2), led us to the question, whether this effect might be related to the continuous turnover of ICE1. The transcriptional activity of this master switch was shown for Arabidopsis thaliana to be modulated by physical interaction with specific members of the JASMONATE ZIM DOMAIN family (Hu et al., 2013). These proteins are negative regulators of jasmonate signalling but are produced in response to jasmonate signalling. If this very specific hallmark of cold signalling would be conserved in tobacco cells, we should see specific changes in the cold response, when the production of jasmonates is disrupted. This can be achieved by phenidone, an inhibitor of lipoxygenase, thus, blocking the conversion of α-linolenic acid into the jasmonate precursor 13-HPOT (Bruinsma et al., 2010a; Bruinsma et al., 2010b). The solvent control, 1% ethanol exerted some effect as well, *VvCBF4* expression was induced by 2-fold at 25°C (Figure. 3A). Likewise, at 25°C *NtERD10d* expression was induced by 4-fold in the *VvCBF4* overexpressor, and even 13-fold in the WT (Figure. 3B). Under cold stress, this effect of the solvent was not noted with exception of a slight, but significant enhancement for the freezing response of *NtERD10d* transcripts. However, the effects of the solvent were minor as compared to the effect seen for phenidone, which already under room temperature stimulated the transcripts of *VvCBF4* by 4-fold (Figure. 3A). The transcripts of *NtERD10d* were elevated as well to a similar degree (in the WT by around 4-fold, in the VvCBF4ox by around 6-fold) (Figure. 3B). Under chilling, phenidone did not yield conspicuous effects, for *VvCBF4*, transcript levels were even exactly the same as in the mock control (Figure. 3A). The situation differed drastically for freezing stress. Here, *VvCBF4* was induced 15-fold (Figure. 3A). For *NtERD10d*, the induction was even more pronounced, but only when *VvCBF4* was overexpressed. Under these conditions, *NtERD10d* was induced more than 50-fold (Figure. 3B), comparing to around 10-fold in the absence of phenidone. Interestingly, this induction was not seen in the wild type (lacking the foreign *VvCBF4* gene and, thus, the induction of *VvCBF4* transcripts by phenidone (Figure. 3A). Taken together, we can show that jasmonates modulate the induction of *VvCBF4* and *NtERD10d* transcripts under freezing stress. This effect of jasmonates is repressive since inhibition of jasmonate synthesis by the lipoxygenase inhibitor phenidone strongly amplifies the induction of both transcripts by freezing stress. The fact that there is no such induction of *ERD10d* seen in the wild type, indicates that the endogenous CBF4 orthologue *Avr9/Cf9* cannot functionally

replace the foreign CBF4 from grapevine.

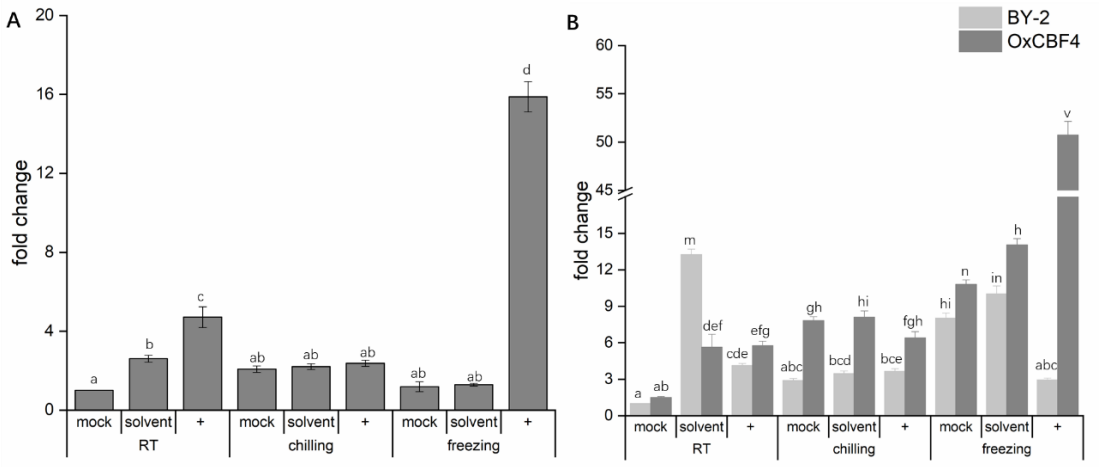

Figure 3. Inhibition of jasminate biosynthesis by phenidone enhances CBF4 expression under cold stress. Cells were incubated with either room temperature or 24h freezing (0°C) or chilling (4°C) after pre-treatment for 30 min with 2mM phenidone (represented by +) or same volume ethanol as solvent control. NtERD10d is based on its mRNA level in none-transformed BY-2 cells. Data are normalised to the expression levels under room temperature without any of the inhibitors and represent means±SE of three independent experiments in technical triplicate. Statistically significant difference is represented as different letters with Fisher's LSD test ($P < 0.01$).

3.2.4 Mitochondrial superoxide accumulation in cold signalling

The tradeoff of mitochondrial reactive oxygen species (ROS) is essential for the integration of plant development and stress response. Superoxides in mitochondria can be florescence-labelled by MitoSOX red to follow the signature of ROS in cold signalling. In general, the VvCBF4ox line showed a slightly stronger response of mitochondrial superoxide than the WT, but with no big difference in trends under chilling and freezing stress (Figure 4). In detail, in both cell lines, a drop of mitochondrial superoxide in the cell was first observed at 3-hour in freezing temperature, then followed by a gradual rise until 12-hour and replaced with a sharp increase of ROS with time (Figure 4A). In contrast to the response of mitochondrial superoxide to freezing stress, the production of mitochondrial superoxide under chilling stress was distinct. The production of mitochondrial superoxide kept dropping in the VvCBF4ox line after 3 h differing to the slight increase from 3 h to 6 h in WT, followed by a rapid accumulation after 6 h of chilling in both lines (Figure 4B). Besides, the big difference observed in mitochondrial superoxides after 48 h of chilling between WT and VvCBF4ox was that superoxides were excluded in WT juxtaposing a continued rise after 48 h in the VvCBF4ox line (Figure 4B). Therefore, mitochondrial superoxide was suggested to be a pivotal conductor to coordinate cold response and development by strict control of ROS generation. Overexpressing CBF4 may mitigate this limitation somehow leading to sustained amplification of cold signal for the

efficient cold hardiness.

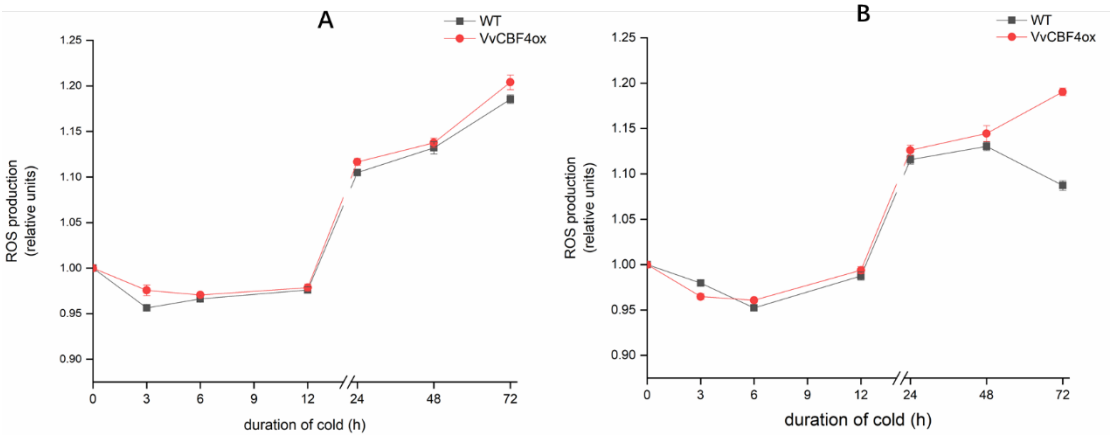

Figure 4. Time-course of mitochondrial superoxide accumulation under freezing (A) and chilling stress (B). Relative fluorescence recorded at constant exposure time (600 ms) was quantified relative to the respective base fluorescence by the Image J software. Data are shown as fluorescence intensity ± Standard Error from three independent experiments with at least 500 individual cells.

3.3 The minus-end Kinesin-14 in grape

3.3.1 *F6HRV2* encodes a putative homologue of Arabidopsis minus-end directed kinesins *ATK5* in Vitis

To assess the grape homologue of *Arabidopsis* minus-end directed ATK5, the amino acid sequence of ATK5 and *OsDLK* were obtained for blasting. According to the result, *Vitis F6HRV2* shows 68.57% amino-acid similarity to *Arabidopsis* kinesin ATK5 and 58.39% to *OsDLK* (UniProtKB/Swiss-Prot accession no. *F6HRV2*). And *F6HRV2* encodes a predicted 89.67kDa protein with 802aa, containing a c-terminal kinesin motor domain (amino acids 440-781), a putative ATP-binding site (aa 498-505), and a putative microtubule-binding site (aa 700-706) (Figure 1A). In the neck region (aa 404-420), it has two critical amino acids (marked in green rectangle, Figure 1B) like ATK5 and *OsDLK*, for kinesin minus-end directed movement. Thus, *F6HRV2* may be a *Vitis* putative homologue of *Arabidopsis* minus-end directed kinesins ATK5.

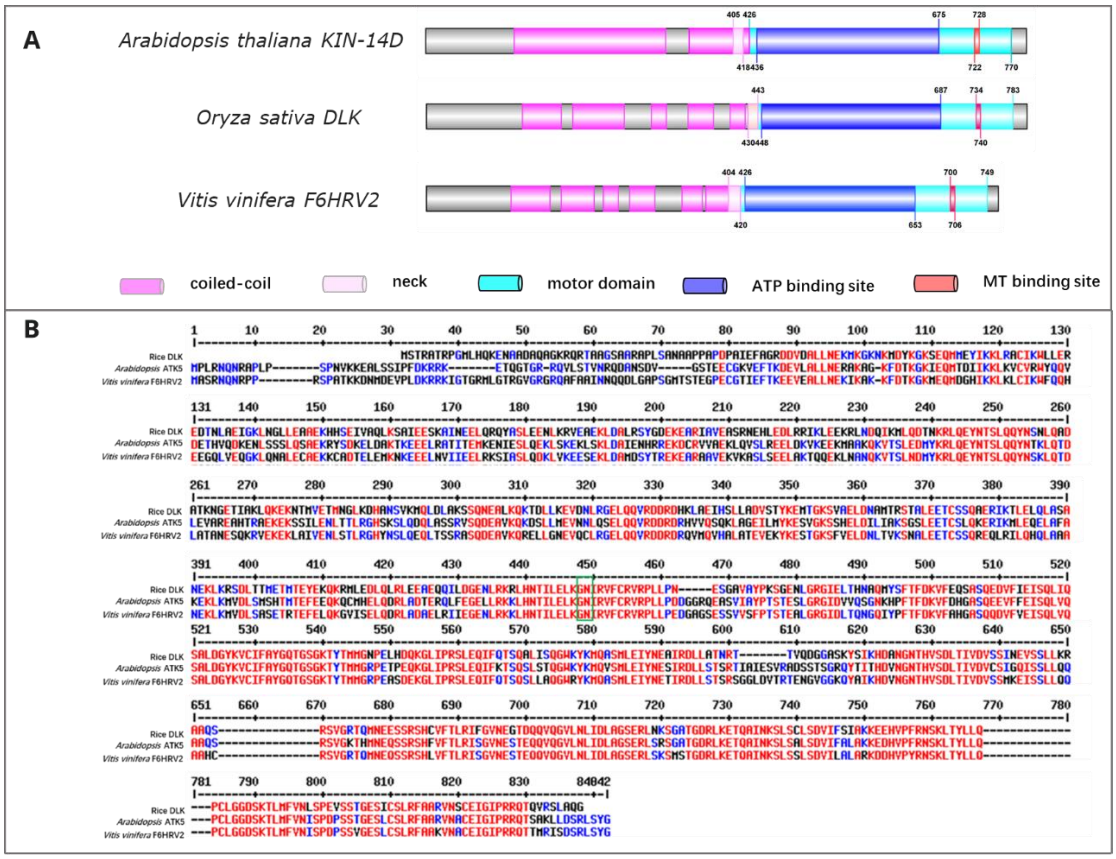

Figure 1. Homogeneous analysis of *Vitis Vinifera F6HRV2*, minus directed kinesin *Arabidopsis ATK5* and rice *OsDLK*. A. Conserved domains in *Vitis Vinifera F6HRV2* comparing to Arabidopsis ATK5 and rice *OsDLK*. B. Amino acid sequence of *Vitis vinifera F6HRV2* aligning with rice *OsDLK* and *Arabidopsis ATK5* . The green rectangle represents the two critical amino acids for kinesins minus-end mobility.

3.3.2 Subcellular localization of overexpressing *F6HRV2* in tobacco BY-2 cells

To investigate the cellular function of *F6HRV2*, the full-length *F6HRV2* was cloned and fused with GFP on its N-terminus to generate the stably overexpressing F6HRV2 line via GATEWAY cloning. In stably transformed calluses, there are two sub-populations with two distinct subcellular localization, which grow clusters by the same sub-populations. Because of this specific localization, we describe this kinesin as dual localization kinesin (*VvDLK* in the following work), which is similar to the rice homologue of *Arabidopsis* ATK1/5 named by *OsDLK*. Among them, one subpopulation shows *VvDLK* not only decorates microtubule but also localizes in the nucleus named as DLK-MTs (D-M for abbreviation), while the other subpopulation shows only nuclear localization of VvF6HRV2 (D-N for abbreviation) (Figure 2).

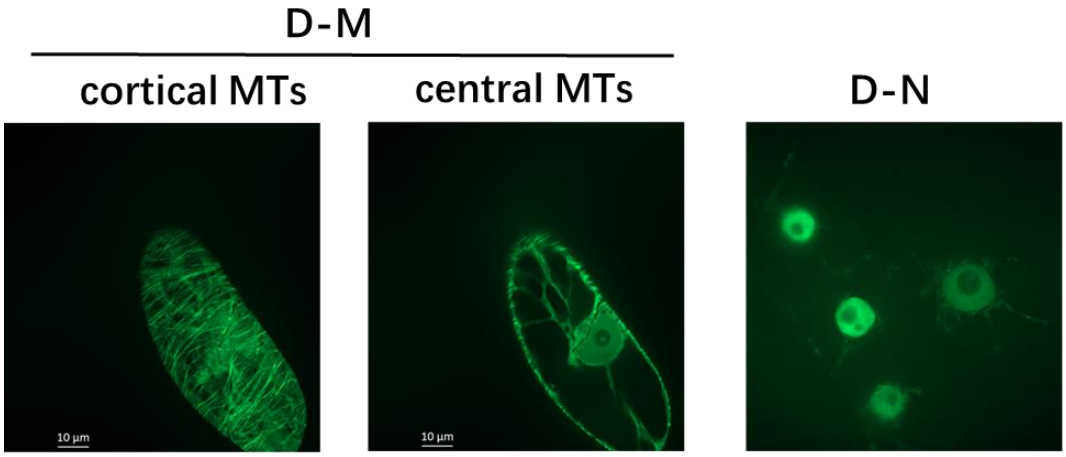

Figure 2. Subcellular localization of two sub-populations stably overexpressing *Vits F6HRV2*, thus Vitis *F6HRV2* is named as *VvDLK*. D-M represents except for localizing in the nucleus, the *VvDLK* tethers with MTs, while D-N implies *VvDLK* appears in nucleaus.

3.3.3 The variation of *VvDLK* transcript and translation in two sub-populations

To understand the molecular mechanisms causing the difference in the two *VvDLK* sub-populations, transcript diversity of *VvDLK* in D-M and D-N lines was first considered. Unexpectedly, *VvDLK* is much more strongly expressed in D-N rather by 400-fold than in D-M, even though both are driven by the CaMV 35S promoter (Figure 3).

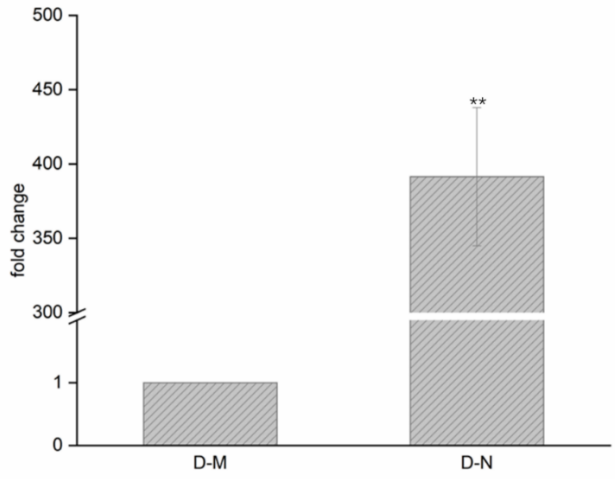

Figure 3. Difference of two *VvDLK* sub-populations (D-M and D-N) in transcript levels. *VvDLK* expression in D-N is normalized to its transcript in D-M. Data are represented by means ± SE from triplicate biological replicates. Asterisk represents a statistically significant difference with Fisher's LSD test (** P <0.01).

Then the difference in those two sub-populations was displayed in protein level blotted by tubulin antibodies because of the correlation of kinesin with MTs. In addition to the expected

55kDa bands for α- and β-tubulin, a mysterious additional band of about 95kDa was also detected by all tubulin antibodies. Only in D-M, this resulted in a significant decrease of tyr-α-tubulin and β-tubulin in D-M compared to the WT (Figure 4A). D-N exhibited the same protein level for tyr-α-tubulin and β-tubulin as the WT with more detyr-α-tubulin than WT and D-M (Figure 4A). The question arose whether this unexpected band tightly binds to MTs to generate a complex. To answer this question, the approach of the ethyl-N-phenylcarbamate (EPC) Sepharose affinity chromatography for purifying tubulins was employed. The purification of α- and β-tubulin was achieved by chromatography on the EPC-Sepharose affinity column in both WT and DLK sub-populations (Figure 4B). The abundance of α- and β-tubulin in both cell lines declined with the ion concentration, which was opposite to the 95kDa protein precipitated gradually from 0.15 M to 0.4 M KCl (Figure 4B). To sum up, difference in two sub-populations of VvDLKox, including extensive *VvDLK* in D-N, a heavy band generating in D-M indicates the diversity function of this minus-end kinesin.

