Cold sensing in grapevine
- how do microtubules function as "thermometer"?

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Abbreviations

MTs: microtubules

MAP: microtubule-associated proteins

ABA: abscisic acid

CRPK1: cold responsive protein kinase 1

CBF: CRT/DRE binding factor

PLD: phospholipase D

MAPK: mitogen-activated protein kinase

ATP: adenosine triphosphate

ROS: reactive oxygen species

P-ATPase: P-type ATPase

ERK: extracellular signal regulated kinase

JNK: jun amino-terminal kinase

Hiks: histidine kinase

BA: benzyl alcohol

DMSO: dimethyl sulfoxide

CNGCs: cyclic nucleotide-gated channels

CaM: calmodulin

CAMTA: calmodulin binding transcription activator

SYT1: a homolog of synaptotagmin

G proteins: guanine nucleotide-binding proteins

NaCl: sodium chloride

PA: phosphatidic acid

H_2O_2: hydrogen peroxide

O_2^-: superoxide anion

$^1O_2$: singlet oxygen

$HO^·$: hydroxyl radical

Abbreviations

SODs: superoxide dismutases

DAGK: diacylglycerol kinase

Hb: haemoglobins

NO: nitric oxide

NR: nitrate reductase

ETH: ethylene

CKs: cytokinins

GAs: gibberellins

SA: salicylic acid

JA: jasmonic acid

MeJA: methyl jasmonate

BR: brassinosteroid

OST1: open stomata 1

LOX2: lipoxygenase 2

COR: cold responsive genes

HATs: histone acetyltransferases

COLD1: chilling-tolerance divergence1

ICE: inducer of CBF expression

EGTA: ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

$GdCl_3$: gadolinium chloride

DPI: diphenyleneiodonium

SNP: sodium nitroprusside

TFP: trifluoperazine

Tween® 20: polyoxyethylene-20-sorbitan monolaurate

PTX: pertussis toxin

$AlF4^-$: aluminium tetrafluoride

LatB: latrunculin B

MDA: malone dialdehyde

TBA: 2-thiobarbituric acid

SDS: sodium dodecyl sulphate

JAZ1: jasmonate-zim-domain protein 1

BZR1: brassi nazole-resistant 1

AtpB: a beta subunit of ATP synthase

Zusammenfassung

Pflanzen können durch Kältebehandlung, jedoch nicht durch Frostbehandlung, Frosttoleranz entwickeln. Um die Kälteakklimatisierung effizient zu aktivieren, müssen niedrige Temperaturen wahrgenommen und schnell weiterverarbeitet werden. Dieser Vorgang steht mit der vorübergehend Eliminierung von Mikrotubuli in Verbindung. In dieser Arbeit wurden verschiedene Arten von Kältestress auf Weinsuspensionszellkulturen ausgeübt. Im Folgenden werden diese Behandlungen Kälteschock (0 °C in Eiswasser) und Kältebehandlung (8 °C) genannt. Wir untersuchten die kälteinduzierte Eleminierung der Mikrotubuli in einer Weinzellkulturlinie, die dauerhaft eine Fusion von GFP und *Arabidopsis* Tubulin 6 (TUB6) exprimiert, was die *in vivo* Beobachtung von Mikrotubuli mittels *Spinning Disc* Mikroskopie und die Quantifizierung der Antwort durch quantitative Bildanalyse ermöglicht. Wir haben Zeitreihenversuche mit mehreren spezifischen pharmakologischen Inhibitoren und Aktivatoren angewendet, um die Signalleitungsereignisse, die innerhalb der Stressantwort oberhalb von Mikrotubuli ablaufen, zu analysieren. Wir beobachteten, dass sich Mikrotubuli 30 Minuten nach Beginn der Kälteeinwirkung auflösten, was auf eine Rolle des Kalziumeinstroms, der Membranverfestigung und der Aktivierung einer NADPH-Oxidase als Faktoren der Signalsuszeption und -amplifikation hinweist. Weiterhin schlussfolgern wir, dass ein G-Protein im Zusammenspiel mit einer Phospholipase D das Signal in Richtung der Mikrotubuli leitet, während Calmodulin nicht involviert zu sein scheint. Darüber hinaus war für eine effiziente Mikrotubuliantwort die Aktivierung des Jasmonatwegs erforderlich. Wir fassen unsere Ergebnisse in einem Arbeitsmodell mit einem komplexen Signalleitungsdrehkreuz am Übergang von Membran und Zellskelett zusammen, das Suszeption, Wahrnehmung und frühe Signalleitung von Kältesignalen zusammenfügt.

Im nächsten Schritt haben wir die Frage gestellt, ob das Kälteakklimatisierungssytem in