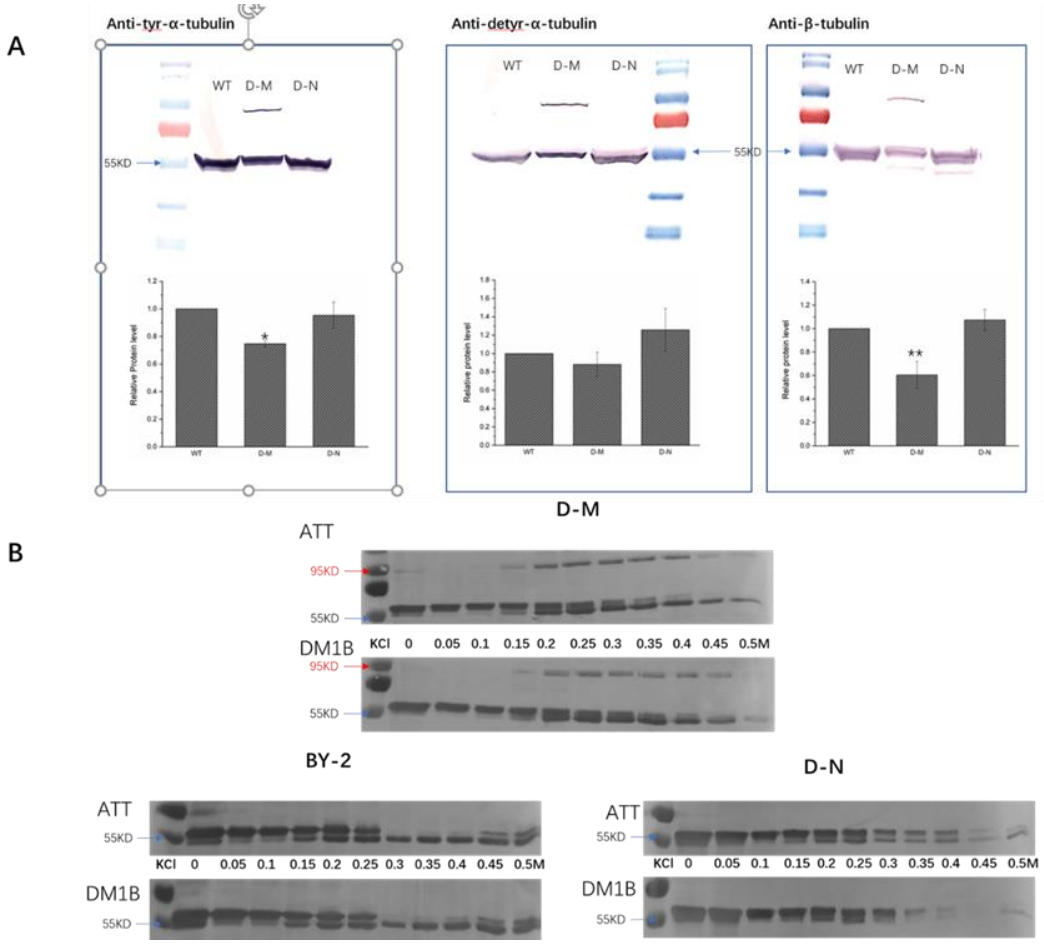

Figure 4. Western blot for protein difference in two sub-populations of VvDLKox. A. Different tubulin proteins immunoprecipitated by different antibodies and quantified protein levels by ImageJ. Data represent means of triplicate biological replicates, and error bars indicate SE. Asterisk represents a statistically significant difference with Fisher's LSD test (* P <0.05, ** P <0.01). B. Isolation of α- and β-tubulin dimers by EPC Sepharose affinity chromatography.

3.4 Different cold responses in two sub-populations of VvDLKox

3.4.1 Two sub-populations of VvDLKox differ in cold tolerance

To address the role of *VvDLK* in cold stress, the time-course of cell mortality of VvDLKox (D-M and D-N) under continuous cold conditions was quantified, as well as of the non-transformed wildtype BY-2 as reference. When the wild type was kept at 0°C, mortality increased steeply from 24 h after induction increasing 3 to 4-fold over the resting level within 72 h. For the two sub-populations of overexpressing *VvDLK*, *VvDLK* localizing with MTs (D-M) had about a 2-fold increase in dead cells for 24 hours and 72 hours of freezing over the non-transformed wild type. The lowest cell mortality under freezing was observed in the *VvDLK* in-nucleus (D-N) line within 72 h, staying at around 3-4 % (Figure 1). When cells were under chilling stress, a significant increase of mortality in wildtype was observed at 72 h with a lower amplitude (less than 2-fold over the resting level at 72 h of freezing). Again, D-M showed higher mortality (2-fold) after 24 h of chilling than the wildtype does, while the D-N line achieved similar cell mortality to the wildtype within 72 h under chilling stress (Figure 2). Thus, two sub-populations of *VvDLK* are referred to as two phenotypes in response to cold stress, cold-tolerant D-N, and cold-sensitive D-M.

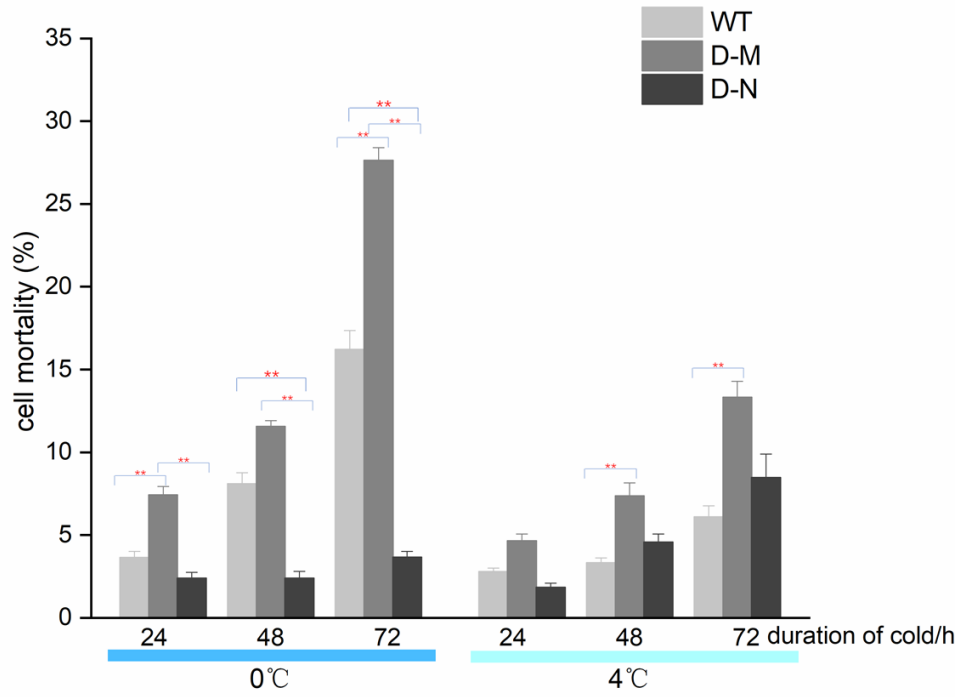

Figure 1. Cell mortality of VvDLKox sub-populations (D-N and D-M), non-transformed BY-2 cells in response to cold treatment (4 ℃ and 0 ℃). Data are shown as means ± SE from three independent biological replicates with 1500 cells each. Asterisk represents a statistically significant difference with Fisher's LSD test (* P <0.05, ** P <0.01).

3.4.2 *VvDLK* expression is not induced by cold stress in D-M but accumulated in D-N by chilling stress.

To assess the role of DLK itself in the cold response, the expression level of *VvDLK* was recorded by time-course of cold treatment. A slight increase of *VvDLK* expression by freezing was observed in D-M, while D-N showed a steady *VvDLK* mRNA level in response to freezing except for an insignificant 100-fold drop at the onset of 24 h freezing (Figure 2). When cells were challenged with chilling stress, D-N performed a remarkably high abundance of *VvDLK* from 24 h until 72 h by about a 250-fold increase, which was distinct from the constant low level of *VvDLK* in D-M (Figure 2), suggesting a putative function of DLK in the nuclear cold signalling transduction.

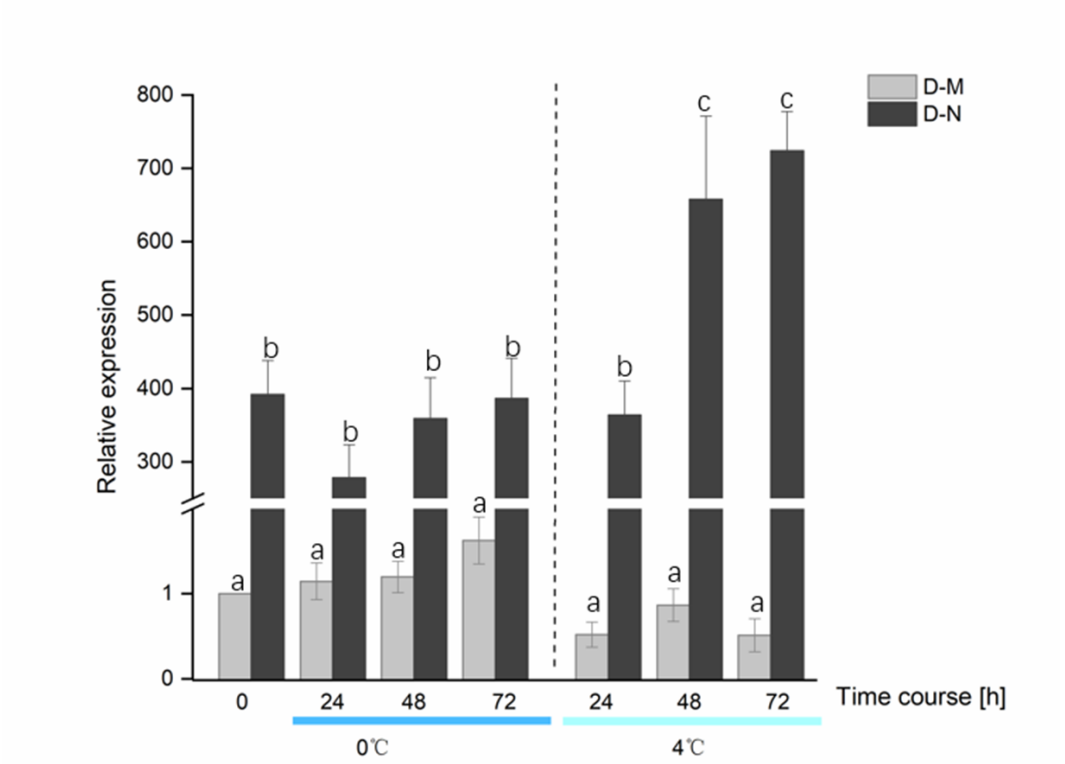

Figure 2. Difference of *VvDLK* transcript level in sub-populations of VvDLKox in response to cold stress. *VvDLK* expression is normalized to it transcript in D-M at room temperature. Different letters indicate the significant difference with Fisher's LSD test ($P < 0.01$) by means ± Standard Error from triplicate biological replicates.

3.4.3 Distinct cold response of two sub-populations is referred to as alteration of endogenous CBF4 by overexpressing *VvDLK*

To understand the difference between two sub-populations overexpressing *VvDLK* in the

expression of cold-induced genes, the expression of endogenous CBF4 (*Avr9/Cf9*) and two COR genes, *NtERD10c*, *NtERD10d* was compared in D-M and D-N in contrast to the wildtype. In general, *Avr9/Cf9* was rapidly and more strongly induced at the first 24 h of freezing than chilling stress in all cells lines and stayed at a high expression within 72 h (Figure 3A). Among those cell lines, D-N was proven as the cold-tolerant phenotype that accumulated much more *Avr9/Cf9* (512-fold) than the other two (64-fold), which exhibited similar expression under freezing stress (Figure 2). The same expression trend of *Avr9/Cf9* was also observed under chilling stress, but at lower *Avr9/Cf9* expression levels (Figure 3A). The difference in a chilling environment was that the *Avr9/Cf9* expression in D-M and D-N exceeded the WT by 4-fold (Figure 1A). For D-M, Avr9/Cf was strongly induced at 24 h of chilling and decreased afterwards, whereas D-N and WT had enhanced *Avr9/Cf9* expression and reached their peaks at the onset of 48 h chilling with a subsequent drop (Figure 3A). Thus, accumulation of DLK in the nucleus facilitates cold-induce *Avr9/Cf9* expression, while this enhanced response of *Avr9/Cf9* is mitigated when DLK has a classical kinesin localization linking with MTs.

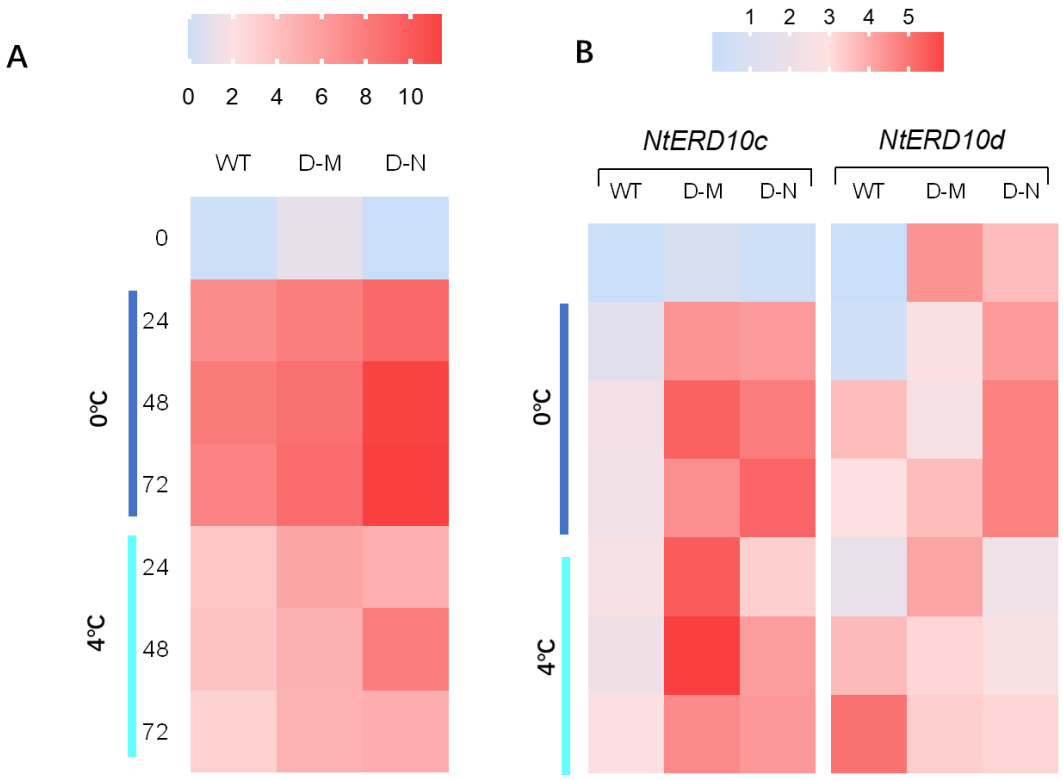

Figure 3. Heatmap of log2 fold change of the steady-state of *Avr9/Cf9*, tobacco ortholog of CBF4 (A) and COR genes *NtERD10c* (B left) and *NtERD10d* (B right) in two sub-populations overexpressing *VvDLK* under cold stress. The colour code represents log2 fold changes in transcript levels normalized with the expression level of noe-transformed wildtype BY-2 under room temperature. Data represent mean values from three biological replicates per genotype and time point.

As mentioned above in the study of the VvCBF4ox line, two COR genes, *NtERD10c* and *NtERD10d* encoding the same protein act downstream of CBF4 and are activated by cold stress

at different levels. To further compare the difference between two sub-populations overexpressing *VvDLK* in cold signalling, the expression of *NtERD10c* and *NtERD10d* was followed in the continuous cold. Compared to the slight increase of *NtERD10c* in WT, D-M and D-N accumulated more *NtERD10c*, especially in D-M the expression of *NtERD10c* was stronger by 8-fold than in the WT (Figure 3B left). The expression of *NtERD10c* in D-N was increased consecutively 32-fold during freezing and varied from 8- to 16-fold in chilling conditions (Figure 3B left). For *NtERD10d* under freezing stress, much more mRNA (varying from 12-fold to 25-fold) was continuously found in D-N than the declined *NtERD10d* transcript levels in D-M by about 4-fold (Figure 3B right), whereas a delayed rise (12-fold) was seen at 48 h of freezing and later followed by a slight drop (Figure 3B right). On the other side, the response of *NtERD10d* to chilling was quite varied in the two sub-populations and WT. The steady decline of *NtERD10d* induction up to 2-fold in D-M differed from the persistent activation of *NtERD10d* from 3- to 32-fold within 72h chilling in WT (Figure 3B right). To sum up, overexpression of *VvDLK* with different localizations leads to an expression difference of endogenous CBFs and COR genes and further impacts the responses to cold stress.

4.4.4 DLK is modulated by translation in temperate conditions, involving in CBF4-independent signalling pathway under cold stress

To investigate the association between CBF4 and *VvDLK*, two inhibitors were used in this work, including CHX (cycloheximide), a protein synthesis inhibitor and AMD (Actinomycin D), a transcription inhibitor and ethanol as the solvent of AMD. By adding the translation inhibitor CHX to cells under room temperature, *VvDLK* expression was abundant in both sub-populations (Figure 4 left). Among them, D-N exhibited more inflexion (32-fold) than D-M (8-fold). Inhibition of the transcription activity in D-N at room temperature alleviated the accumulation of *VvDLK* by addition of ethanol, while no difference in *VvDLK* expression was observed in D-M treated with AMD (Figure 4 left). Once cells were put under 0℃， no expression change of *VvDLK* in the mRNA level was shown in both sub-populations (Figure 4 left). Taken together, when DLK binds to MTs, DLK activity is regulated by translation while DLK localizing in the nucleus alters DLK regulation in both the transcriptional and translational levels but not in cold stress, suggesting that DLK itself possibly is not a cold signalling molecule. Given the data in *VvDLK*, the question was asked whether DLK plays a role in cold signalling by regulating CBF4. By treating the cells with CHX at room temperature, *Avr9/Cf9* transcript levels were increased to 8192-fold in both sub-populations when compared to no effect caused by the transcriptional inhibitor AMD (Figure 4 right). Once cells were treated with cold, *Avr9/Cf9* was strongly induced in two sub-populations by 128 folds, whereas adding AMD caused a reduction of *Avr9/Cf9* expression in two sub-populations, especially in D-M by 3-fold

compared to its expression in the respective solvent control under cold conditions (Figure 4 right). Thus, *Avr9/Cf9* is likely suppressed by some genes regulated in a translation manner in no direct relation to DLK.

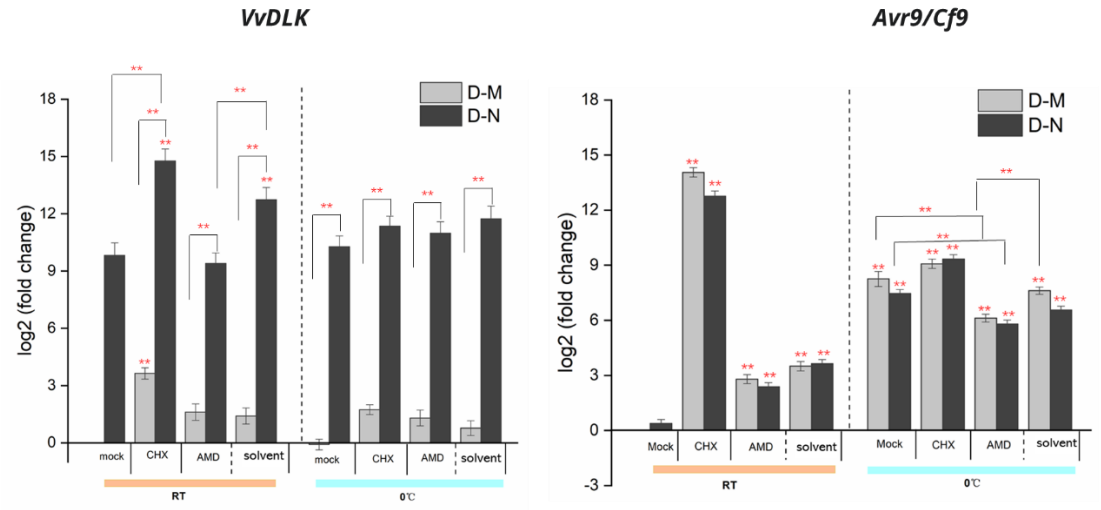

Figure 4. Role of transcription for expression of *VvDLK* and tobacco ortholog CBF4 Avr9/Cf9. Steady-state transcript levels for the foreign *VvDLK* and the endogenous CBF4 transcript *NtAvr9/Cf9* were measured after 24h incubation of VvDLK overexpressor cells D-M and D-N either at 25°C (RT), or freezing (0°C) stress after pre-treatment for 2 h with either the transcription inhibitor Actinomycin D (AMD, 50 µg/ml) or the translation inhibitor Cycloheximide (CHX, 100 µg/ml). The solvent control of AMD was 2.5% EtOH, while CHX was diluted in an aequous stock. Data are normalized to the expression levels under room temperature without any of the inhibitors and represent means±SE of three independent experiments in technical triplicate. Statistically significant difference are represented as different letters with Fisher's LSD test (P <0.01).

4.4.5 DLK's distinct expression between different *Vitis* genotypes

To understand DLK's function in the cold response, the transcript levels of DLK were followed in different grapevine varieties with divergent cold tolerance under freezing (-20℃) and chilling (4℃). In general, the DLK level of all varieties in response to cold stress was quite dynamic (Figure 5). When Pinot Noir was subjected to freezing temperature (-20°C), the transcript level of DLK in vivo was gradually enhanced and peaked at 8-fold at 6 h of freezing before the mild drop to 2-fold at 24 h after the onset of severe cold stress (0°C) (Figure 5). Under chilling stress, expression of DLK in Pinot Noir initially declined about 2-fold at 1 h, and kept increasing afterwards until 24 h by 8-fold (Figure 5). Lower DLK mRNA levels were detected in the cold-tolerant *V. amurensis* than in Pinot Noir under moderate conditions, while DLK in *V. amurensis* was strongly and rapidly induced by 1 h of freezing and stayed at a high induction at 16-fold by 3 h (Figure 5). Interestingly, DLK induction dropped at 6 h of freezing （4-fold） but dramatically rose to 127-fold by 12 h of freezing followed by a drop to normal expression levels at 24 h (Figure 5). Compared to the response to freezing, DLK expression was

continuously high under chilling varying from 4-fold at 6 h to 16-fold at 1 h (Figure 5). Except for the earlier decline of DLK expression in *V. coignetiae* at 3 h freezing (2-fold), DLK transcript in *V. coignetiae* for the remaining time point was gradually rising peaking at 24 h of freezing (127-fold) (Figure 5). The transcriptional changes in *V. coignetiae* under chilling were quite dynamic (Figure 5). An initial increase of DLK was observed at 1 h of chilling (32-fold) and followed by a sharp drop to 1.5-fold at 3 h (Figure 5). Afterwards, the DLK gradually induced until it peaked at 32-fold at 24 h of chilling (Figure 5). Thus, DLK could function as a cold-induced signal molecule for early cold stress, playing a positive regulator in cold tolerance.

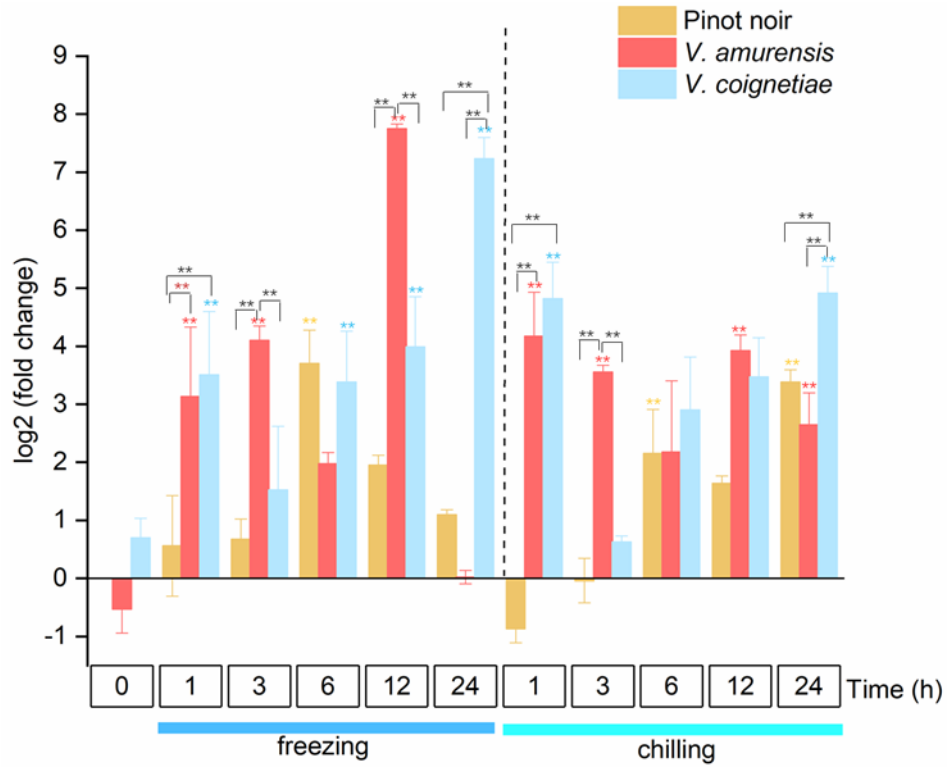

Figure 5. log2 fold changes in steady-state transcript levels of *VvDLK* in different Vitis genotypes in response to cold stress. Plants with the similar growth were challenged by chilling (4℃) and freezing (-20℃). The leaves were sampled at specific time points after cold stress. Data are normalized to the expression level of Pinot Noir under room temperature. Data represent means of triplicate biological replicates, and error bars indicate SE. Asterisk represents a statistically significant difference with Fisher's LSD test (** P <0.01).

4. Discussion

4.1 Chapter 1: CBF4's role in cold stress

To better understand the molecular function of CBF4 in cold signalling, the discussion will be addressed by the following questions:

1. What drives CBF4 different from the other CBF transcript factors?
2. By what mechanism is cold signalling perceived and translated into downstream responses?
3. Which events are shared between chilling and freezing signals and where do they diverge?
4. What is the explanation for CBFs in cold response and plant development?

4.1.1 CBF4, a unique CBF transcript factor

The subcellular localization of the proteins of interest provides the first sight of the estimated function. According to our findings, 35S driven *VvCBF4* fused with GFP localizes at the nuclear rim but not the nucleus in suspension tobacco BY-2, which is contrary to expectations and any previous work in CBFs (Vonapartis *et al.*, 2022; Carlow *et al.*, 2017; Hu *et al.*, 2013; Hua, 2016; Jia *et al.*, 2016; Kidokoro *et al.*, 2015; Li *et al.*, 2017b; Liu *et al.*, 2017; Marozsán-Tóth *et al.*, 2015; Medina *et al.*, 2011; Novillo *et al.*, 2004; Novillo *et al.*, 2007; Song *et al.*, 2021; Stockinger *et al.*, 1997; Sun *et al.*, 2021; Tillett *et al.*, 2012; Xiao *et al.*, 2006; Zhou *et al.*, 2017). This rather contradictory result may be due to gene duplication or promoter scrambling via the random insert of exogeneous *VvCBF4* in cells during the process of GATEWAY cloning (Haake *et al.*, 2002). However, given the nuclear localization signal motif in *VvCBF4*, CBF4 was found to translocate into the nucleus, thus improving cold tolerance or coordinating the stress response with development. This phenomenon of nuclear import is rare but also found in other transcription factors in animals and plants, which are often involved in the signal transduction (Mütze *et al.*, 2007; Smirnova *et al.*, 2000). For example, translocation of the transcription factor STAT5 was suggested to act as an intracellular mediator of leptin signalling in the rat hypothalamus (Mütze *et al.*, 2007). Besides, a cold-induced VaWRKY12 enhancing plant tolerance showed subcellular localization in the nucleus and cytoplasm in temperate environments but only nuclear localization after cold treatment (Zhang *et al.*, 2019). In this study, the nuclear import of CBF4 facilitates the expression of tobacco homologous CBF4 and DREB3 under freezing stress. On the contrary, DREB1 was repressed by CBF4 in freezing response, while the induction of COR genes ERD10c and ERD10d under chilling stress was also

alleviated by CBF4. Previous work suggested BREB1 genes are mainly involved in cold signalling, and DREB3 is possibly connected to osmotic stress in ABA-dependent signalling (Novillo *et al.*, 2004; Vonapartis *et al.*, 2022; Akhtar *et al.*, 2012; Nasreen *et al.*, 2013). Here, DREB3 as a cold-induced transcription factor as well as DREB1, the negative regulator interplay with CBF4 to improve freezing tolerance, while enhanced CBF4 expression act as a negative feedback loop to negatively regulate COR genes, in return attenuating stress responses in mild cold condition, which is consistent with recent work by Vonapartis (Vonapartis *et al.*, 2022). However, several questions are remaining unanswered in our work, such as experimental evidence for an interaction of DREBs, and CBF4's role in plant development, which needs to be further investigated.

In previous studies, *Arabidopsis* CBF4 unlike CBF1, 2 and 3 was induced by drought stress but not at low temperature, however, constitutively overexpressing CBF4 retained higher expression of COR genes without cold or drought stimulus and conferred more cold and drought tolerance (Haake *et al.*, 2002). Conversely, the identification of CBF4 in grape showed CBF4 was activated by cold rather than drought or salt stress (Xiao *et al.*, 2008). Subsequently, CBF4 from *V. riparia* overexpressed in *Arabidopsis* lines represented highly freezing and drought-tolerant similar to VrCBF1 transgenics. Better membrane stability indicated a positive role of CBF4 in cold hardiness (Siddiqua and Nassuth, 2011). Moreover, CBF4 from *Populus euphratica* exhibited greater tolerance to multiple stresses, including osmotic, salt and cold stress, while overexpressing *Solanum tuberosum* CBF4 displayed higher tolerance than the CBF1 overexpressor (Tian *et al.*, 2017; Song *et al.*, 2021). In fact, at first sight, those inconsistent results illustrate the plant's sophisticated mechanisms when responding to abiotic stress with substantial overlaps in the downstream signalling, such as a redundancy of CBFs and regulons (Lamers *et al.*, 2020; Zhang *et al.*, 2020). Thereby, CBF4s are likely recruited for various abiotic stresses.

Differing from CBF1-3, CBF4 is induced by severe cold and stays at a relative high for longer than days, especially when compared to the several hours of high expression of CBF1 and CBF2 (Xiao *et al.*, 2008; Akhtar *et al.*, 2012; Carlow *et al.*, 2017). The difference in the spatial-temporal expression of CBFs has been demonstrated under cold stress. In poplar, CBF1 and PtCBF3 were detected in many tissues including leaves, stems and dormant buds, differing from the leaves-specific expression pattern of CBF2 and CBF4 (Sterky *et al.*, 2004; Benedict *et al.*, 2006). *Vitis* CBF1-3 activation was only detected in young leaves and buds, while CBF4 accumulated in both young and old leaves (Xiao *et al.*, 2008). In *Arabidopsis*, CBF2 negatively regulates CBF1 and CBF3, which can concertedly induce all CBF regulons (Novillo *et al.*, 2007). In our work, CBF4 expression was weakly induced by chilling and stayed at low transcript levels under freezing, which contrasted with cold tolerance. This discrepancy could be attributed to

posttranscriptional regulation of CBF4 as observed in prior work in DREB2 (Liu *et al.*, 1998; Shinozaki and Yamaguchi-Shinozaki, 2000). On the other hand, we also found that foreign *VvCBF4* interplay with other tobacco endogenous CBFs. There are two possible explanations for this result. A possible explanation might be the interference of native interacting proteins with endogenous CBF4 by ectopically overexpressing *VvCBF4*. The other one is ectopic and overexpression of *VvCBF4* may cause the imbalance of stoichiometric, such as insufficiency of availability of previous interactors. Therefore, loss-of-function mutants of CBF4 accompanied with overexpressing CBF4 may offer more adequate evidence of CBF4 function *in vivo* in cold signalling.

4.1.2 Signal perception of chilling and freezing

More is known about cold transduction, but the mechanisms of how plants sense fluctuating ambient temperature and precisely trigger the respective signalling cascades remain elusive. In plant cells, the components of the cytoskeleton such as MTs, plasma membrane as well as plasma membrane-localized Ca^{2+} sensors and ion channels are intimately interconnected to interpret extracellular signals (Conde *et al.*, 2011). The transient microtubules disassembly is required and proven as the initial response of cold signal perception (Wang and Nick, 2017). Those events intricately overlap and interweave with one another to transduce the cold signal from the extracellular space. For instance, by application of pharmacology, membrane rigidity has proven to be required and sufficient to trigger MTs disassembly, whereas NADPH oxidase activity and Ca^{2+} influx are necessary for cold-induced disassembly of MTs (Wang and Nick, 2017; Wang *et al.*, 2019). DMSO as the solvent control in this study can induce COR genes at moderate temperatures because DMSO can pharmacologically mimic cold stress to cause depolymerization of microtubules (Wang *et al.*, 2019). Besides, the unaltered expression of CBF4 is consistent with previous study that CBF4 expression is independent of MTs (Wang *et al.*, 2019). Damage in the membrane integrity at low temperatures can trigger Ca^{2+} influx coupled with the opening of ion channels mediated by H^+-ATPases (Wang *et al.*, 2020). The tight link between Ca^{2+} activity and CBF4 expression has been verified before (Wang *et al.*, 2019). To explain the calcium signalling in cold perception, studies have found several Ca^{2+} channels (Guo *et al.*, 2018; Ding *et al.*, 2020). Among them, in rice, the COLD1/RGA1 complex, a cold sensor has been used to uncover that plasma the membrane-localized COLD1 interacts with the G-protein to activate the Ca^{2+} channel for chilling signal sensing. In *Arabidopsis*, CRPK1 interacting with receptor-like kinase (RLK) forms a complex to perceive the freezing signal and modulates CBF genes (Ma *et al.*, 2015; Liu *et al.*, 2017). In our study, lower apoplastic alkalinization by overexpressing CBF4 indicates enhanced freezing tolerance and membrane integrity. Moreover, cytosolic [Ca^{2+}] measurements further confirm the intensity of the cold stress and the magnitude of cytosolic [Ca^{2+}] are directly correlated (Umezawa *et al.*, 2010). On

the contrary, the dynamic nuclear [Ca^{2+}] in response to freezing in CBF4ox shows CBF4's positive role in nuclear freezing signalling, while various responses of wildtype BY-2 and CBF4ox to chilling imply that CBF4 may modulate Ca^{2+} signalling in return. Recently found of nuclear-localized cyclic nucleotide-gated channels shed light on studying the link between CBF4-dependent signalling and nuclear Ca^{2+} (Charpentier *et al.*, 2016). In conclusion, there still is plenty of potential to find more proteins or channels that contribute to the exquisite sensing systems in plants.

4.1.3 Two distinct signalling pathways in plants under chilling and freezing stress

The capacity of cold tolerance in plants shows diversity between not only species but also varieties, for example, the lethal temperature for cucumber is +10℃, while winter wheat can survive starting at -10℃ of ambient temperature (Wang *et al.*, 2020). Moreover, in grapevine, as one of the most important cultivated fruits, 50% of bud damage is caused from -15℃ to -23℃ varying in different cultivars. However, *V. amurensis* can withstand harsh winters at -40°C (https://wine.wsu.edu/extension/weather/cold-hardiness/) (Zhang *et al.*, 2012). Thus, understanding how plants adapt and acquire various cold tolerance will shed light on facilitating plant development via improved hardiness.

Prior studies have classified cold stress into two distinct signalling pathways based on two typical plants, rice as a cold-sensitive crop to study chilling response and the insensitive *Arabidopsis* as material for freezing signalling, which results in intricate and even contradictory conclusions (Guo *et al.*, 2018). In this study, to compare CBF4-dependent chilling and freezing signalling pathways in the same model, tobacco has been chosen as the organism to investigate the different signal transductions. Enhanced freezing tolerance in the ectopic overexpressing *VvCBF4* in this study corroborates these earlier findings, which have shown the positive role of CBF4 in freezing tolerance, which is further confirmed by higher expression of CBF4 in leaves of cold-resistant *V. amurensis* than the cold susceptible *V. coignetiae* under freezing stress (Song *et al.*, 2021; Sun *et al.*, 2021; Tillett *et al.*, 2012; Xiao *et al.*, 2008; Zhang *et al.*, 2016). Thus, CBF4 is more likely involved in freezing than chilling signalling. Upstream of CBF4 expression, prior work showed that a Ca^{2+} influx is responsible for CBF4 activation under freezing stress (Wang *et al.*, 2019). In addition to calcium, this work showed ROS and MAPK may be involved in freezing signalling, however, they are independent of CBF4. The mitochondrial superoxide in CBF4ox under freezing was accumulated more than in non-transformed BY-2, which is contrary to existing studies showing scavenging cytosolic ROS in cold-tolerant plants (Peng *et al.*, 2016; Zuo *et al.*, 2020). The MAPK cascade is a complex pathway that integrates external signals and internal cell responses in multi-stress scenarios

(He *et al.*, 2020). Recent studies established MPK3 and MPK6 being activated by freezing can negatively regulate CBF1-3 expression by phosphorylating and degrading ICE1, while MPK4 constitutively suppresses MPK3 and MPK6 activity (Zhao *et al.*, 2017b). However, whether CBF4 expression depends on the MAPK cascade remains unknown.