Zusammenfassung

Weinpflanzen mit dem vorübergehenden Abbau kortikaler Mikrotubuli und dem *Downstream*-Signalweg, wie etwa der Induktion des Transkriptionsfaktors *VvCBF4*, in Verbindung steht. Um diese Hypothese zu verifizieren, haben wir auch die oben beschriebenen transgenen Zelllinien verwendet und mit verschiedenen Mikrotubuli auflösenden oder stabilisierenden Verbindungen vorbehandelt, um Zellmortalität, Mikrotubuliveränderungen und das Expressionsniveau von *VvCBF4* unter Kältetress zu überprüfen. Wir zeigten, dass die Zelltodrate nach 72-stündiger Kälteschockbehandlung, ohne vorherige Kälteakklimatisierung durch Kältebehandlung, auf nahezu 40% anstieg, begleitet von einer vollständig Auflösung der kortikalen Mikrotubuli und der Induktion von *VvCBF4*. Wohingegen eine Kältevorbehandlung den kälteinduzierten Zelltod abschwächte, gefolgt von einem verzögerten Abbau der Mikrotubuli. Taxol und Pronamid konnten die Kältebehandlung hinsichtlich der Auflösung der Mikrotubuli nachahmen, aber induzierte nicht einen Anstieg der Expression von *VvCBF4*. Darüber hinaus konnte die Zellmortalität durch Erhöhung der Membranfluidität, Einstrom von Kalziumionen und Applikation exogener Jasmonsäure über Manipulation des Mikrotubulizustands oder durch Induktion von *VvCBF4*-abhängigen oder -unabhängigen Wegen während der Kälteaklimatisierung abgeschwächt werden. Gesteigerte Membranfluidität konnte teilweise der Auflösung von Mikrotubuli entgegenwirken und die Kälteakklimatisierung modulieren, jedoch unabhängig vom *VvCBF4*-Weg. Jasmonsäure förderte hingegen die Auflösung der Mikrotubuli unabhängig vom *VvCBF4*-Weg und modulierte so die Kälteakklimatisierung. Der zentrale Faktor sind Kalziumionen, die in verschiedenen funktionellen Zweigen der Kälteakklimatisierung, einem Mikrotubuli abhängigen und einem *VvCBF4* abhängigen Zweig, zwingend erforderlich sind.

Abstract

Plants can acquire freezing tolerance in response to cold, but non-freezing temperatures. To efficiently activate this cold acclimation, low temperature has to be sensed and processed swiftly, a process that is linked with a transient elimination of microtubules (MTs). In this thesis different types of cold stress were applied to grapevine suspension cell cultures. Subsequently, those different cold regimes are referred to as cold shock (0 ℃ in an ice bath) and chilling (8 ℃). First we addressed cold shock-induced MTs elimination in a grapevine cell line stably expressing a GFP fusion of *Arabidopsis* Tubulin6 (TuB6), which allowed to follow the response of MTs *in vivo* by spinning disc microscopy and to quantify this response by quantitative image analysis. We used time-course studies with several specific pharmacological inhibitors and activators to dissect the signalling events acting upstream of MTs elimination. We found that MTs disappeared within 30 minutes after the onset of cold stress, and provided evidence for a role of calcium influx, membrane rigidification, and activation of a NADPH oxidase as factors in signal susception and amplification. Furthermore, we conclude that a G-protein in concert with a phospholipase D conveyed the signal towards MTs, whereas calmodulin seemed not to be involved. Moreover, activation of the jasmonate pathway in response to cold was required for an efficient microtubule response. We summarize our findings in a working model on a complex signalling hub at the membrane-cytoskeleton interphase that assembles the susception, perception, and early transduction of cold signals.

In the next step, we asked whether the cold acclimation system in grapevine might be linked with the transient disassembly of cortical microtubules and the downstream signalling pathway, using the transcriptional regulation of the transcription factor *VvCBF4* as a read-out. In order to further verify this hypothesis, we also used above mentioned transgenic cell line and pretreated them with some microtubule destabilizing or stabilizing compounds to observe cell mortality, microtubule changes and the

expression profile of *VvCBF4* under cold stress. We illustrated that the cell mortality increased to nearly 40% in 72h cold shock treatment, without prior cold acclimation by chilling, accompanied by the total disassembly of cortical MTs and the induction of *VvCBF4*, but chilling pretreatment could mitigate cold-induced cell death followed by gradual disassembly of cortical MTs. Taxol and pronamide could mimic chilling treatment with respect to partial MTs elimination, but did not induce the elevation of *VvCBF4* expression levels. Furthermore, the increment of membrane fluidity, the influx of calcium ions and the application of exogenous jasmonic acid could retrieve cell mortality through the manipulation of the status of cortical MTs or the induction of *VvCBF4* dependent or independent pathways during the cold acclimation. Increasing membrane fluidity could partially prevent the disassembly of MTs, but independent of the *VvCBF4* pathway, to modulate cold acclimation. In contrast jasmonic acid could promote the disassembly of MTs independently of the *VvCBF4* pathway, to adjust cold acclimation. The central factor is calcium which plays imperative role in cold acclimation by diverse functional branches, one depends on MTs and another depends on *VvCBF4*.

1. Introduction

1.1 The concepts and effects of cold stress and cold acclimation

In nature, plants always suffer from various biotic and abiotic stresses, adversely affecting plant development and productivity. However, plants as sessile organisms cannot run away when facing abiotic or biotic stresses and in order to avoid an impairment of their growth and development, they must adjust themselves to adapt to the environment which requires a rapid response of several intra- or intercellular and interconnected signalling pathways, such as the perception, reaction and adaption，to protect them from being killed quickly.

Cold stress, one of the primary abiotic stresses, is a major constraint for many crop plants, leading to chilling injury (below 10 ℃, but above the freezing points) (Lyons 1973) and freezing injury (below 0 ℃) (Sanghera *et al.* 2011), resulting in the adverse influence on the morphological, physiological growth and development which restrict the spatial distribution of plant species (Repo *et al.* 2008). Cold stress takes place with other side effect stresses, such as the cold-induced osmotic (because of chilling-induced inhibition of water uptake and freezing-induced ice formation to cause the cellular severe dehydration) and oxidative stresses (Steponkus *et al.* 1993, Chinnusamy *et al.* 2007), directly reducing and limiting some protein activities or inhibiting the expression of some related genetic genes to damage plants. Furthermore, when plants respond to chilling and freezing temperatures, especially diverse kinds of crops in tropical or subtropical areas, many cellular dysfunctions and physiological alterations happen and lead to the development of a variety of chilling injury symptoms, such as surface lesions, water-soaking of the tissue, membrane lipid composition changes, de-coloration, tissue breakdown, accelerated senescence, changes in hormone levels, imbalance of oxidative and ionic homeostasis and failure to ripen normally (Lyons and Raison 1970, Wang and

Adams 1982, Sharma *et al.* 2005). However, chilling tolerant species could protect themselves and survive well in chilling temperature. Thus, it is important to improve the plants' cold tolerance ability.