In CBFs-dependent signalling, plenty of works on ICE1 have established the essential effect of ICE1 on CBF1, 2 and 3, however, here ICE1 is also proven to act upstream of CBF4, positively modulating CBF4 expression in both freezing and chilling signalling (Ding *et al.*, 2015a; Feng *et al.*, 2013; Li *et al.*, 2017a; Zhan *et al.*, 2015; Zhang *et al.*, 2017; Zhao *et al.*, 2017b). Furthermore, the treatment with phenidone, an inhibitor of JAZ biosynthesis reveals CBF4 activation is strongly suppressed by JAZ under room temperature and freezing stress. A possible explanation for this might be the interaction of JAZ1 and JAZ4 with ICE1 to repress its transcriptional activity (Makhazen *et al.*, 2021; Hu *et al.*, 2013) (Makhazen *et al.*, 2021; Hu *et al.*, 2013). Or JA directly or indirectly acts upstream of CBF4 to participates in regulating CBF4 expression. As downstream target genes, *NtERD10a*, *NtERD10c* and *NtERD10d* encoding ERD10 (early response to dehydration 10) proteins, also known as group 2 LEA (late embryogenesis-abundant) proteins belong to the dehydrin family, which confers the plant tolerance under various stresses by diverse biochemical activities including sequestering ions, stabilizing membranes, buffering water or as chaperones (Rorat, 2006; Kovacs *et al.*, 2008). In this study, COR genes *NtERD10c* and *NtERD10d* but not *NtERD10a* were activated by both chilling and freezing stress. However, CBF4 may restrain its activation in chilling response due to ERD10's function in tradeoff the stress response with the development in mild cold conditions (Kim and Nam, 2010). Hence, the module of the ICE1-CBF4-COR genes has been proven in this work. However, to get insight into the interaction of CBF4 and ICE1, additional studies on the ICE1-CBF4 module *in vitro* and *in vivo* will be needed. Moreover, there are about 4000 COR genes induced after 4 °C for 24-h in *Arabidopsis thaliana* (Zhao *et al.*, 2016). Among them, about 90% are regulated by CBF1-3, which are partly redundant in regulating similar COR genes, indicating that differential expression patterns of CBFs may play an essential role in the plant cold defence (Liu *et al.*, 2019). To have a further insight into the mechanism of plant cold response, it's necessary to find more upstream regulators of CBF-independent COR genes.

In chilling signalling, Ca^{2+}, ROS and MAPK act upstream of CBF4 leading to the activation of CBF4 and thus inducing the expression of CBF4 downstream COR genes *NtERD10c* and *NtERD10d*, which is consistent with previous studies (Wang *et al.*, 2019; Wang and Hua, 2009; Hussain *et al.*, 2018; Ruelland and Collin, 2012; Cheng *et al.*, 2007; Kasuga *et al.*, 2004). However, higher accumulation of mitochondrial superoxide in constitutive expression of CBF4 contradicts previous work about attenuated ROS generation in cold-tolerant plants, perhaps

because superoxide is rather a signal molecule rather than leading to oxidative stress causing damage in the cell (Bai *et al.*, 2018; Ma *et al.*, 2021; Wei *et al.*, 2016). On the other hand, staying at high levels of superoxide after 48h chilling with down-regulated CBF4 expression indicates CBF4 may be a positive feedback signalling of ROS.

4.1.4 The tradeoff of CBFs for plant cold response and development

Cold stress delays plant growth by affecting several cell homeostases, such as oxygenic photosynthesis. PHYTOCHROME B (phyB) perceiving red and far-red wavelengths can rapidly translocate from the cytoplasm to the nucleus to directly interact with PHYTOCHROME-INTERACTING FACTORs (PIFs), leading to the initiation of a phytochrome-mediated light response (Jiang *et al.*, 2020). To control plant growth during cold stress, cold-induced CBFs interact with PIF 3 and repress the co-degradation of the PIF3–phyB module (Jiang *et al.*, 2020). On the other side, PIF3, PIF4, and PIF7 can also restrict the expression of CBF genes under low temperatures to avoid an excessive cold response as well as growth repression (Jiang *et al.*, 2020). Moreover, the accumulation of intracellular abscisic acid (ABA) in response to abiotic stresses has been studied as an important event for plant physiological adaptations, including stomatal closure, growth inhibition and activation of stress-inducible genes (Knight *et al.*, 2004). Therefore, the application of ABA is sufficient for the expression of CBF1-3 and cold-induced COR genes, whereas the CBF4 level is also elevated by ABA as well as drought stress (Haake *et al.*, 2002; Knight *et al.*, 2004). In addition, the miR396 growth-regulating factors (GRF), participate in plant growth and development for almost all of the life cycle and extensively coordinate with other hormones such as gibberellin (GA), auxin as well as brassinosteroids (BRs) during abiotic stress (Zhang *et al.*, 2020). In a previous study, GRF7 directly can bind to DREB2A and repress its activation in response to osmotic and heat stress (Kim *et al.*, 2012). For GA, CBF3 has been proven to delay plant growth by resulting in an accumulation of DELLA proteins, which are key negative elements of GA signalling, while DELLAs positively regulate CBF1-3 induced by cold in the JA-dependent signalling (Zhou *et al.*, 2017). BRs, the steroid plant hormones for plant growth-promoting can modulate plant growth during stress, for example, BRs enhanced freezing tolerance by partly activating CBFs and downstream COR genes (Eremina *et al.*, 2016). Subsequently, two pivotal transcript factors, brassinazole-resistant1 (BZR1) and BRI1-EMS-suppressor 1 (BES1) directly bind to the promoters of CBF1 and CBF2 *in vitro and in vivo* to positively regulate their expression, thus leading to an improved cold tolerance (Li *et al.*, 2017c). However, as a special CBF gene, the study of CBF4 had focused on its effect on abiotic stress response. Thus, for a better understanding of CBF4 in future work it is urgent to explain the crosstalk of CBF4 and plant growth in a tissue- and temporal-specific manner.

4.2 Chapter 2: Does DLK play a role in cold signalling?

DLK, as a kinesin-14 motor protein in plants seems be unique and essential because of its unconventional localization and modulation of CBF4(Xu *et al.*, 2022). In our work, the questions were arisen and anwered in below:

1. What's the localization of DLK homologue in grape (VvDLK)?
2. What's the functional difference caused by different localizations of kinesin proteins?
3. Why does VvDLK appear into two localizations?
4. Does VvDLK involve in cold signalling? How?

4.2.1 The heterogeneity of kinesins' subcellular localization confers functional diversity

Study on chicken kinesins have indicated a diversity of kinesin distribution differing extensively between cell and tissue types, whereas 68% of fibroblast kinesins are soluble and 32% are membrane- or organelle-associated kinesins, but none in cytoskeletal fractions (Hollenbeck, 1989). And the subcellular localization of kinesin with calponin homology (KCH) belonging to the kinesin-14 family has been found to vary with the species, for example perinuclear actin filaments and phragmoplast in *Arabidopsis*, actin and cortical microtubule filaments in cotton and rice perinuclear actin filaments in tobacco (Xu *et al.*, 2007; Preuss *et al.*, 2004; Frey *et al.*, 2010; Klotz and Nick, 2012). The plant kingdom is comprised of 13 kinesin families from classified around 17 families, including kinesin-1, -4, -5, -7, -12, -13, -14 and so on (Ali and Yang, 2020). Among them, plant kinesin-5 and kinesin-14 subfamilies are mitotic kinesins, which share similar functions with their orthologs in fungi and animals, while, others are plant-specific (Li *et al.*, 2012). Animal kinesin family member 12 (Kif12) such as Kif12 and Kif15, display primary functions in mitosis, including mediating the assembly of the bipolar spindle and chromosome movement (Sturgill *et al.*, 2014; Drechsler and McAinsh, 2016; Lakshmikanth *et al.*, 2004). Unlike their animal orthologs, plant kinesin-12 PHRAGMOPLAST ORIENTING KINESINS (POKs) mainly related to cell division are expanded and heterogeneous, localizing in the midzone of the cytokinetic apparatus, and/or the cortical division zone (Müller and Livanos, 2019). *Arabidopsis* Kin12A, Kin12B and POK2/Kin12D decorate the midzone of the phragmoplast during late anaphase and vanish until the phragmoplast disassembles at the end of cytokinesis (Herrmann *et al.*, 2018; Pan *et al.*, 2004). In addition to that, those Kin12 localizing to the midzone of the phragmoplast also interact with microtubule-associated proteins 65-3 (MAP65-3), which is essential for MTs stability and phragmoplast integrity

(Gaillard *et al.*, 2008; Ho *et al.*, 2012; Müller *et al.*, 2004). In this work, the *Vitis* homologue of ATK1,5 in *Arabidopsis* (*VvDLK*) exhibits uncanny dual localization, on MTs (D-M) and/or in the nucleus (D-N). Some cases in animals showed an indirect link between kinesin, MTs and nuclear localization. For example, the depolymerization of MTs but not actin inhibited the nuclear delivery of human HHV-8 viral DNA and infection, implying the relationship between MTs and virus-induced nuclear trafficking (Naranatt *et al.*, 2005). The plus-end directed Kinesin 1, 3 as well as cytoplasmic dynein interacted with nesprin-1 and -2 to modulate nuclear positioning (Tsai *et al.*, 2010; Wilson and Holzbaur, 2015; Fridolfsson and Starr, 2010). Moreover, kinesin1 and dynein were required for the early infection stage of human HIV1, which binds with kinesin-1 adaptor FEZ1 for moving to the nucleus, indicating the implicative link between kinesin and nucleus (Malikov *et al.*, 2015). Beyond that, several studies on the kinesin-6, -8 and -14 families showed nuclear sequestration during interphase, whereas the Kinesin-7 motor CENPE was restricted in the cytoplasm until the nuclear envelope breakdown (Goshima and Vale, 2005; Liu and Erikson, 2007; Cai *et al.*, 2009; Brown *et al.*, 1996). The explanation for this nuclear sequestration is probably their interactions with nuclear import proteins such as importin-α and importin-β (Verhey and Hammond, 2009). In yeast, kinesin-8 Klp5 and Klp6 were exported to the cytoplasm but entered into the nucleus during mitosis (Unsworth *et al.*, 2008). Furthermore, a rice homologue of the *Arabidopsis* kinesin-14 *ATK1* and ATK5 *OsDLK* had cortical MT-localization, but shifted into the nucleus in interphase, providing more evidence for the kinesin's nuclear localization in higher plants (Xu *et al.*, 2018b). However, the mechanism for this phenomenon remains unknown, which is assumed to be linked to signal transduction.

4.2.2 Dual localization of VvDLK endows two phenotypes

Unlike the permanent single localization of general genes, more and more genes with dual localization or translocations are gradually studied in multiple species. In the human genome, the TOP3 α gene was found to encode a DNA topoisomerase III α in mitochondria and the nucleus, just like the gene encoding the human RNA helicase MDDX28 (Valgardsdottir *et al.*, 2001; Wang *et al.*, 2002). This dual localization was also observed in the yeast system, which is essential for cell survival (Nakai *et al.*, 2001). In the plant kingdom, a maize DWARF1 encoding the GA 3-oxidase presented the dual localization in cytosol and nucleus for bioactive GA synthesis (Chen *et al.*, 2014). Here, we pointed out that DLK refers to two sub-populations: D-N, D-M with two different localizations, DLK in the nucleus and DLK in the nucleus and merging with MTs, respectively. This gives the two sub-populations two phenotypes, cold-tolerant D-N and cold-susceptible D-M. One explanation might be that DLK can be recognized by two receptors on MTs and the nuclear envelope, as learnt from prior work (Addya *et al.*, 1997). DLK contains an MT-binding motif, allowing co-localization with MTs, which explains D-

M's location. On the other side, DLK also has two predicted NLS for a nuclear destination. However, DLK as a kinesin is supposed to bind with MTs, which is not observed in D-N. Therefore, for entering the nucleus, DLK could be spliced in D-N. Based on this assumption, the nuclear protein fraction has been tried, but unfortunately failed. Thus, further work should be done by extracting nuclear proteins from D-M and D-N comparing them to the cytoskeleton protein in D-M to see the difference in molecular weight. And the translocation through nuclear membranes into nucleus should be supported by specific import machineries, which also need further investigation.

According to previous studies, the molecular explanation for this difference can be on the mRNA level (transcription initiation or alternative splicing), translation initiation or post-translational modification (PTM) (Yogev and Pines, 2011; Karniely and Pines, 2005). The enormous difference between D-N and D-M was obtained in transcript and translation levels: about 400-fold DLK mRNA in D-N compared to D-M and unknown 90kD bands in D-M detected by tubulin antibodies. The difference in transcript levels was partly consistent with previous work, which proved the difference in localizations was coupled with the content of DNA (Wang *et al.*, 2002). The mysterious 90kD fraction of soluble proteins could be a stubborn complex comprised of part DLK and MAPs or MTs dimers. The difference of amino acids in 90kD by overexpressing *VvDLK* between D-N and D-M was compared to non-transformed BY-2, by which some proteins with distinct or specific expression have been addressed. In future investigations, it might be possible to select interacting proteins and follow their function. Besides, DLK binding to MTs leads to a decrease of tyrosinated α-tubulin as well as β-tubulin in D-M and an increase of detyrosinated α-tubulin. This phenomenon was also observed in a rice tubulin tyrosine ligase (TTL), which can modulate phospholipase D (PLD) activity and tubulin synthesis (Zhang *et al.*, 2022; Zhang *et al.*, 2021). However, the mechanism for this change is still lacking.

4.2.3 The possible crosstalk of kinesin and plant development and signalling pathway

The kinesin-14 family, a rare kinesin family in plants with a minus-end direction, may substitute the function of dynein motors, which are absent in plants and responsible for intracellular long-distant transport of multiple molecules. The mRNA level of KP1, a plant-specific kinesin-14 family motor, was significantly increased from quite a low level in *Arabidopsis* by cold treatment, implying the furtive association between the kinesin-14 family and the stress response (Li *et al.*, 2008; Ni *et al.*, 2005). Subsequently, *AtKP1* specifically co-localizes and interacts with voltage-dependent anion channel 3 (VDAC3) proteins on the outer membrane of mitochondria to regulate the ATP level, thus modulating mitochondrial respiratory and alternative oxidase (AOX) during seed germination (Yang *et al.*, 2011). In prior studies on fungi

and animal cells, VDACs could form large aqueous pores through membranes to regulate the transport between the cytosol and mitochondria by switching between two stages, such as a flux of ATP/ADP at the closed stage, Ca^{2+}, and other metabolites at the open stage (Lemasters and Holmuhamedov, 2006; Rostovtseva and Colombini, 1996). Moreover, tubulin could reduce the respiratory rate of mitochondria by the reversible blockage of VDACs (Rostovtseva *et al.*, 2008). Given the crucial role of Ca^{2+} and ROS during abiotic stress, those results indicate a probable crosslink between kinesin-14 KP1 and the abiotic stress response. In our work, two sub-populations represented opposite cold tolerances correlating to different CBF4 expression, DLK decorating MTs is cold-resistant while DLK only in the nucleus conferred extremely high cold hardiness. The DLK transcript was up-regulated by long-term chilling, even DLK itself is regulated on the translational level regardless of the localization. Higher transcript levels of DLK with an earlier peak were found in cold-tolerant *V. amurensis*, suggesting DLK functions in cold signalling. However, there are still questions remaining, for example why *VvDLK* is in the nucleus, whether *VvDLK* itself is a signal molecule and how and what *VvDLK* interacts with in cold signalling. Those questions are worthy of further investigation.

The previous works have offered some ideas about kinesin-14 in the cellular processes, which may answer those questions. In animal cells, the activation of MAPK signalling has negative implications for the interaction of Kinesin-1, 2 motors with their cargoes (Verhey *et al.*, 2001; Byrd *et al.*, 2001; Horiuchi *et al.*, 2007; Bengs *et al.*, 2005; Berman *et al.*, 2003; Burghoorn *et al.*, 2007). Furthermore, works in mammalian neurons have established that Ca^{2+}/calmodulin-dependent protein kinase II promoted the dissociation of Kinesin-2 motors from their cargoes and also Kinesin-4 motor KIF4 from its binding partner, which resulted in the entering of the binding partner into the nucleus for participating in DNA repair as well as transcriptional regulation (Guillaud *et al.*, 2008; Midorikawa *et al.*, 2006). Besides, the high abundance of OsKCH1 was found in the young meristem implying the possible function of KCH1 in cell division and development (Frey *et al.*, 2010). The expression of *OsDLK* was modulated by red light and linked with JA and auxin (Xu *et al.*, 2018a). *OsBC12*, a rice cytoplasmic kinesin with localization of both cytoplasm and nucleus belonging to the kinesin-4 family has been proven as a transcriptional regulator for gibberellic acid (GA) biosynthesis, which was consistent with the short grain length (SGL), a kinesin-like protein that mainly localizes in the nucleus (Li *et al.*, 2011; Wu *et al.*, 2014b; Zhang *et al.*, 2010). The addition of phenidone, an inhibitor of JA biosynthesis in this work showed no effect on the DLK transcript levels (supplementary figure 2). Given by DLK is regulated protein level, the possible explanation is the interaction of JA and DLK may be indirect and purified DLK protein is needed to test this interaction. Besides, DLK in nucleus could be a good material to learn the domains' function in nuclear transport and find the transporters or channels for translocation from cytosol to nucleus.

4.3 Chapter 3: What's the correlation of DLK and CBF4?

In this work, we showed the different expression level of tobacco CBF4 *NtAvr9/Cf9* may contribute to the distinct cold tolerance between D-N and D-M, which suggest the VvDLK's subcellular localization may alter the cold-induced transcript factor CBF4, resulting the difference of COR gene expression (results **3.4**.**3**). On the other hand, comparing the differential expression of DLK in different grape genotypes (increased high DLK in cold tolerant *V. amuransis* under freezing stress) to CBF4 expression in grape leaves indicates a potential regulation of DLK in cold response achieving by CBF4 (results **3.1.8**). The previous work on OsDLK also showed that Overexpression of OsDLK suppressed CBF4's expression under chilling stress (Xu *et al.*, 2022).

How does DLK changes CBF4 expression? Recombinant expression of OsDLK bound to a specific DNA motif, a target for transcription factor OPAQUE 2, which is a transcriptional key regulator of zein genes, moving into the nucleus after ubiquitination by a E3 ubiquitin ligase RFWD2 (Li *et al.*, 2020). Besides, RFWD2 is phosphorylated and degraded by the sucrose-responsive kinase SnRK1, which interacts with with other signaling molecules to regulate stress response and development (Martínez-Barajas and Coello, 2020). In cold signalling, the phosphatases ABI1 and PP2CA negatively regulate SnRK1, resulting in the release of the kinase OST1 from the membrane into cytosol to activate ICE1, inducing the expression of CBFs and downstream COR genes in the nucleus (Rodrigues *et al.*, 2013; Ding *et al.*, 2015b). Moreover, different translocational proteins has been found such as 14-3-3 and OST1 and proved indirectedly or directedly regulate CBFs expression, which shed light of the essential function of the proteins with multiple intracellular localization in external stumuli response.

5. Conclusion and Perspectives

The main aim of this study was to uncover the molecular function of CBF4 in cold stress and elucidate the distinction between chilling and freezing signalling. Thus, an overexpressor of CBF4 from *Vitis Vinifera*. CV Pinot Noir in tobacco BY-2 was generated and fused the GFP at its C-terminus. First of all, *VvCBF4* showed the unexpected non-nucleus localization (cytoplasm) but can shift into the nucleus in present of low temperature, which is unlike other nuclear-localized CBFs. Moreover, this translocation of VvCBF4 was proven to be required to regulate endogenous CBFs and cold responsive (COR) genes ERD10s. For cold tolerance, *VvCBF4* mitigates mortality inflicted by cold stress with enhanced expression under chilling stress. On the contrary, CBF4 orthologue in tobacco *Avr9/Cf9* is strongly induced by freezing than chilling, indicating the different expression patterns between foreign *VvCBF4* and native CBF4. Further results supported function of *VvCBF4* in cold signaling is irreplaceable by *Avr9/Cf9*. Besides, the transcript differences of endogenous CBFs and COR genes between VvCBF4 overexpressor and non-transformed wildtype BY-2 revealed the ectopic overexpression of *VvCBF4* altered their expression patterns. Taken together, these findings suggest that CBF4 achieves its role in the chilling response by interplaying with other CBFs and activating specific downstream COR genes. One the other side, our data suggested there are two distinct signalling pathways, consisting of different signal molecules. Unfortunately, the study lacked the evidence to explain whether CBF4's unusual localization is attributed to the alteration of endogenous genes by ectopic overexpression of CBF4. To answer this question, it's necessary to overexpress *VvCBF4* in various systems. The question was also raised whether CBF4 directly interacts with other CBFs to regulate their expression in response to different qualities of cold. Meanwhile, to further establish CBF4's role in cold stress, the establishment of a mutant of CBF4 is required.

Given by the CBF4's function in cold tolerance, to make sure the activation of CBF4 is sufficient and precise, the external cold requires to be conveyed from cytoskeleton to the nucleus the plasma membrane. DLK in rice, as a motor protein moves along MTs and regulate CBF4 expression under chilling stress, may provide a possibility for integration of cold perception and transduction in plant.

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The putative *OsDLK* (homology of Kinesin-14, ATK1, 5), *F6HRV2* was found in *Vitis* with two distinct localizations, creating two phenotypes, thus named *VvDLK*. One is in the nucleus (D-N), behaving resistant to cold stress. The other is a *VvDLK* that decorates microtubules and appears inside of the nucleus (D-M), proving to be a cold-sensitive phenotype. To figure out the possible reasons for this phenomenon, the mRNA levels, the protein expression of DLK as well as various cold-induced genes were investigated. D-N harbours extremely high DLK mRNA

levels, while a mysterious heavy band (90kD) is tightly bound to microtubules in D-M, indicating a molecular variety in two sub-populations caused by different subcellular localizations of this kinesin. On the other hand, when DLK is expressed only in the nucleus high amounts of tobacco CBF4 is accumulated in response to severe cold, which results in high expression of the COR gene *ERD10d*. But in contrast, D-M exhibits the same CBF level and lower *ERD10d* than the wildtype. As a kinesin, *VvDLK* is modulated by translation with or without cold treatment. Taken together, we explained the variation between two sub-populations of *VvDLK* and presented distinct cold responses by two localizations. Meanwhile, this research has surfaced many questions in need of further investigation. First, what is the 90kD band strongly binding to microtubules? Is it a complex? Nano-HPLC/nano-ESI-LTQ-Orbitrap-MS/MS analysis could properly answer this question. As to identify target proteins of DLK signalling, we have conducted a comparative nuclear proteome analysis of overexpression DLK lines (D-N and D-M) and non-transformed wildtype, leading to several interesting proteins that show strong differences in abundance (Figure S3). Among those proteins, a novel pentatricopeptide repeat-containing protein LOC107786373 (XP_016463327.1.) is of special interests, because it exhibits extremely high protein abundance (8.6-fold) in D-N line compared to wildtype. This protein has been proven that mediate various gene expression not only in organelles also in the nucleus, which may explain the different CBF4 or COR genes in VvDLK overexpressor (Manna, 2015). Future studies will be pursued in to get insight into the functional relevance of the DLK-dependent signalling pathway.and find out the potential reasons for DLK different phenotypes. The next question should be what is the function of this microtubule-specific protein in cold signalling? Can it indirectly or directly interact with CBF4? Besides, what is DLK doing in the nucleus? Recently, we have purified nuclear and cytosolic proteins from D-N and D-M and realized DLK may be alternatively spliced or splited in nucleus. Whether it links with cold response needs to be further investigated. At last, more work will be needed to investigate the interplay of kinesin in the abiotic or biotic response.

6. Appendix

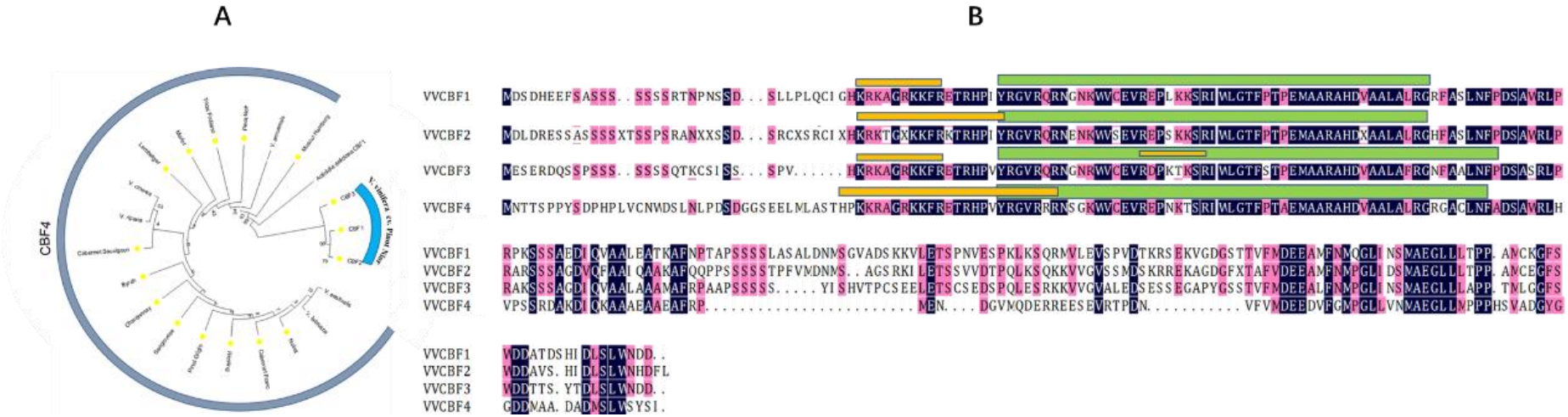

Figure S1. Sequence comparisons for Cold Box Factor 4 (CBF4) from Vitis vinifera. A Phylogenetic relationship between CBF4 from different varieties of V. vinifera (yellow circles) along with some wild species of Vitis, compared to the other CBF proteins from V. vinifera cv. Pinot Noir as outgroup (blue circle). B Alignment of the CBFs from V. vinifera cv. Pinot Noir. Identities are highlighted in black, similarities in magenta. The AP2/ERF DNA-binding domain is marked by the green rectangle, while the orange rectangles indicate the predicted nuclear localisation signals (NLS).

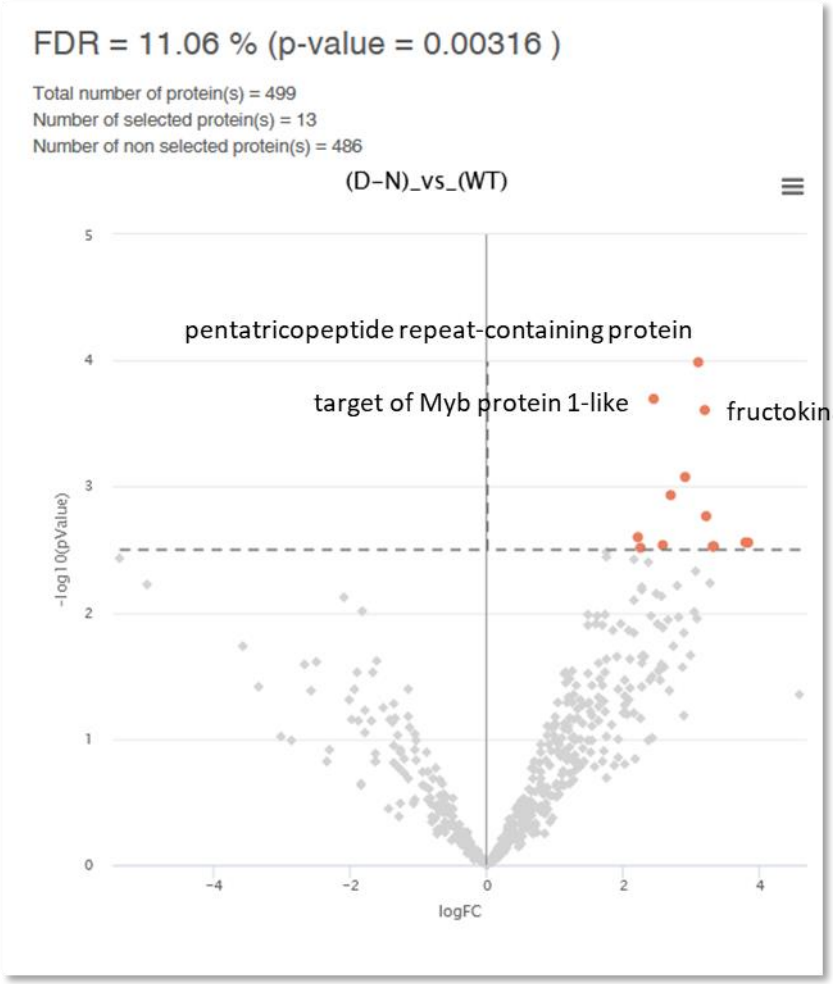

Figure S2. Proteomics analysis of protein differential expression in D-N compared to non-transformed wildtype. A total of 486 proteins were present with a filter of at least 4/4 values in one condition. 13 proteins are differentialy expressed (only overexpressed proteins here) with a FDR >11%, which are marked in red.

Application	Gene		Sequence	TA℃
clone of full-length cDNA	*VvCBF4*	forward	ATGAATACTACTTCTCCACC	55
		reverse	AATAGAGTAACTCCATAACG	
	VvDLK	forward	ATGGCTTCCCGTAATCAGAAC	55
		reverse	AGCTCAAGCGAGAATCTGAAA	
GATEWAY clone	*VvCBF4* C-terminal	forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAATACTACTTCTCCACCATATTC	55
		reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCAATAGAGTAACTCCATAACGACA	
	VvDLK N-terminal	forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCTTCCCGTAATCAGAA	55
		reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAGCCATAGCTCAAGCGAG	
RT-qPCR	*NtL25*	forward	GTTGCCAAGGCTGTCAAGTCAGG	57-61.5
		reverse	GCACTAATACGAGGGTACTTGGGG	
	VvCBF4	forward	AGGCGTTTAGGCCAATGGAG	61.5
		reverse	AACACATTATCCGGCGTCCT	
	Avr9/Cf9	forward	AAGAGGAATTCAGACAAGTG	57
		reverse	AAAGTTCAAGCAAGCAGAAC	
	NtDREB1	forward	CAGGTAAGTGGGTGTGTGAAGTG	61.5
		reverse	TGCGATCTCGGCTGTTAGG	
	NtDREB3	forward	TACAGGGGAGTGAGGAAGAGGA	61.5
		reverse	GCAGAAGGGAAAGTGCCAAG	
	NtERD10a	forward	TGAGAAGAAGGGAATTATGGACAAG	61.5
		reverse	CGCAGCAGATTTTCTAGTGGTG	
	NtERD10c	forward	CAACAAACTCAGAGGAAATAG	59
		reverse	CACGAGGGAAGAAGAGAAGG	
	NtERD10d	forward	GAGGACACGGCTGTACCAGT	61.5
		reverse	GCGCCACTTCCTCTGTCTT	

Application	Gene		Sequence	TA℃
RT-qPCR	*VvDLK*	forward	AACGGGAGCAGTTACGCATC	60
		reverse	CTGCATCTGCCAAACGATCT	
	Vitis actin	forward	CTCTATATGCCAGTGGGCGTAC	59-61.5
		reverse	CTGAGGAGCTGCTCTTTGCAG	
	VaCBF4	forward	GATTCCCTGAATTTGCCTGA	59
		reverse	ACTTCTTCCTCCCAGCTCGT	
	VcCBF4	forward	CGAGGTAAGGGAGCCCAACAA	61.5
		reverse	GCCACGCAGAGTCCGCAAAAT	

Table S1 Primers used in this work.

7. Reference

Addya, S et al., (1997). Targeting of NH2-terminal–processed microsomal protein to mitochondria: a novel pathway for the biogenesis of hepatic mitochondrial P450MT2. *The Journal of cell biology* **139**(3): 589-599.

Agarwal, P et al., (2020). MYB4 transcription factor, a member of R2R3-subfamily of MYB domain protein, regulates cadmium tolerance via enhanced protection against oxidative damage and increases expression of PCS1 and MT1C in Arabidopsis. *Plant Science* **297**: 110501.

Agrawal, G et al., (2002). Isolation of novel rice (Oryza sativa L.) multiple stress responsive MAP kinase gene, OsMSRMK2, whose mRNA accumulates rapidly in response to environmental cues. *Biochemical and biophysical research communications* **294**(5): 1009-1016.

Akhtar, M et al., (2012). DREB1/CBF transcription factors: their structure, function and role in abiotic stress tolerance in plants. *Journal of genetics* **91**(3): 385-395.

Ali, I. & Yang, W.-C. (2020). Why are ATP-driven microtubule minus-end directed motors critical to plants? An overview of plant multifunctional kinesins. *Functional Plant Biology* **47**(6): 524-536.

Ali, R et al., (2007). Death don't have no mercy and neither does calcium: Arabidopsis CYCLIC NUCLEOTIDE GATED CHANNEL2 and innate immunity. *The Plant Cell* **19**(3): 1081-1095.

Ambrose, C et al., (2011). A CLASP-modulated cell edge barrier mechanism drives cell-wide cortical microtubule organization in Arabidopsis. *Nature communications* **2**(1): 1-12.

Ambrose, C. & Wasteneys, G. O. (2014). Microtubule initiation from the nuclear surface controls cortical microtubule growth polarity and orientation in Arabidopsis thaliana. *Plant and Cell Physiology* **55**(9): 1636-1645.

Ambrose, J. C. & Cyr, R. (2007). The kinesin ATK5 functions in early spindle assembly in Arabidopsis. *The Plant Cell* **19**(1): 226-236.

Ambrose, J. C et al.,. (2005). A minus-end–directed kinesin with plus-end tracking protein activity is involved in spindle morphogenesis. *Molecular biology of the cell* **16**(4): 1584-1592.

Ambrose, J. C et al.,. (2007). The Arabidopsis CLASP gene encodes a microtubule-associated protein involved in cell expansion and division. *The Plant Cell* **19**(9): 2763-2775.

An, J.-P et al.,.. (2017). MdHY5 positively regulates cold tolerance via CBF-dependent and CBF-independent pathways in apple. *Journal of plant physiology* **218**: 275-281.

Appert-Rolland, C et al.,. (2015). Intracellular transport driven by cytoskeletal motors: General mechanisms and defects. *Physics Reports* **593**: 1-59.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology* **141**(2): 391-396.

Bagur, R. & Hajnóczky, G. (2017). Intracellular Ca2+ sensing: its role in calcium homeostasis and signaling. *Molecular cell* **66**(6): 780-788.

Bai, L. et al., (2018). Heterotrimeric G-protein γ subunit CsGG3. 2 positively regulates the expression of CBF genes and chilling tolerance in cucumber. *Frontiers in Plant Science* **9**: 488.

Barkla, B. J. & Pantoja, O. (2011).Plasma membrane and abiotic stress. In *The plant plasma membrane*, 457-470: Springer.

Bartoli, C. G et al.,. (2004). Mitochondria are the main target for oxidative damage in leaves of wheat

(Triticum aestivum L.). *Journal of experimental botany* **55**(403): 1663-1669.

Benedict, C et al.,. (2006). The CBF1-dependent low temperature signalling pathway, regulon and increase in freeze tolerance are conserved in Populus spp. *Plant, cell & environment* **29**(7): 1259-1272.

Bengs, F et al.,.. (2005). LmxMPK9, a mitogen-activated protein kinase homologue affects flagellar length in Leishmania mexicana. *Molecular microbiology* **55**(5): 1606-1615.

Bensaude, O. (2011). Inhibiting eukaryotic transcription. Which compound to choose? How to evaluate its activity? Which compound to choose? How to evaluate its activity? *Transcription* **2**(3): 103-108.

Berman, S. A et al.,. (2003). A novel MAP kinase regulates flagellar length in Chlamydomonas. *Current Biology* **13**(13): 1145-1149.

Bhabha, G et al.,. (2016). How dynein moves along microtubules. *Trends in biochemical sciences* **41**(1): 94-105.

Bienert, G. P. & Chaumont, F. (2014). Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide. *Biochimica et Biophysica Acta (BBA)-General Subjects* **1840**(5): 1596-1604.

Bloom, G. S et al.,. (1988). Native structure and physical properties of bovine brain kinesin and identification of the ATP-binding subunit polypeptide. *Biochemistry* **27**(9): 3409-3416.

Bose, J et al.,. (2011). Calcium efflux systems in stress signaling and adaptation in plants. *Frontiers in Plant Science* **2**: 85.

Bourdais, G et al., (2015). Large-scale phenomics identifies primary and fine-tuning roles for CRKs in responses related to oxidative stress. *PLoS Genetics* **11**(7): e1005373.

Brodersen, P et al., (2006). Arabidopsis MAP kinase 4 regulates salicylic acid-and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. *The Plant Journal* **47**(4): 532-546.

Brown, K. D et al., (1996). The kinesin-like protein CENP-E is kinetochore-associated throughout poleward chromosome segregation during anaphase-A. *Journal of Cell Science* **109**(5): 961-969.