Grapevine, a fruit crop of global importance and high economic yield, is mostly growing in temperate regions and also cultivated over broad areas that strongly differ in climatic conditions. Some wild species of grapevine as well as some cultivars could tolerate even freezing conditions. For instance, grapevines in Europe are more susceptible to disease and less cold tolerance than American grapevines. It is widely believed that *V. riparia* and *V. labrusca* from north America are cold hardy species and *V. vinifera* is the cold-tender cultivars (Fuller and Telli 1999), causing many detrimental effects on wine growing in cold regions of the eastern and mid-western United States. Moreover, *V. vinifera* could easily attain damages in the low temperature of later spring because of the fast de-acclimation (Stafne 2007, Zabadal *et al.* 2007). For example, in the Finger Lakes region, almost half of the *V. vinifera* plants lost their yield in 2004 just because of a single freezing event happening in January (Martinson and White 2004). However, *V. amurensis* is another well-known cold tolerance cultivar which could survive at temperature falling to -40 ℃ (Fennell 2004, Ma *et al.* 2010). Even though, viticulture is still strongly limited by temperature condition, not only with respect to freezing during the winter, but also associated with the chilling damages in young leaves and flowers during late spring. In late spring, the sudden low temperature could harm the early bud-break by the ice formation in the tissues, cause fall down of flowers, damage tendrils, and result in the reduction of the whole year yield, and even worse could induce plant death (Fuller and Telli 1999). In the United Kingdom, when the temperature decreases to a minimum of -2 ℃ to -5 ℃, the buds are prone to break (Fuller and Le Grice 1998). Likewise, the temperature at -26 ℃ and -23 ℃ could kill half of dormant buds in *V. labrusca* and *V. vinifera*, respectively (Proebsting *et al.* 1980). In addition, cold stress affecting grapevines may disturb carbon metabolism in the inflorescence, leading to ovule abortion and reducing fruit set (Ebadi *et al.* 1995). Moreover, low temperature could reduce water up-take, which brings about the

desiccation and kill young and old grapevines by a long-dry winter. Thus, low temperature stress really puts a vital impact on grapevine growth and development even though there seems to be genetic variation in freezing tolerance. But it is still unclear how grapevine can recognize low temperature and acquire cold tolerance (Takuhara *et al.* 2011).

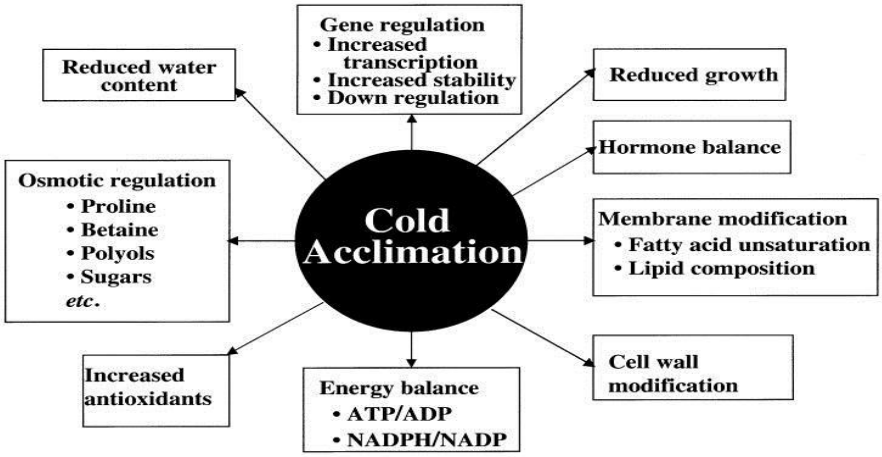

Figure 1. The morphological, physiological and molecular changes of plants under cold acclimation (Xin and Browse 2000), such as the increment of ABA content, the accumulation of osmolytes, cell membrane modification, increment of antioxidants and induction of some cold responsive genes, etc.

Plants are sessile but they are smart as well, as they could adapt themselves to various stresses. Pretreatment with some natural or synthetic compounds could improve the plants ability to cope better with various environmental changes resulting in greater survival, a process called priming (Tanou *et al.* 2012). Cold acclimation is considered as one of the priming processes utilized by plants to protect themselves from cold damages. For a cold sensitive cultivar, exposure to non-freezing temperature prior to freezing temperature could effectively improve the cold tolerance. This phenomenon is named cold acclimation. The research on rye is an illustrative example as non-acclimated rye is killed by freezing at about -5 ℃, but after a period of exposure to low non-freezing temperature they can survive freezing temperature down to about -30 ℃ (Thomashow 1999). Cold acclimation has evolved as a crucial factor that allows plants to extend their habitats because of various physiological and biochemical changes, such as the cessation of plant growth, the reduction of water content, the transient disassembly of MTs, the increment of abscisic acid (ABA), cell membrane modifications and the