Burghoorn, J et al., (2007). Mutation of the MAP kinase DYF-5 affects docking and undocking of kinesin-2 motors and reduces their speed in the cilia of Caenorhabditis elegans. *Proceedings of the National Academy of Sciences* **104**(17): 7157-7162.

Buschmann, H et al., (2015). Arabidopsis KCBP interacts with AIR9 but stays in the cortical division zone throughout mitosis via its MyTH4-FERM domain. *Journal of Cell Science* **128**(11): 2033-2046.

Byrd, D. T et al., (2001). UNC-16, a JNK-signaling scaffold protein, regulates vesicle transport in C. elegans. *Neuron* **32**(5): 787-800.

Cai, S et al., (2009). Kinesin-14 family proteins HSET/XCTK2 control spindle length by cross-linking and sliding microtubules. *Molecular biology of the cell* **20**(5): 1348-1359.

Cai, X. (2007). Molecular evolution and structural analysis of the Ca2+ release-activated Ca2+ channel subunit, Orai. *Journal of molecular biology* **368**(5): 1284-1291.

ÇAKIR AYDEMİR, B. & KILIÇKAYA, O. (2015). Mitogen-activated protein kinase cascades in Vitis vinifera. *Frontiers in Plant Science* 6.

Canty, J. T. & Yildiz, A. (2020). Activation and regulation of cytoplasmic dynein. *Trends in biochemical sciences* **45**(5): 440-453.

Carlow, C. E et al., (2017). Nuclear localization and transactivation by Vitis CBF transcription factors are regulated by combinations of conserved amino acid domains. *Plant Physiology and Biochemistry* **118**: 306-319.

Case, R. B et al., (1997). The directional preference of kinesin motors is specified by an element outside

of the motor catalytic domain. *Cell* **90**(5): 959-966.

Castro, B et al., (2021). Stress-induced reactive oxygen species compartmentalization, perception and signalling. *Nat Plants* **7**(4): 403-412.

Chan, J et al., (2003). EB1 reveals mobile microtubule nucleation sites in Arabidopsis. *Nature cell biology* **5**(11): 967-971.

Charpentier, M et al., (2016). Nuclear-localized cyclic nucleotide–gated channels mediate symbiotic calcium oscillations. *Science* **352**(6289): 1102-1105.

Chen, X et al., (2016). Environmental and endogenous control of cortical microtubule orientation. *Trends in cell biology* **26**(6): 409-419.

Chen, et al., (2014). The maize DWARF1 encodes a gibberellin 3-oxidase and is dual localized to the nucleus and cytosol. *Plant Physiology* **166**(4): 2028-2039.

Cheng, C et al., (2007). An early response regulatory cluster induced by low temperature and hydrogen peroxide in seedlings of chilling-tolerant japonica rice. *BMC genomics* **8**(1): 1-18.

Chinnusamy, V et al., (2003). ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. *Genes & development* **17**(8): 1043-1054.

Clapham, D. E. (1995). Calcium signaling. *Cell* 80(2): 259-268.

Colcombet, J. & Hirt, H. (2008). Arabidopsis MAPKs: a complex signalling network involved in multiple biological processes. *Biochemical Journal* **413**(2): 217-226.

Collins, S. R. & Meyer, T. (2011). Evolutionary origins of STIM1 and STIM2 within ancient Ca2+ signaling systems. *Trends in cell biology* **21**(4): 202-211.

Conde, A., Chaves, M. M. & Gerós, H. (2011). Membrane transport, sensing and signaling in plant adaptation to environmental stress. *Plant and Cell Physiology* **52**(9): 1583-1602.

Cook, B. I. & Wolkovich, E. M. (2016). Climate change decouples drought from early wine grape harvests in France. *Nature Climate Change* **6**(7): 715-719.

Cristina, M. S., Petersen, M. & Mundy, J. (2010). Mitogen-activated protein kinase signaling in plants. *Annual review of plant biology* **61**: 621-649.

Dai, X et al., (2007). Overexpression of an R1R2R3 MYB gene, OsMYB3R-2, increases tolerance to freezing, drought, and salt stress in transgenic Arabidopsis. *Plant Physiology* **143**(4): 1739-1751.

Danquah, A et al., (2015). Identification and characterization of an ABA-activated MAP kinase cascade in Arabidopsis thaliana. *The Plant Journal* **82**(2): 232-244.

de Zelicourt, A., Colcombet, J. & Hirt, H. (2016). The Role of MAPK Modules and ABA during Abiotic Stress Signaling. *Trends Plant Sci* **21**(8): 677-685.

Desikan, R et al., (2004a). Hydrogen peroxide is a common signal for darkness-and ABA-induced stomatal closure in Pisum sativum. *Functional Plant Biology* **31**(9): 913-920.

Desikan, R et al., (2004b). ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. *Journal of experimental botany* **55**(395): 205-212.

Desikan, R et al., (2001). Harpin induces activation of the Arabidopsis mitogen-activated protein kinases AtMPK4 and AtMPK6. *Plant Physiology* **126**(4): 1579-1587.

Devendrakumar, K. T., Li, X. & Zhang, Y. (2018). MAP kinase signalling: interplays between plant PAMP- and effector-triggered immunity. *Cellular and molecular life sciences* **75**(16): 2981-2989.

Di Capite, J. & Parekh, A. B. (2009). CRAC channels and Ca2+ signaling in mast cells. *Immunological reviews* **231**(1): 45-58.

Ding, Y et al., (2015a). OST1 kinase modulates freezing tolerance by enhancing ICE1 stability in Arabidopsis. *Developmental cell* **32**(3): 278-289.

Ding, Y. **et al.,** (2015b). OST1 kinase modulates freezing tolerance by enhancing ICE1 stability in Arabidopsis. *Dev Cell* **32**(3): 278-289.

Ding, Y., Shi, Y. & Yang, S. (2019). Advances and challenges in uncovering cold tolerance regulatory mechanisms in plants. *New Phytol* **222**(4): 1690-1704.

Ding, Y., Shi, Y. & Yang, S. (2020). Molecular Regulation of Plant Responses to Environmental Temperatures. *Mol Plant* **13**(4): 544-564.

Dong, C.-H et al., (2006). The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. *Proceedings of the National Academy of Sciences* **103**(21): 8281-8286.

Dou, L et al., (2018). Ethylene signaling modulates cortical microtubule reassembly in response to salt stress. *Plant Physiology* **176**(3): 2071-2081.

Downing, K. H. & Nogales, E. (1998). Tubulin and microtubule structure. *Current opinion in cell biology* **10**(1): 16-22.

Drechsler, H. & McAinsh, A. D. (2016). Kinesin-12 motors cooperate to suppress microtubule catastrophes and drive the formation of parallel microtubule bundles. *Proceedings of the National Academy of Sciences* **113**(12): E1635-E1644.

Edel, K. H. & Kudla, J. (2015). Increasing complexity and versatility: how the calcium signaling toolkit was shaped during plant land colonization. *Cell Calcium* **57**(3): 231-246.

Edel, K. H et al., (2017). The Evolution of Calcium-Based Signalling in Plants. *Curr Biol* **27**(13): R667-R679.

Endow, S. A. & Waligora, K. W. (1998). Determinants of kinesin motor polarity. *Science* **281**(5380): 1200-1202.

Eremina, M et al., (2016). Brassinosteroids participate in the control of basal and acquired freezing tolerance of plants. *Proceedings of the National Academy of Sciences* **113**(40): E5982-E5991.

Felle, H. (2001). pH: signal and messenger in plant cells. *Plant biology* **3**(06): 577-591.

Feng, H. L **et al.,** (2013). A novel tomato MYC-type ICE1-like transcription factor, SlICE1a, confers cold, osmotic and salt tolerance in transgenic tobacco. *Plant Physiol Biochem* **73**: 309-320.

Fernandez-Gonzalez, R et al., (2009). Myosin II dynamics are regulated by tension in intercalating cells. *Developmental cell* **1**7(5): 736-743.

Feske, S et al., (2006). A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *nature* **441**(7090): 179-185.

Fitter, A. & Fitter, R. (2002). Rapid changes in flowering time in British plants. *Science* **296**(5573): 1689-1691.

Frey, N., Klotz, J. & Nick, P. (2010). A kinesin with calponin-homology domain is involved in premitotic nuclear migration. *Journal of experimental botany* **61**(12): 3423-3437.

Fridolfsson, H. N. & Starr, D. A. (2010). Kinesin-1 and dynein at the nuclear envelope mediate the bidirectional migrations of nuclei. *Journal of Cell Biology* **191**(1): 115-128.

Frietsch, S et al., (2007). A cyclic nucleotide-gated channel is essential for polarized tip growth of pollen. *Proceedings of the National Academy of Sciences* **104**(36): 14531-14536.

Fuller, M. & Telli, G. (1999). An investigation of the frost hardiness of grapevine (Vitis vinifera) during bud break. *Annals of Applied Biology* **135**(3): 589-595.

Gadde, S. & Heald, R. (2004). Mechanisms and molecules of the mitotic spindle. *Current Biology* **14**(18): R797-R805.

Gaillard, J et al., (2008). Two microtubule-associated proteins of Arabidopsis MAP65s promote antiparallel microtubule bundling. *Molecular biology of the cell* **19**(10): 4534-4544.

Gao, M et al., (2008). MEKK1, MKK1/MKK2 and MPK4 function together in a mitogen-activated protein kinase cascade to regulate innate immunity in plants. *Cell research* **18**(12): 1190-1198.

Geilfus, C.-M. (2017). The pH of the apoplast: dynamic factor with functional impact under stress. *Molecular plant* **10**(11): 1371-1386.

Gilmour, S. J et al., (2000). Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiology* **124**(4): 1854-1865.

Goodwin, S. S. & Vale, R. D. (2010). Patronin regulates the microtubule network by protecting microtubule minus ends. *Cell* **143**(2): 263-274.

Goshima, G. & Vale, R. D. (2005). Cell cycle-dependent dynamics and regulation of mitotic kinesins in Drosophila S2 cells. *Molecular biology of the cell* **16**(8): 3896-3907.

Greenberg, M. J. & Ostap, E. M. (2013). Regulation and control of myosin-I by the motor and light chain-binding domains. *Trends in cell biology* **23**(2): 81-89.

Guillaud, L., Wong, R. & Hirokawa, N. (2008). Disruption of KIF17–Mint1 interaction by CaMKII-dependent phosphorylation: a molecular model of kinesin–cargo release. *Nature cell biology* **10**(1): 19-29.

Gundersen, G. G. & Cook, T. A. (1999). Microtubules and signal transduction. *Current opinion in cell biology* **11**(1): 81-94.

Guo, K. M et al., (2010). The cyclic nucleotide-gated channel AtCNGC10 transports Ca2+ and Mg2+ in Arabidopsis. *Physiologia Plantarum* **139**(3): 303-312.

Guo, X., Liu, D. & Chong, K. (2018). Cold signaling in plants: Insights into mechanisms and regulation. *J Integr Plant Biol* **60**(9): 745-756.

Guy, C. L. (1990). Cold acclimation and freezing stress tolerance: role of protein metabolism. *Annual review of plant biology* **41**(1): 187-223.

Haake, V et al., (2002). Transcription factor CBF4 is a regulator of drought adaptation in Arabidopsis. *Plant Physiology* **130**(2): 639-648.

Hafner, A. E et al., (2016). Run-and-pause dynamics of cytoskeletal motor proteins. *Scientific reports* **6**(1): 1-12.

Hammer, J. A. & Sellers, J. R. (2012). Walking to work: roles for class V myosins as cargo transporters. *Nature reviews Molecular cell biology* **13**(1): 13-26.

He, X et al., (2020). The Function of MAPK Cascades in Response to Various Stresses in Horticultural Plants. *Front Plant Sci* **1**1: 952.

Heald, R. & Nogales, E. (2002). Microtubule dynamics. *Journal of Cell Science* **115**(1): 3-4.

Heidarvand, L. & Amiri, R. M. (2010). What happens in plant molecular responses to cold stress? *Acta Physiologiae Plantarum* **32**(3): 419-431.

Hendricks, A. G. et al., (2012). Dynein tethers and stabilizes dynamic microtubule plus ends. *Current Biology* **22**(7): 632-637.

Herrmann, A et al., (2018). Dual localized kinesin-12 POK 2 plays multiple roles during cell division and interacts with MAP 65-3. *EMBO reports* **19**(9): e46085.

HIRAMOTO, K. et al., (1996). Effect of plant phenolics on the formation of the spin-adduct of hydroxyl radical and the DNA strand breaking by hydroxyl radical. *Biological and Pharmaceutical Bulletin* **19**(4): 558-563.

Hirokawa, N. & Noda, Y. (2008). Intracellular transport and kinesin superfamily proteins, KIFs: structure, function, and dynamics. *Physiological reviews* **88**(3): 1089-1118.

Hirokawa, N et al., (2009). Kinesin superfamily motor proteins and intracellular transport. *Nature reviews Molecular cell biology* **10**(10): 682-696.

Ho, C.-M. et al., (2012). Arabidopsis microtubule-associated protein MAP65-3 cross-links antiparallel microtubules toward their plus ends in the phragmoplast via its distinct C-terminal microtubule binding domain. *The Plant Cell* **24**(5): 2071-2085.

Hollenbeck, P. J. (1989). The distribution, abundance and subcellular localization of kinesin. *The Journal of cell biology* **108**(6): 2335-2342.

Holzinger, A. & Lütz-Meindl, U. (2002). Kinesin-like proteins are involved in postmitotic nuclear migration of the unicellular green alga Micrasterias denticulata. *Cell Biology International* **26**(8): 689-697.

Horiuchi, D et al., (2007). Control of a kinesin-cargo linkage mechanism by JNK pathway kinases. *Current Biology* **17**(15): 1313-1317.

Hou, X. et al., (2012). Crystal structure of the calcium release–activated calcium channel Orai. *Science* **338**(6112): 1308-1313.

Hsieh, T et al., (2002). Tomato plants ectopically expressing Arabidopsis CBF1 show enhanced resistance to water deficit stress. *Plant Physiology* **130**(2): 618-626.

Hu, W et al., (2008). Changes in electron transport, superoxide dismutase and ascorbate peroxidase isoenzymes in chloroplasts and mitochondria of cucumber leaves as influenced by chilling. *Photosynthetica* **46**(4): 581-588.

Hu, Y et al., (2013). Jasmonate regulates the inducer of CBF expression–c-repeat binding factor/DRE binding factor1 cascade and freezing tolerance in Arabidopsis. *The Plant Cell* **25**(8): 2907-2924.

Hua, J. (2016). Defining roles of tandemly arrayed CBF genes in freezing tolerance with new genome editing tools. *New Phytologist* **212**(2): 301-302.

Huang, H et al., (2019). Mechanisms of ROS regulation of plant development and stress responses. *Frontiers in Plant Science* **10**: 800.

Huang, J et al., (2012). Lis1 acts as a "clutch" between the ATPase and microtubule-binding domains of the dynein motor. *Cell* **150**(5): 975-986.

Hussain, H et al., (2018). Chilling and drought stresses in crop plants: implications, cross talk, and potential management opportunities. *Frontiers in Plant Science* **9**: 393.

Ichimura, K. et al., (2000). Various abiotic stresses rapidly activate Arabidopsis MAP kinases ATMPK4 and ATMPK6. *The Plant Journal* **24**(5): 655-665.

Inaba, M et al., (2003). Gene-engineered rigidification of membrane lipids enhances the cold inducibility of gene expression in Synechocystis. *Journal of Biological Chemistry* **278**(14): 12191-12198.

J. CYR, R. (1991). Calcium/calmodulin affects microtubule stability in lysed protoplasts. *Journal of Cell Science* **100**(2): 311-317.

Jaglo-Ottosen, K. R et al., (1998). Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science* **280**(5360): 104-106.

Jaillon, O et al., (2007). The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *nature* **449**(7161): 463-467.

Janská, A et al., (2010). Cold stress and acclimation–what is important for metabolic adjustment? *Plant biology* **12**(3): 395-405.

Jia, Y et al., (2016). The cbfs triple mutants reveal the essential functions of CBFs in cold acclimation and allow the definition of CBF regulons in Arabidopsis. *New Phytol* **212**(2): 345-353.

Jiang, B et al., (2020). Cold-induced CBF–PIF3 interaction enhances freezing tolerance by stabilizing the phyB thermosensor in Arabidopsis. *Molecular plant* **13**(6): 894-906.

Jonak, C et al., (2002). Complexity, cross talk and integration of plant MAP kinase signalling. *Current opinion in plant biology* **5**(5): 415-424.

Kadota, Y et al., (2004). Identification of putative voltage-dependent Ca2+-permeable channels involved in cryptogein-induced Ca2+ transients and defense responses in tobacco BY-2 cells. *Biochemical and biophysical research communications* **317**(3): 823-830.

Kao, Y.-L. et al., (2000). Bundling of microtubules by motor and tail domains of a kinesin-like calmodulin-binding protein from Arabidopsis: regulation by Ca2+/calmodulin. *Biochemical and biophysical research communications* **267**(1): 201-207.

Kärkönen, A. & Kuchitsu, K. (2015). Reactive oxygen species in cell wall metabolism and development in plants. *Phytochemistry* **112**: 22-32.

Karniely, S. & Pines, O. (2005). Single translation—dual destination: mechanisms of dual protein targeting in eukaryotes. *EMBO reports* **6**(5): 420-425.

Kasuga, M et al., (2004). A combination of the Arabidopsis DREB1A gene and stress-inducible rd29A promoter improved drought-and low-temperature stress tolerance in tobacco by gene transfer. *Plant and Cell Physiology* **45**(3): 346-350.

Khokon, M. A et al., (2015). Two guard cell mitogen-activated protein kinases, MPK 9 and MPK 12, function in methyl jasmonate-induced stomatal closure in Arabidopsis thaliana. *Plant biology* **17**(5): 946-952.

Kidokoro, S et al., (2015). Soybean DREB 1/CBF-type transcription factors function in heat and drought as well as cold stress-responsive gene expression. *The Plant Journal* **81**(3): 505-518.

Kim, J.-S et al., (2012). Arabidopsis GROWTH-REGULATING FACTOR7 functions as a transcriptional repressor of abscisic acid–and osmotic stress–responsive genes, including DREB2A. *The Plant Cell* **24**(8): 3393-3405.

Kim, J. C et al., (2001). A novel cold-inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plants. *The Plant Journal* **25**(3): 247-259.

Kim, S. H et al., (2017). Phosphorylation of the transcriptional repressor MYB15 by mitogen-activated protein kinase 6 is required for freezing tolerance in Arabidopsis. *Nucleic Acids Research* **45**(11): 6613-6627.