synthesis of sugars or cryoprotective proteins, allowing to circumvent freezing damage **(Figure 1)** (Xin and Browse 2000, Zhao *et al.* 2009, Janska *et al.* 2010). Hence, from the upstream signal transduction to downstream gene expression and protein synthesis important factors are involved which could affect cold acclimation. Numerous researches in the last decades elucidate that the plasma membrane is the first place to perceive low temperature, accompanied by changes of membrane proteins, calcium influxes and activation of protein kinases to acquire cold acclimation (Chinnusamy *et al.* 2007, Takahashi *et al.* 2013). This is the major upstream signalling transduction, but it still remains equivocal. For the downstream gene expression and protein synthesis effects, a family of transcription factors, named CBF/DREB1 proteins, has been identified in *Arabidopsis* which imply key roles in cold acclimation. The C-repeat (CRT) dehydration responsive element (DRE), which has a conserved core sequence of CCGAC, controls the expression of a regulon of cold-induced genes that increases plant freezing tolerance (Thomashow 2001). However, to efficiently activate cold acclimation, low temperature has to be sensed and processed swiftly.

Cold acclimation phenomena could also be observed on grapevines and were firstly discovered on dormant grapevines against the low temperature damages during autumn and winter (Levitt 1980). In nature, shorten day length could inhibit the photosynthesis process to prolong the dormant period which could induce cold acclimation (Weiser 1970). However, several other factors are involved in plants to influence the cold acclimation, such as genetic, environmental and viticulture conditions (Jackson 2000). Regarding the genetic factor, different *Vitis* species differ in the respective favourite climates. Regarding the environmental factor two stages are involved to induce cold acclimation in grapevines: The first stage is induced by non-freezing temperature and happens in late summer/early fall. In the meanwhile, the grapevine during this stage does not reach maximum cold hardiness. The second stage is induced by temperatures below freezing and cold hardiness increases largely. During midwinter, grapevine reaches its maximum hardiness (Zabadal *et al.* 2007). However, the third stage is called de-acclimation; this process takes place faster than acclimation. It usually occurs in the

spring, but warm temperatures in winter can result in de-acclimation leading to severe damages when cold weather returns (Stafne 2007). Therefore, cold acclimation and preventing the de-acclimation in the early spring is important for some cold tender grapevine cultivars and utilization of cold acclimation could facilitate the development of strategies to improve cold tolerance of grapevines.

1.2 The perception and signal transduction of cold stress

As we discussed above, cold stress is one of the important environmental factors influencing plant growth and development and plants have to evolve a mechanism, called cold acclimation by altering their physical and biochemical modifications to adapt or enhance tolerance to low temperature stress. In addition, low temperature could induce the cellular response with the alterations in the expression of some cold related genes and the synthesis of key protein products for plants' survival. However, the critical question is that cold stress must be firstly sensed, perceived and subsequently translated into molecular signals to be transduced in the cellular condition. This progress is sophisticated and still ambiguous because of the shortage of an internal intrinsic "thermometer" in plants (Ruelland and Zachowski 2010). However, recently a cold responsive protein kinase 1 (CRPK1) located on plasma membrane, which could phosphorylate 14-3-3 proteins to transduce the signal from the cytosol to the nucleus to destabilize the CBFs via the 26S proteasome pathway in response to low temperature (Liu *et al.* 2017) is found in *Arabidopsis* and it is thought to be a "thermometer" to perceive low temperature. However, there must be other potential molecules functioning as "thermometer" in plants to sense low temperature. In general, the genetic signal transduction pathway to various stimuli includes the following stages: the signal is initially perceived by some plasma membrane receptor, leading to the activation of some second messengers such as calcium ions, after that a cascade of protein phosphorylation or de-phosphorylation takes place and ultimately induces the regulation of some specific transcription factors or stress related genes to make the plants achieve the tolerance (Tuteja and Mahajan 2007). Current researches have discerned some

factors that might be involved in cold perception, such as the cytoskeleton depolymerization, calcium influx, membrane rigidification, activations of phospholipid D (PLD), generation of reactive oxygen species (ROS), protein conformation and other metabolic reactions. All of these are potentially molecules involved in the upstream signal transduction against cold stress. Moreover, the MAPK (mitogen-activated protein kinase) cascade also attends in the regulation of cold signalling, in particular, as the activated MKK2 overexpressing plants exhibits cold tolerance through the up-regulation of CBF transcription factors (Teige *et al.* 2004). To sum up, all the signal transduction which are up regulated by cold stress including carbohydrate metabolism (ATP formation), calcium ions, ROS scavenging, redox adjustment, cell wall remodelling, cytoskeleton rearrangement, cryoprotection, defence/detoxification, MAP kinases and CBF transcription factors, etc. (**Figure 2**) (Janmohammadi *et al.* 2015).

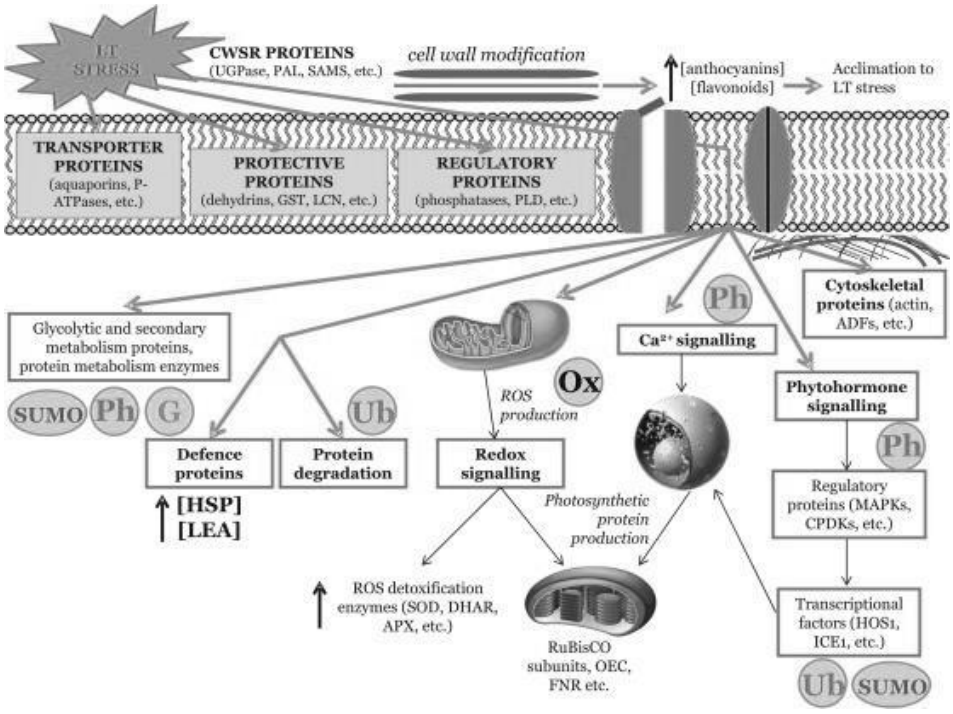

Figure 2. The schematic diagram shows potential protein molecules response to cold stress (Janmohammadi *et al.* 2015). Low temperature is perceived by the plasma membrane by inducing the protein changes in the membrane, such as the P-ATPase, dehydrins and PLD, etc., and then activating different intercellular signalling cascades, including the changes of cytoskeleton, phytohormones, calcium signalling, redox signalling and finally resulting the cold responsive gene expression and protective proteins synthesis to acquire cold tolerance. Abbreviations: CWSR, cell wall synthesis related; UGPase, UDP-glucose pyrophosphorylase; PAL, phenylalanine

ammonia-lyase; SAMS, S-adenosylmethionine synthetase; GST, glutathione S-transferase; LCN, Lipocalin; PLD, phospholipase D; ADF, Actin Depolymerizing Factor; MAPK, Mitogen-activated protein kinases; CDPK, Calcium-dependent protein kinase; HOS1, high expression of osmotically responsive geneS1; ICE1, inducer of CBF expression 1; GRP2, Glycine Rich Protein 2; AOX, alternative oxidase; OEC, Oxygen-evolving complex; FNR, ferredoxin NADPH oxidoreductase; LEA, late embryogenesis abundant; HSP, heat shock protein; DHAR, dehydroascorbate reductase; APX, Ascorbate peroxidase; SOD, Superoxide dismutase; Ph, phosphorylation; G, glycosylation; SUMO, Sumoylation; Ox, oxidation.

However, to understand cold sensing is far from trivial, because, in contrast to the sensing of a chemical ligand, the sensing of a physical stimulus must proceed in two steps. In the first step, physical energy is transformed into a signal in a process that has been named ‘susception’ (Bjorkman 1988). Only in a second step, perception in the strict sense is triggered by this transformed input and will generate a chemical signal. This ‘susception’ can be a relatively passive event, for instance, when gravity is transformed into a displacement of amyloplasts. However, in case of cold, susception must be more sophisticated. Although it is generally accepted that the decreasing fluidity of the membrane is important for cold sensing, the forces generated by these fluidity changes are minor and it will be difficult for the cell to discriminate them against the noise generated by Brownian movement. Therefore, cold susception must require efficient signal amplification mechanisms (Nick 2013).

1.2.1 Microtubules may function as plant cells’ “Thermometer” under cold stress

Microtubules (MTs) are important cytoskeleton filaments in plant cells and have various functions during plant development, such as the formation of cellulose synthesis, the role in cell division and expansion and the signal transduction in response to different biotic and abiotic stresses. MTs are synthesized by α-β tubulin dimers and remain

dynamic states by modulating the balance between the shrinking phase and regrowth phase. This property is called the dynamic stability of MTs which fulfils the critical role of MTs to participate in the signal transduction. In addition, MTs provide a linear scaffold and widely spread in the cytoplasm in two different types which are referred to as radial MTs, connecting the nucleus through transvacuolar strands with the periphery, and as cortical MTs which are oriented in parallel bundles perpendicular to the long cell axis. As a matter of fact, numerous protein binding surfaces are present along MTs. Therefore, the dynamics and reorganization of MTs in combination with other complex molecules may contribute to the signal transduction process. Due to the property of MTs they provide directional transport machinery by guiding the polar movement of molecular motors (Gundersen and Cook 1999, Etienne-Manneville 2010). Many evidences have revealed the signal transduction are exactly involved in the changes of MTs, such as the identification of molecules involved in *JNK* and *ERK* kinase pathways on MTs (Gundersen and Cook 1999). In addition, one of the important mechanisms of MTs involving signal transduction is that MTs could sequestrate and release molecules involved in transduction (Gundersen and Cook 1999). In this respect, the microtubule cytoskeleton might endue importance for the signal transduction because of cold-related properties of MTs, such as disassembly of cortical MTs and accumulation of tubulins in the nucleus.