Kim, S. Y. & Nam, K. H. (2010). Physiological roles of ERD10 in abiotic stresses and seed germination of Arabidopsis. *Plant cell reports* **29**(2): 203-209.

Kim, Y et al., (2013). Roles of CAMTA transcription factors and salicylic acid in configuring the low-temperature transcriptome and freezing tolerance of A rabidopsis. *The Plant Journal* **75**(3): 364-376.

Kimura, S et al., (2020). CRK2 and C-terminal phosphorylation of NADPH oxidase RBOHD regulate reactive oxygen species production in Arabidopsis. *The Plant Cell* **32**(4): 1063-1080.

Kincaid, R. L. & Vaughan, M. (1986). Direct comparison of Ca2+ requirements for calmodulin interaction with and activation of protein phosphatase. *Proceedings of the National Academy of Sciences* **83**(5): 1193-1197.

Klotz, J. & Nick, P. (2012). A novel actin–microtubule cross-linking kinesin, NtKCH, functions in cell expansion and division. *New Phytologist* **193**(3): 576-589.

Knight, H et al., (2004). Abscisic acid induces CBF gene transcription and subsequent induction of cold-regulated genes via the CRT promoter element. *Plant Physiology* **135**(3): 1710-1717.

Komis, G., Illés, P., Beck, M. & Šamaj, J. (2011). Microtubules and mitogen-activated protein kinase signalling. *Current opinion in plant biology* **14**(6): 650-657.

Kong, Q et al., (2012). The MEKK1-MKK1/MKK2-MPK4 kinase cascade negatively regulates immunity mediated by a mitogen-activated protein kinase kinase kinase in Arabidopsis. *Plant Cell* **24**(5): 2225-2236.

Kovacs, D., Kalmar, E., Torok, Z. & Tompa, P. (2008). Chaperone activity of ERD10 and ERD14, two disordered stress-related plant proteins. *Plant Physiology* **147**(1): 381-390.

Kozielski, F. et al., (1997). The crystal structure of dimeric kinesin and implications for microtubule-dependent motility. *Cell* **91**(7): 985-994.

Krtkova, J et al.,. (2012). Hsp90 binds microtubules and is involved in the reorganization of the microtubular network in angiosperms. *J Plant Physiol* **169**(14): 1329-1339.

Kurusu, T. et al., (2005). Identification of a putative voltage-gated Ca2+ channel as a key regulator of elicitor-induced hypersensitive cell death and mitogen-activated protein kinase activation in rice. *The Plant Journal* **42**(6): 798-809.

Lacombe, B et al., (2001). The identity of plant glutamate receptors. *Science* **292**(5521): 1486-1486.

Lakshmikanth, G. S et al., (2004). A mitotic kinesin-like protein required for normal karyokinesis, myosin localization to the furrow, and cytokinesis in Dictyostelium. *Proceedings of the National Academy of Sciences* **101**(47): 16519-16524.

Lam, H.-M et al., (1998). Glutamate-receptor genes in plants. *nature* **396**(6707): 125-126.

L**amers, J et al.,** (2020). How plants sense and respond to stressful environments. *Plant Physiology* **182**(4): 1624-1635.

Lemasters, J. J. & Holmuhamedov, E. (2006). Voltage-dependent anion channel (VDAC) as mitochondrial governator—thinking outside the box. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* **1762**(2): 181-190.

Leng, Q et al., (1999). Cloning and first functional characterization of a plant cyclic nucleotide-gated cation channel. *Plant Physiology* **121**(3): 753-761.

Li, C et al., (2020). A SnRK1-Zm RFWD3-Opaque2 signaling axis regulates diurnal nitrogen accumulation in maize seeds. *Plant Cell* **32**(9): 2823-2841.

Li, H et al., (2017a). MPK3- and MPK6-Mediated ICE1 Phosphorylation Negatively Regulates ICE1 Stability and Freezing Tolerance in Arabidopsis. *Dev Cell* **43**(5): 630-642 e634.

Li, H et al., (2017b). BZR1 Positively Regulates Freezing Tolerance via CBF-Dependent and CBF-Independent Pathways in Arabidopsis. *Mol Plant* **10**(4): 545-559.

Li, H et al., (2009). Arabidopsis microtubule-associated protein AtMAP65-2 acts as a microtubule stabilizer. *Plant Molecular Biology* **69**(3): 313-324.

Li, J et al., (2011). Mutation of rice BC12/GDD1, which encodes a kinesin-like protein that binds to a GA biosynthesis gene promoter, leads to dwarfism with impaired cell elongation. *The Plant Cell* **23**(2): 628-640.

Li, J., Lee, W.-L. & Cooper, J. A. (2005). NudEL targets dynein to microtubule ends through LIS1. *Nature cell biology* **7**(7): 686-690.

Li, J., Xu, Y. & Chong, K. (2012). The novel functions of kinesin motor proteins in plants. *Protoplasma* **249**(2): 95-100.

Li, Y et al., (2008). Genome-wide identification of osmotic stress response gene in Arabidopsis thaliana. *Genomics* **92**(6): 488-493.

Liang, Y.-J. & Yang, W.-X. (2019). Kinesins in MAPK cascade: how kinesin motors are involved in the

MAPK pathway? *Gene* **684**: 1-9.

Lin, L., Liu, X. & Yin, R. (2018). PIF3 Integrates Light and Low Temperature Signaling. *Trends in plant science* **23**(2): 93-95.

Lippmann, R et al., (2019). Development of Wild and Cultivated Plants under Global Warming Conditions. *Curr Biol* **29**(24): R1326-R1338.

Liu, Q et al., (1998). Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought-and low-temperature-responsive gene expression, respectively, in Arabidopsis. *The Plant Cell* **10**(8): 1391-1406.

Liu, X. & Erikson, R. L. (2007). The nuclear localization signal of mitotic kinesin-like protein Mklp-1: effect on Mklp-1 function during cytokinesis. *Biochemical and biophysical research communications* **353**(4): 960-964.

Liu, Y., Dang, P., Liu, L. & He, C. (2019). Cold acclimation by the CBF–COR pathway in a changing climate: Lessons from Arabidopsis thaliana. *Plant cell reports* **38**(5): 511-519.

Liu, Z. et al., (2017). Plasma membrane CRPK1-mediated phosphorylation of 14-3-3 proteins induces their nuclear import to fine-tune CBF signaling during cold response. *Molecular cell* **66**(1): 117-128. e115.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods* **25**(4): 402-408.

Locke, J et al., (2017). Structural basis of human kinesin-8 function and inhibition. *Proceedings of the National Academy of Sciences* **114**(45): E9539-E9548.

Logan, B. A et al., (2006). The role of antioxidant enzymes in photoprotection. *Photosynthesis research* **88**(2): 119-132.

Lu, L et al., (2005). An internal motor kinesin is associated with the Golgi apparatus and plays a role in trichome morphogenesis in Arabidopsis. *Molecular biology of the cell* **16**(2): 811-823.

Lu, W. & Gelfand, V. I. (2017). Moonlighting motors: kinesin, dynein, and cell polarity. *Trends in cell biology* **27**(7): 505-514.

Lwin, K. M., Li, D. & Bretscher, A. (2016). Kinesin-related Smy1 enhances the Rab-dependent association of myosin-V with secretory cargo. *Molecular biology of the cell* **27**(15): 2450-2462.

Lyons, J. M. (1973). Chilling injury in plants. *Annual review of plant physiology* **24**(1): 445-466.

Ma, X et al.,. (2021). CaCIPK13, a CBL-interacting protein kinase positively regulates defense mechanism against cold stress in pepper (Capsicum annuum L.). *Journal of experimental botany*.

Ma, Y et al., (2015). COLD1 confers chilling tolerance in rice. *Cell* **160**(6): 1209-1221.

Makhazen, D et al., (2021). Inhibition of the JAZ1 gene causes activation of camalexin biosynthesis in Arabidopsis callus cultures. *Journal of Biotechnology* **342**: 102-113.

Malikov, V et al.,. (2015). HIV-1 capsids bind and exploit the kinesin-1 adaptor FEZ1 for inward movement to the nucleus. *Nature communications* **6**(1): 1-13.

Mandelkow, E. & Mandelkow, E.-M. (1994). Microtubule structure. *Current Opinion in Structural Biology* **4**(2): 171-179.

Mann, B. J. & Wadsworth, P. (2019). Kinesin-5 regulation and function in mitosis. *Trends in cell biology* **29**(1): 66-79.

Manna, S. (2015). An overview of pentatricopeptide repeat proteins and their applications. *Biochimie* **113**: 93-99.

Mao, T et al., (2005). Two microtubule-associated proteins of the Arabidopsis MAP65 family function differently on microtubules. *Plant Physiology* **138**(2): 654-662.

Marcus, A. I et al., (2002). Arabidopsis thaliana protein, ATK1, is a minus-end directed kinesin that exhibits non-processive movement. *Cell motility and the cytoskeleton* **52**(3): 144-150.

Marozsán-Tóth, Z et al., (2015). The cold response of CBF genes in barley is regulated by distinct signaling mechanisms. *Journal of plant physiology* **181**: 42-49.

Martínez-Barajas, E. & Coello, P. (2020). How do SnRK1 protein kinases truly work? *Plant Science* **291**: 110330.

Matsuoka, D et al., (2015). An abscisic acid inducible Arabidopsis MAPKKK, MAPKKK18 regulates leaf senescence via its kinase activity. *Plant Molecular Biology* **87**(6): 565-575.

McNally, F. (2000). Capturing a ring of samurai. *Nature cell biology* 2(1): E4-E6.

Medina, J., Catalá, R. & Salinas, J. (2011). The CBFs: three Arabidopsis transcription factors to cold acclimate. *Plant Science* **180**(1): 3-11.

Mennella, V et al., (2005). Functionally distinct kinesin-13 family members cooperate to regulate microtubule dynamics during interphase. *Nature cell biology* **7**(3): 235-245.

Merkle, T et al., (1996). A plant in vitro system for the nuclear import of proteins. *The Plant Journal* **10**(6): 1177-1186.

Mhamdi, A. & Van Breusegem, F. (2018). Reactive oxygen species in plant development. *Development* **145**(15): dev164376.

Midorikawa, R., Takei, Y. & Hirokawa, N. (2006). KIF4 motor regulates activity-dependent neuronal survival by suppressing PARP-1 enzymatic activity. *Cell* **125**(2): 371-383.

Miki, H., Okada, Y. & Hirokawa, N. (2005). Analysis of the kinesin superfamily: insights into structure and function. *Trends in cell biology* **15**(9): 467-476.

Miki, H., Setou, M., Kaneshiro, K. & Hirokawa, N. (2001). All kinesin superfamily protein, KIF, genes in mouse and human. *Proceedings of the National Academy of Sciences* **98**(13): 7004-7011.

Miller-Rushing, A. J. & Primack, R. B. (2008). Global warming and flowering times in Thoreau's Concord: a community perspective. *Ecology* **89**(2): 332-341.

Mittler, R. (2017). ROS Are Good. *Trends Plant Sci* **22**(1): 11-19.

Mitula, F et al., (2015). Arabidopsis ABA-activated kinase MAPKKK18 is regulated by protein phosphatase 2C ABI1 and the ubiquitin–proteasome pathway. *Plant and Cell Physiology* **56**(12): 2351-2367.

Miura, K et al., (2007). SIZ1-mediated sumoylation of ICE1 controls CBF3/DREB1A expression and freezing tolerance in Arabidopsis. *The Plant Cell* **19**(4): 1403-1414.

Mizoguchi, T et al., (1996). A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences* **93**(2): 765-769.

Mizuno, K., Koyama, M. & Shibaoka, H. (1981). Isolation of plant tubulin from azuki bean epicotyls by ethyl N-phenylcarbamate-sepharose affinity chromatography. *The Journal of Biochemistry* **89**(1): 329-332.

Möller, M. N et al., (2019). Diffusion and transport of reactive species across cell membranes. *Bioactive Lipids in Health and Disease*: 3-19.

Müller, S. & Livanos, P. (2019). Plant kinesin-12: Localization heterogeneity and functional implications. *International journal of molecular sciences* **20**(17): 4213.

Müller, S et al.,. (2004). The plant microtubule-associated protein AtMAP65-3/PLE is essential for cytokinetic phragmoplast function. *Current Biology* **14**(5): 412-417.

Mütze, J., Roth, J., Gerstberger, R. & Hübschle, T. (2007). Nuclear translocation of the transcription factor STAT5 in the rat brain after systemic leptin administration. *Neuroscience letters* **417**(3): 286-291.

Muzi, C et al., (2016). Cold stress affects H+-ATPase and phospholipase D activity in Arabidopsis. *Plant Physiology and Biochemistry* **108**: 328-336.

Nagy, S. K et al., (2015). Activation of AtMPK9 through autophosphorylation that makes it independent of the canonical MAPK cascades. *Biochemical Journal* **467**(1): 167-175.

Nakai, Y et al., (2001). Nuclear localization of yeast Nfs1p is required for cell survival. *Journal of Biological Chemistry* **276**(11): 8314-8320.

Naranatt, P. P et al., (2005). Kaposi's sarcoma-associated herpesvirus modulates microtubule dynamics via RhoA-GTP-diaphanous 2 signaling and utilizes the dynein motors to deliver its DNA to the nucleus. *Journal of virology* **79**(2): 1191-1206.

Nasreen, S., Amudha, J. & Pandey, S. (2013). Isolation and characterization of Soybean DREB 3 transcriptional activator. *Journal of Applied Biology and Biotechnology* **1**(2): 0-1.

Ng, S et al., (2013). A membrane-bound NAC transcription factor, ANAC017, mediates mitochondrial retrograde signaling in Arabidopsis. *The Plant Cell* **25**(9): 3450-3471.

Ni, C. Z et al.,. (2005). AtKP1, a kinesin-like protein, mainly localizes to mitochondria in Arabidopsis thaliana. *Cell research* **15**(9): 725-733.

Nick, P. (2012). Microtubules and the tax payer. *Protoplasma* 249 Suppl 2: S81-94.

Nishihama, R et al., (2002). Expansion of the cell plate in plant cytokinesis requires a kinesin-like protein/MAPKKK complex. *Cell* **109**(1): 87-99.

Novillo, F et al., (2004). CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in Arabidopsis. *Proceedings of the National Academy of Sciences* **101**(11): 3985-3990.

N**ovillo, F., Medina, J. & Salinas, J.** (2007). Arabidopsis CBF1 and CBF3 have a different function than CBF2 in cold acclimation and define different gene classes in the CBF regulon. *Proceedings of the National Academy of Sciences* **104**(52): 21002-21007.

Odronitz, F. & Kollmar, M. (2007). Drawing the tree of eukaryotic life based on the analysis of 2,269 manually annotated myosins from 328 species. *Genome biology* **8**(9): 1-23.

Oh, S.-J et al., (2005). Arabidopsis CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. *Plant Physiology* **138**(1): 341-351.

Örvar, B. L. et al.,. (2000). Early steps in cold sensing by plant cells: the role of actin cytoskeleton and membrane fluidity. *The Plant Journal* **23**(6): 785-794.

Palmgren, M. G. (2001). Plant plasma membrane H+-ATPases: powerhouses for nutrient uptake. *Annual review of plant biology* **52**(1): 817-845.

Pan, R., Lee, Y.-R. J. & Liu, B. (2004). Localization of two homologous Arabidopsis kinesin-related proteins in the phragmoplast. *Planta* **220**(1): 156-164.

Pei, Y et al., (2017). Kinesin family member 11 contributes to the progression and prognosis of human breast cancer. *Oncology letters* **14**(6): 6618-6626.

Peng, T et al., (2016). Overexpression of a Mei (Prunus mume) CBF gene confers tolerance to freezing and oxidative stress in Arabidopsis. *Plant Cell, Tissue and Organ Culture (PCTOC)* **126**(3): 373-385.

Pilling, A. D et al., (2006). Kinesin-1 and Dynein are the primary motors for fast transport of mitochondria in Drosophila motor axons. *Molecular biology of the cell* 17(4): 2057-2068.

Pittman, J. K. (2011). Vacuolar Ca2+ uptake. *Cell Calcium* **50**(2): 139-146.

Pitzschke, A et al., (2009). A major role of the MEKK1–MKK1/2–MPK4 pathway in ROS signalling. *Molecular plant* **2**(1): 120-137.

Pospíšil, P et al., (2004). Hydroxyl radical generation by photosystem II. *Biochemistry* **43**(21): 6783-6792.

Prakriya, M. et al.,. (2006). Orai1 is an essential pore subunit of the CRAC channel. *nature* **443**(7108): 230-233.

Preuss, M. L. et al.,. (2004). A plant-specific kinesin binds to actin microfilaments and interacts with cortical microtubules in cotton fibers. *Plant Physiology* **136**(4): 3945-3955.

Rahman, A. et al., (1999). Defective kinesin heavy chain behavior in mouse kinesin light chain mutants. *The Journal of cell biology* **146**(6): 1277-1288.

Ranty, B. et al., (2016). Calcium sensors as key hubs in plant responses to biotic and abiotic stresses. *Frontiers in Plant Science* **7**: 327.

Reddy, A. S. & Day, I. S. (2001). Kinesins in the Arabidopsis genome: a comparative analysis among eukaryotes. *BMC genomics* **2**(1): 1-13.

Ren, C. et al.,. (2021). Characterization of Chromatin Accessibility and Gene Expression upon Cold Stress Reveals that the RAV1 Transcription Factor Functions in Cold Response in Vitis Amurensis. *Plant and Cell Physiology* **62**(10): 1615-1629.

Rice, S et al.,. (1999). A structural change in the kinesin motor protein that drives motility. *nature* **402**(6763): 778-784.

Richardson, D. N., Simmons, M. P. & Reddy, A. S. (2006). Comprehensive comparative analysis of kinesins in photosynthetic eukaryotes. *BMC genomics* **7**(1): 1-37.

Roberts, A. J. et al., (2012). ATP-driven remodeling of the linker domain in the dynein motor. *Structure* **20**(10): 1670-1680.

Rodrigues, A et al., (2013). ABI1 and PP2CA phosphatases are negative regulators of Snf1-related protein kinase1 signaling in Arabidopsis. *The Plant Cell* **25**(10): 3871-3884.

Rorat, T. (2006). Plant dehydrins—tissue location, structure and function. *Cellular and Molecular Biology Letters* **11**(4): 536-556.

Ross, J. L., Ali, M. Y. & Warshaw, D. M. (2008). Cargo transport: molecular motors navigate a complex cytoskeleton. *Current opinion in cell biology* **20**(1): 41-47.

Rostovtseva, T. & Colombini, M. (1996). ATP flux is controlled by a voltage-gated channel from the mitochondrial outer membrane. *Journal of Biological Chemistry* **271**(45): 28006-28008.