Many abiotic stresses could cause the mechanical transformation on plants and plants must evolve a mechanical sensor to perceive the signal and translate it into chemical molecules to trigger the defence mechanism. For instance, freezing stress always induces the formation of ice crystals which could cause mechanical stress on the cell surface accompanied by the side effect of osmotic and oxidative stress. In bacteria, a cold sensor named *DesK* which is an integral membrane-associated transmembrane histidine kinase (Hiks) could regulate the changes in membrane structure, like inducing rigidification, to sense low temperature (Aguilar *et al.* 2001, Saita *et al.* 2016). However, the perfect mechanical sensor does not exist in plants (Nick 2008). Recent studies surmise that MTs may function as this kind of sensor involved in signal transduction,

because the important property of a sensor could amplify the signal and convey a signal over considerable distance (Nick 2013). MTs account all of these requirements which could function as these input amplifiers. Likewise, the property of MTs polarity could lead MTs to guide the signal factors to the specific sites in the cell along with microtubule surface (Gundersen and Cook 1999). Wang *et al.* (2009) disclosed that the cytoskeleton could rapidly transfer the signal to the nucleus under the process of direct propagation in animal cells. Grishchuk *et al.* (2005) explained the substantial forces generated by the depolymerization of MTs were about tenfold higher than those caused by microtubule motors. Therefore, MTs maybe really function as a sensor under mechanical stresses. However the molecular mechanism how MTs perform as a sensor still remains elusive. MTs deformation and mechanosensitive ion channels are presumed to be the main mechanism involved in this process (Nick 2008).

Under cold stress, the role of MTs for cold sensing in plants is supported by different observations. The common occurrence is the disassembly of MTs in both animal and plant cells in response to low temperature. The relationship between the disassembly of MTs and the cold sensitivity and cold acclimation is also observed in different organisms. MTs are extremely cold-sensitive in chilling sensitive species, whereas they persist at low temperatures in chilling-tolerant species (Jian *et al.* 1989). ABA could induce cold acclimation and stabilize cortical MTs against low temperature (Sakiyama and Shibaoka 1990, Wang and Nick 2001). In cotton, a chilling-sensitive species, anti-microtubular drugs significantly accelerate and enhance chilling injury, whereas a pretreatment with ABA prevents chilling injury and counteracts the sensitizing effect of anti-microtubular drugs. Chilling injury is accompanied by a destruction of the microtubular network, and ABA prevents this microtubular destruction (Rikin *et al.* 1980, 1983). In an activation-tagged transgenic tobacco line MTs were cold stable, indicated by cold resistant leaf expansion (Ahad *et al.* 2003). Cold acclimation could be detected on the level of MTs as well (Nick 2012). The MTs in cold acclimated cells in some species [*Spinacia oleracea* (spinach) mesophyll, (Bartolo and Carter 1991); *Secale cereal* (rye) roots, (Pihakaski‐Maunsbach and Puhakainen 1995); *Triticum* spp.

(wheat) roots, (Abdrakhamanova *et al.* 2003)] are more resistant against cold stress. In winter wheat, a transient elimination of cortical MTs has been shown to be necessary and sufficient to initiate cold adaptation, which is accompanied by subsequent formation of stable microtubule bundles (Abdrakhamanova *et al.* 2003). A role of MTs for cold signalling has also been demonstrated by a study, where the activation of the *Brassica napus* BN115 promoter had been mapped by pharmacological manipulations (Sangwan *et al.* 2001). Here, oryzalin, a compound that eliminates MTs by sequestering tubulin heterodimers, could mimic the effect of low temperature, whereas taxol could suppress the activation of this promoter by cold. Likewise, gadolinium ions and benzyl alcohol (BA) could prevent the induction of this reporter by low temperature, and, conversely, the calcium ionophore A23187, or DMSO could activate the reporter in the absence of cold treatment (Sangwan *et al.* 2001). These researches denote the rigidification of plasma membrane, the calcium influxes and the disassembly of MTs are mutual affected with each other to participate in cold signal transduction. Due to their close relationship with the plasma membrane as the major platform for signal perception and transduction, MTs and microfilaments have been suggested as downstream targets of various signalling pathways including cold stress (Heidarvand and Amiri 2010). Since MTs and actin filaments can modulate the opening of cold-responsive Ca^{2+} channels (Thion *et al.* 1996, Örvar *et al.* 2000), they apparently participate in the generation or transduction of a cold signal. In summary, all these observations may reach to the conclusion that MTs show high cold sensing function strongly associated with other potential molecular factors, such as calcium.

1.2.2 Calcium influx involved in cold stress

Calcium has been known for a long time (Marme 1985) as a central second messenger and it plays an important role in cellular homeostasis and plant development. Due to the great concentration gradient of calcium in the cytosol, extracellular and intracellular stores which are ~100nM and ~mM, respectively, at normal condition (Knight 2000),

the influx of Ca^{2+} into the cytoplasm takes place rapidly when plants respond to different stimuli, such as light, pathogenic elicitor, drought, salt or cold stress. In addition, the influx of calcium has been observed in many plant cells, triggering wide-ranging intracellular biochemical consequences (for instance, (Gilroy *et al.* 1990, Pineros and Tester 1997)) and further transducing the signal to various Ca^{2+}-binding proteins, such as calmodulin (CaM), consequently inducing a panel of different intracellular responses (reviewed in (Bush 1995)). While the diversity of Ca^{2+} pumps and H^{+}-coupled Ca^{2+} co-transporters also regulate the homogenous stasis of Ca^{2+} (Catal á *et al.* 2003). For example, in *Arabidopsis*, the cyclic nucleotide-gated channels (CNGCs) are responsible for the influx of Ca^{2+} in response to various stresses (Swarbreck *et al.* 2013). Moreover, the Ca^{2+} signalling pathway and the resultant changes in gene transcription are widely studied in plant cells (Ma *et al.* 2009). The relationship between MTs and calcium has been verified. For instance, calcium is involved in mitosis which is required for the formation of MTs and in turn MTs are essential for anaphase movements of chromosomes (Tuteja and Mahajan 2007).

Calmodulin (CaM) is a small acidic adaptor protein that amplifies Ca^{2+}' diminutive size to the scale of proteins (Clapham 2007) and the targeted enzyme activity is regulated by the conformational changes of CaM upon binding to the Ca^{2+} (Hoeflich and Ikura 2002). In animal or plant cells the MTs state is influenced by CaM/Ca^{2+} complexes (Hepler 2005). CaM/Ca^{2+} complexes also regulate the redox signalling by positively regulating H_2O_2 levels through the activation of NADPH oxidase or down regulation of catalase activity (Keller *et al.* 1998, Yang and Poovaiah 2002). Recent studies reveal that CaM/Ca^{2+} complexes could regulate CAMTAs transcription factors in response to various stimuli. For example, under the regulation of cold stress, plants could enhance their cold tolerance by Ca^{2+} signalling via CAMTA1 and CAMTA3 binding to the promoter region of *CBF2* gene (Doherty *et al.* 2009).

In fact, a transient influx of Ca^{2+} into the cytoplasm is among the earliest events triggered by a cold shock (Monroy and Dhindsa 1995, Örvar *et al.* 2000). However,

another event is the transient disassembly of cortical MTs (Abdrakhamanova *et al.* 2003). And the transient influx of Ca^{2+} and disassembly of MTs play essential roles in cold acclimation. The disassembly of MTs has been ascribed to the activation of CaM by cold-triggered calcium influx, but the quality of the microtubular response to CaM seems to be qualitatively different depending on the concentration (Fisher *et al.* 1996), which indicates that there exist several and partially antagonistic pathways. In addition to direct interaction of CaM with MTs, modulation of microtubule-associated proteins (MAPs) by CaM, known to occur in neural cells (Pirollet *et al.* 1992) should be kept in mind. Irrespective of these complexities that are far from being understood, not only the microtubular response, but also the modulation of Ca^{2+} is necessary to initiate cold acclimation as concluded from experiments, where calcium chelators and channel blockers were found to suppress cold adaptation in alfalfa and *Arabidopsis* (Knight *et al.* 1996, Tahtiharju *et al.* 1997). Pharmacological elimination of MTs or actin filaments was shown to amplify cold induced Ca^{2+} influx (Mazars *et al.* 1997), suggesting that MTs act not only downstream of calcium, but might also fulfil a second function very early in cold signalling. In addition, the rapid calcium influx into the cytoplasm could also be induced by the changes of membrane properties through membrane rigidification, and activation of mechanosensitive or ligand-activated Ca^{2+} channels (Komatsu *et al.* 2007). A calcium sensor named *SYT1* (a homolog of synaptotagmin) could prevent ice formation damages by resealing of membranes which are punctured by ice crystals (Yamazaki *et al.* 2008). All these researches demonstrate plasma membrane, together with calcium and MTs plays an imperative role in cold signal transduction pathway and cold protection.

1.2.3 Changes of membrane fluidity response to cold stress

Under cold stress, the plasma membrane is always considered as the first part of cells to perceive the low temperature, manifested by membrane rigidification. With its own structure property, the plasma membrane has critical impact on maintaining the ion homeostasis, proteomic activity, signal transduction and uptake of nutrients. In addition,

the intracellular space and extracellular stimuli is separated by plasma membrane, for the reason that the proteins located in the plasma membrane may be responsible to translate the signals to the downstream activities in the nucleus where the transcriptome is altered to prevent the plants from various damages (Takahashi *et al.* 2013). Membrane fluidity is affected by several factors, such as the changes of ambient environments, and the composition of membranes (Gennis 2013). High temperature providing more energy could increase membrane fluidity, whereas low temperature decreases membrane fluidity (Alonso *et al.* 1997). When the temperature falls, the main change of membrane is the alteration of the fatty acid component of lipids (Murata and Ishizaki-Nishizawa 1992). Saturated fatty acid decelerated by the genetic manipulation of fatty acid desaturases which induce changes in fatty acid and lipid class compositions of membrane lipids (Tasaka *et al.* 1996). For instance, rigidification of plasma membranes by Pd-catalysed hydrogenation leads to the induction of the cold-inducible gene, *desA*, encoding a fatty acid desaturase in the cyanobacterium, *Synecocystis* (Vigh *et al.* 1993). Likewise, mutants affected in desaturases show enhanced cold responses such as cold activation of diacylglycerol kinase (Vaultier *et al.* 2006) and the increment of cytosolic Ca^{2+} is induce by the membrane rigidification opening the ligand activated calcium channels (Komatsu *et al.* 2007). Unsaturated fatty acids have double C=C bond, “kinks” in the fatty acid chains, low temperature melting point and are not well packed in comparison with saturated fatty acid (Sinensky 1974, Wang *et al.* 2006) and the increment of unsaturated fatty acid and the changes of phospholipid contents and contents of solutes in plasma membrane are crucial for cold tolerance (Moellering *et al.* 2010). Thus, it has been proposed that low temperature could be perceived by actually measuring these changes in membrane rigidity (Nishida and Murata 1996, Vigh *et al.* 2007). Two implications of this membrane rigidity model have been confirmed experimentally: rigidification of the membrane by DMSO can mimic cold responses that could induce the expression of a cold-activated promoter in the absence of cold stress, and, conversely, cold responses are inhibited, when the membrane is rendered more fluid by chemicals such as benzyl alcohol (BA) (Sangwan *et al.* 2001).