Rostovtseva, T. K et al.,. (2008). Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration. *Proceedings of the National Academy of Sciences* **105**(48): 18746-18751.

Rothberg, B. S., Wang, Y. & Gill, D. L. (2013). Orai channel pore properties and gating by STIM: implications from the Orai crystal structure. *Science signaling* **6**(267): pe9-pe9.

Ruelland, E. & Collin, S. (2012). Chilling stress. *Plant stress physiology*: 94-117.

Sablin, E. P **et al.,** (1998). Direction determination in the minus-end-directed kinesin motor ncd. *nature* **395**(6704): 813-816.

Salam, M. A et al., (2012). MAP kinases, MPK9 and MPK12, regulate chitosan-induced stomatal closure. *Bioscience, biotechnology, and biochemistry*: 120228.

Salina, D et al., (2002). Cytoplasmic dynein as a facilitator of nuclear envelope breakdown. *Cell* **108**(1): 97-107.

Sánchez-Madrid, F. & Serrador, J. M. (2007). Mitochondrial redistribution: adding new players to the

chemotaxis game. *Trends in immunology* **28**(5): 193-196.

Sangwan, V et al., (2002). Opposite changes in membrane fluidity mimic cold and heat stress activation of distinct plant MAP kinase pathways. *The Plant Journal* **31**(5): 629-638.

Scandalios, J. G. (1993). Oxygen stress and superoxide dismutases. *Plant Physiology* 101(1): 7.

Schneider-Poetsch, T et al., (2010). Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. *Nature chemical biology* **6**(3): 209-217.

Scholey, J. M et al., (1989). Identification of globular mechanochemical heads of kinesin. *nature* **338**(6213): 355-357.

Sherman, T. & Fromm, H. (2009).Physiological roles of cyclic nucleotide gated channels in plants. In *Signaling in plants*, 91-106: Springer.

Shewfelt, R. (1992).Response of plant membranes to chilling and freezing. In *Plant membranes*, 192-219: Springer.

Shi, Y., Ding, Y. & Yang, S. (2018). Molecular Regulation of CBF Signaling in Cold Acclimation. *Trends in plant science* **23**(7): 623-637.

Shi, Y et al.,. (2017). The precise regulation of different COR genes by individual CBF transcription factors in Arabidopsis thaliana. *Journal of Integrative Plant Biology* **59**(2): 118-133.

Shi, Y et al., (2012). Ethylene signaling negatively regulates freezing tolerance by repressing expression of CBF and type-A ARR genes in Arabidopsis. *The Plant Cell* **24**(6): 2578-2595.

Shigaki, T et al., (2006). Identification of three distinct phylogenetic groups of CAX cation/proton antiporters. *Journal of Molecular Evolution* **63**(6): 815-825.

Shinozaki, K. & Yamaguchi-Shinozaki, K. (2000). Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Current opinion in plant biology* **3**(3): 217-223.

Siddiqua, M. & Nassuth, A. (2011). Vitis CBF1 and Vitis CBF4 differ in their effect on Arabidopsis abiotic stress tolerance, development and gene expression. *Plant, cell & environment* **34**(8): 1345-1359.

Smirnova, I. V et al.,. (2000). Zinc and cadmium can promote rapid nuclear translocation of metal response element-binding transcription factor-1. *Journal of Biological Chemistry* **275**(13): 9377-9384.

Smith, D. S et al., (2000). Regulation of cytoplasmic dynein behaviour and microtubule organization by mammalian Lis1. *Nature cell biology* **2**(11): 767-775.

Song, Q et al., (2021). CBF1 and CBF4 in Solanum tuberosum L. differ in their effect on low-temperature tolerance and development. *Environmental and Experimental Botany* **185**: 104416.

Spalding, E. P. & Harper, J. F. (2011). The ins and outs of cellular Ca2+ transport. *Current opinion in plant biology* **14**(6): 715-720.

Stenoien, D. & Brady, S. (1997). Immunochemical analysis of kinesin light chain function. *Molecular biology of the cell* **8**(4): 675-689.

Sterky, F et al., (2004). A Populus EST resource for plant functional genomics. *Proceedings of the National Academy of Sciences* **101**(38): 13951-13956.

Stockinger, E. J et al.,. (1997). 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. *Proceedings of the National Academy of Sciences* **94(**3): 1035-1040.

Strompen, G. et al., (2002). The Arabidopsis HINKEL gene encodes a kinesin-related protein involved in

cytokinesis and is expressed in a cell cycle-dependent manner. *Current Biology* **12**(2): 153-158.

Sturgill, E. G et al., (2014). Kinesin-12 Kif15 targets kinetochore fibers through an intrinsic two-step mechanism. *Current Biology* **24**(19): 2307-2313.

Suarez-Rodriguez, M. C et al., (2007). MEKK1 is required for flg22-induced MPK4 activation in Arabidopsis plants. *Plant Physiology* **143**(2): 661-669.

Sun, J., Ma, Q. & Mao, T. (2015). Ethylene regulates the Arabidopsis microtubule-associated protein WAVE-DAMPENED2-LIKE5 in etiolated hypocotyl elongation. *Plant Physiology* **169**(1): 325-337.

Sun, M et al., (2017). Global identification, classification, and expression analysis of MAPKKK genes: functional characterization of MdRaf5 reveals evolution and drought-responsive profile in apple. *Scientific reports* **7**(1): 1-14.

Sun, S et al., (2021). The AaCBF4-AaBAM3.1 module enhances freezing tolerance of kiwifruit (Actinidia arguta). *Hortic Res* **8**(1): 97.

Suzuki, N., Koussevitzky, S., Mittler, R. & Miller, G. (2012). ROS and redox signalling in the response of plants to abiotic stress. *Plant, cell & environment* **35**(2): 259-270.

Sweeney, H. L. & Houdusse, A. (2010). Myosin VI rewrites the rules for myosin motors. *Cell* **141**(4): 573-582.

Takahashi, D et al., (2013). Plant plasma membrane proteomics for improving cold tolerance. *Frontiers in Plant Science* **4**: 90.

Takahashi, F et al.,. (2007). The mitogen-activated protein kinase cascade MKK3–MPK6 is an important part of the jasmonate signal transduction pathway in Arabidopsis. *The Plant Cell* **19**(3): 805-818.

Teige, M et al., (2004). The MKK2 pathway mediates cold and salt stress signaling in Arabidopsis. *Molecular cell* **15**(1): 141-152.

Tian, Q et al., (2017). Overexpression of a Populus euphratica CBF4 gene in poplar confers tolerance to multiple stresses. *Plant Cell, Tissue and Organ Culture (PCTOC)* **128**(2): 391-407.

Tian, W et al., (2019). A calmodulin-gated calcium channel links pathogen patterns to plant immunity. *nature* **572**(7767): 131-135.

Tillett, R. L et al., (2012). The Vitis vinifera C-repeat binding protein 4 (VvCBF4) transcriptional factor enhances freezing tolerance in wine grape. *Plant biotechnology journal* **10**(1): 105-124.

Titus, M. A. (2000). Cytoskeleton: Getting to the point with myosin VI. *Current Biology* **10**(8): R294-R297.

Tokai, N et al., (1996). Kid, a novel kinesin-like DNA binding protein, is localized to chromosomes and the mitotic spindle. *The EMBO Journal* **15**(3): 457-467.

Traynelis, S. F et al., (2010). Glutamate receptor ion channels: structure, regulation, and function. *Pharmacological reviews* **62**(3): 405-496.

Tsai, J.-W et al., (2010). Kinesin 3 and cytoplasmic dynein mediate interkinetic nuclear migration in neural stem cells. *Nature neuroscience* **13**(12): 1463-1471.

Tulin, A et al., (2012). Single-molecule analysis of the microtubule cross-linking protein MAP65-1 reveals a molecular mechanism for contact-angle-dependent microtubule bundling. *Biophysical journal* **102**(4): 802-809.

Ulm, R et al., (2002). Distinct regulation of salinity and genotoxic stress responses by Arabidopsis MAP kinase phosphatase 1. *The EMBO Journal* **21**(23): 6483-6493.

Umezawa, T et al., (2010). Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. *Plant and Cell Physiology* **51**(11): 1821-1839.

Unsworth, C et al., (2008). Fission yeast kinesin-8 Klp5 and Klp6 are interdependent for mitotic nuclear

retention and required for proper microtubule dynamics. *Molecular biology of the cell* **19**(12): 5104-5115.

Vale, R. D. (2003). The molecular motor toolbox for intracellular transport. *Cell* 112(4): 467-480.

Valgardsdottir, R et al., (2001). Cloning and characterization of MDDX28, a putative dead-box helicase with mitochondrial and nuclear localization. *Journal of Biological Chemistry* **276**(34): 32056-32063.

Van Damme, D et al., (2004). In vivo dynamics and differential microtubule-binding activities of MAP65 proteins. *Plant Physiology* **136**(4): 3956-3967.

Vannini, C et al.,. (2004). Overexpression of the rice Osmyb4 gene increases chilling and freezing tolerance of Arabidopsis thaliana plants. *The Plant Journal* **37**(1): 115-127.

Varga, V et al., (2009). Kinesin-8 motors act cooperatively to mediate length-dependent microtubule depolymerization. *Cell* **138**(6): 1174-1183.

Verhey, K. J. & Hammond, J. W. (2009). Traffic control: regulation of kinesin motors. *Nature reviews Molecular cell biology* **10**(11): 765-777.

Verhey, K. J et al., (1998). Light chain–dependent regulation of kinesin's interaction with microtubules. *The Journal of cell biology* **143**(4): 1053-1066.

Verhey, K. J. et al., (2001). Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules. *The Journal of cell biology* **152**(5): 959-970.

Verret, F et al., (2010). Calcium channels in photosynthetic eukaryotes: implications for evolution of calcium-based signalling. *New Phytologist* **187**(1): 23-43.

Visconti, S., D et al., (2019). Overexpression of 14-3-3 proteins enhances cold tolerance and increases levels of stress-responsive proteins of Arabidopsis plants. *Plant Science* **289**: 110215.

Vonapartis, E. et al., (2022). CBF4/DREB1D represses XERICO to attenuate ABA, osmotic and drought stress responses in Arabidopsis. *The Plant Journal*.

Wade, R. H. & Hyman, A. A. (1997). Microtubule structure and dynamics. *Current opinion in cell biology* **9**(1): 12-17.

Wang, L. & Nick, P. (2017). Cold sensing in grapevine—which signals are upstream of the microtubular "thermometer". *Plant, cell & environment* **40**(11): 2844-2857.

Wang, L et al., (2021). Microtubules monitor calcium and reactive oxygen species signatures in signal transduction. *Plant Science* **304**: 110589.

Wang, L., Sadeghnezhad, E. & Nick, P. (2020). Upstream of gene expression: what is the role of microtubules in cold signalling? *Journal of experimental botany* **71**(1): 36-48.

Wang, L et al., (2018). Microtubule dynamics modulate sensing during cold acclimation in grapevine suspension cells. *Plant Science*.

Wang, L et al., (2019). Microtubule dynamics modulate sensing during cold acclimation in grapevine suspension cells. *Plant Sci* **280**: 18-30.

Wang, Y. & Hua, J. (2009). A moderate decrease in temperature induces COR15a expression through the CBF signaling cascade and enhances freezing tolerance. *The Plant Journal* **60**(2): 340-349.

Wang, Y., Lyu, Y. L. & Wang, J. C. (2002). Dual localization of human DNA topoisomerase IIIα to mitochondria and nucleus. *Proceedings of the National Academy of Sciences* **99**(19): 12114-12119.

Wei, L., Zhang, W., Liu, Z. & Li, Y. (2009). AtKinesin-13A is located on Golgi-associated vesicle and involved in vesicle formation/budding in Arabidopsis root-cap peripheral cells. *BMC plant biology* **9**(1): 1-8.

Wei, T et al., (2016). Arabidopsis DREB1B in transgenic Salvia miltiorrhiza increased tolerance to drought stress without stunting growth. *Plant Physiology and Biochemistry* **104**: 17-28.

Wheeler, G. L. & Brownlee, C. (2008). Ca2+ signalling in plants and green algae–changing channels. *Trends in plant science* **13**(9): 506-514.

Wilson, M. H. & Holzbaur, E. L. (2015). Nesprins anchor kinesin-1 motors to the nucleus to drive nuclear distribution in muscle cells. *Development* **142**(1): 218-228.

Woźniak, M. J. & Allan, V. J. (2006). Cargo selection by specific kinesin light chain 1 isoforms. *The EMBO Journal* **25**(23): 5457-5468.

Wu, J et al., (2014a). Genome-wide identification of MAPKK and MAPKKK gene families in tomato and transcriptional profiling analysis during development and stress response. *PloS one* **9**(7): e103032.

Wu, T et al., (2014b). Gene SGL, encoding a kinesin-like protein with transactivation activity, is involved in grain length and plant height in rice. *Plant cell reports* **33**(2): 235-244.

Wudick, M et al., (2015). Subcellular redistribution of root aquaporins induced by hydrogen peroxide. *Molecular plant* **8**(7): 1103-1114.

Xiao, H et al., (2006). Three grape CBF/DREB1 genes respond to low temperature, drought and abscisic acid. *Plant, cell & environment* **29**(7): 1410-1421.

Xiao, H et al., (2008). CBF4 is a unique member of the CBF transcription factor family of Vitis vinifera and Vitis riparia. *Plant, cell & environment* **31**(1): 1-10.

Xiong, L. & Yang, Y. (2003). Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid–inducible mitogen-activated protein kinase. *The Plant Cell* **15**(3): 745-759.

Xu, J. & Zhang, S. (2015). Mitogen-activated protein kinase cascades in signaling plant growth and development. *Trends in plant science* **20**(1): 56-64.

Xu, T et al., (2007). Cotton GhKCH2, a plant-specific kinesin, is low-affinitive and nucleotide-independent as binding to microtubule. *BMB Reports* **40**(5): 723-730.

Xu, X et al., (2022). The Minus-End-Directed Kinesin OsDLK Shuttles to the Nucleus and Modulates the Expression of Cold-Box Factor 4. *International journal of molecular sciences* **23**(11): 6291.

Xu, X et al., (2018). A rice class-XIV kinesin enters the nucleus in response to cold. *Sci Rep* **8**(1): 3588.

Yang, T et al., (2010). Calcium/calmodulin-regulated receptor-like kinase CRLK1 interacts with MEKK1 in plants. *Plant signaling & behavior* **5**(8): 991-994.

Yang, X.-Y et al., (2011). Arabidopsis kinesin KP1 specifically interacts with VDAC3, a mitochondrial protein, and regulates respiration during seed germination at low temperature. *The Plant Cell* **23**(3): 1093-1106.

Yogev, O. & Pines, O. (2011). Dual targeting of mitochondrial proteins: mechanism, regulation and function. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1808**(3): 1012-1020.

Zepeda-Jazo, I et al., (2011). Polyamines interact with hydroxyl radicals in activating Ca2+ and K+ transport across the root epidermal plasma membranes. *Plant Physiology* **157**(4): 2167-2180.

Zhan, X., Zhu, J. K. & Lang, Z. (2015). Increasing freezing tolerance: kinase regulation of ICE1. *Dev Cell* **32**(3): 257-258.

Zhang, H., Zhao, Y. & Zhu, J. K. (2020). Thriving under Stress: How Plants Balance Growth and the Stress Response. *Dev Cell* **55**(5): 529-543.

Zhang, J et al., (2012). Cold-resistance evaluation in 25 wild grape species. *Vitis* 51(4): 153-160.

Zhang, K et al., (2022). A rice tubulin tyrosine ligase like 12 regulates phospholipase D activity and

tubulin synthesis. *Plant Science* **316**: 111155.

Zhang, K et al., (2021). A rice tubulin tyrosine ligase-like 12 protein affects the dynamic and orientation of microtubules. *Journal of Integrative Plant Biology* **63**(5): 848-864.

Zhang, L et al., (2019). Overexpression of VaWRKY12, a transcription factor from Vitis amurensis with increased nuclear localization under low temperature, enhances cold tolerance of plants. *Plant Molecular Biology* **100**(1): 95-110.

Zhang, M et al., (2018). Conveying endogenous and exogenous signals: MAPK cascades in plant growth and defense. *Current opinion in plant biology* **45**: 1-10.

Zhang, M et al., (2010). Brittle Culm 12, a dual-targeting kinesin-4 protein, controls cell-cycle progression and wall properties in rice. *The Plant Journal* **63**(2): 312-328.

Zhang, M. & Zhang, S. (2018). Mitogen-activated protein kinase cascades in plant signaling. *Journal of Integrative Plant Biology*.

Zhang, Z et al., (2016). Opposing control by transcription factors MYB61 and MYB3 increases freezing tolerance by relieving C-repeat binding factor suppression. *Plant Physiology* **172**(2): 1306-1323.

Zhang, Z et al., (2017). OsMAPK3 phosphorylates OsbHLH002/OsICE1 and inhibits its ubiquitination to activate OsTPP1 and enhances rice chilling tolerance. *Developmental cell* **43**(6): 731-743. e735.

Zhao, C et al., (2017a). Temperature increase reduces global yields of major crops in four independent estimates. *Proceedings of the National Academy of Sciences* **114**(35): 9326-9331.

Zhao, C et al., (2017b). MAP Kinase Cascades Regulate the Cold Response by Modulating ICE1 Protein Stability. *Dev Cell* **43**(5): 618-629 e615.

Zhao, C et al., (2016). Mutational evidence for the critical role of CBF transcription factors in cold acclimation in Arabidopsis. *Plant Physiology* **171**(4): 2744-2759.

Zhou, M et al., (2017). Arabidopsis CBF3 and DELLAs positively regulate each other in response to low temperature. *Sci Rep* **7**: 39819.

Zhu, C. & Dixit, R. (2012). Functions of the Arabidopsis kinesin superfamily of microtubule-based motor proteins. *Protoplasma* **249**(4): 887-899.

Zhu, J et al., (2004). An Arabidopsis homeodomain transcription factor gene, HOS9, mediates cold tolerance through a CBF-independent pathway. *Proceedings of the National Academy of Sciences* **101**(26): 9873-9878.

Zhu, J et al., (2005). HOS10 encodes an R2R3-type MYB transcription factor essential for cold acclimation in plants. *Proceedings of the National Academy of Sciences* **102**(28): 9966-9971.

Zimmermann, S et al., (1997). Receptor-mediated activation of a plant Ca2+-permeable ion channel involved in pathogen defense. *Proceedings of the National Academy of Sciences* **94**(6): 2751-2755.

Zuo, Z.-F et al., (2020). A novel basic helix-loop-helix transcription factor, ZjICE2 from Zoysia japonica confers abiotic stress tolerance to transgenic plants via activating the DREB/CBF regulon and enhancing ROS scavenging. *Plant Molecular Biology* **102**(4): 447-462.