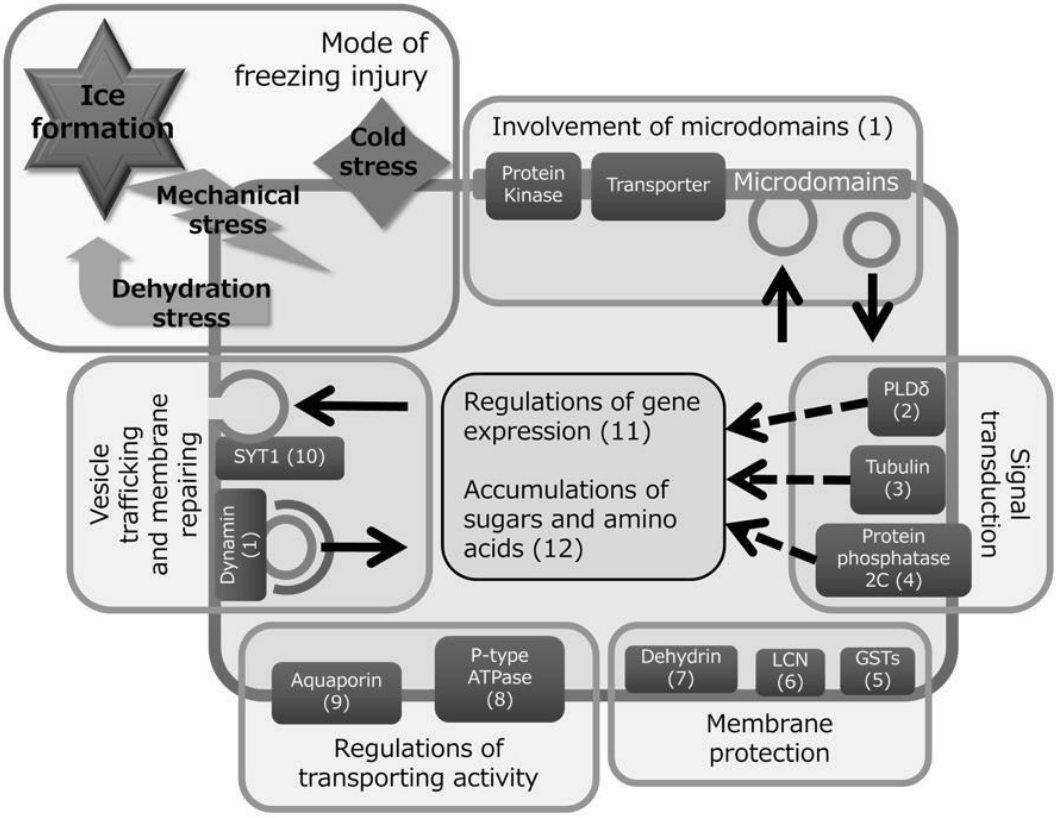

Figure 3. The general model of molecules involved in plasma membrane in response to cold stress (Takahashi *et al.* 2013). Cold stress could also lead to dehydration and mechanical stresses to injure the cells. In order to protect themselves, the protein changes on the membrane parts take place firstly to counter these stresses with cold-evoked membrane protection system, such as the increment of P-type ATPase activity, disassembly of MTs, accumulation of several dehydrin family proteins, involvement of micro-domains, activation of aquaporin and PLD activity.

Besides the rigidification during the process of cold acclimation, the protein composition in the plasma membrane transforms largely, including the increments of P-type Ca^{2+} ATPase activity which could pumps Ca^{2+} against a concentration gradient (Lee 2002), the disassembly of MTs, the synthesis of dehydrin related proteins, some micro-domains and the activation of PLD etc. **(Figure 3)** (Takahashi *et al.* 2013). And the PLD activity involved with G proteins might play the central role between changes of MTs and plasma membrane under cold stress.

1.2.4 The signalling function of phospholipase D and G protein

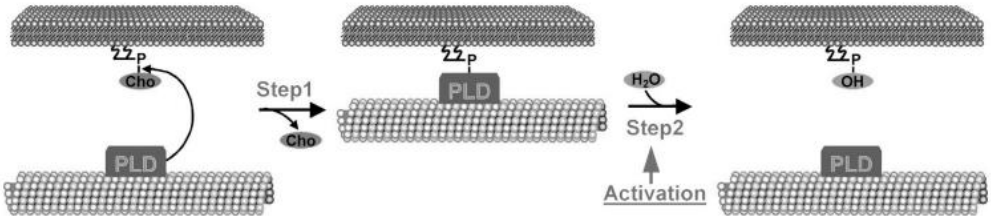

Figure 4. Model of PLD activated reorganization of plant MTs (Dhonukshe *et al.* 2003). Step 1, the remove of choline makes the left PLD-microtubule and phosphatidyl moiety complexes to attach to membrane. Step 2, the PLD-microtubule complex is released by the process of transferring the phosphatidyl group to water. Abbreviations: PLD, Phospholipase D; Cho, Choline.

The early players discussed above (membrane rigidification, calcium influx, MTs) must converge somehow to generate and adjust the signal(s) inducing the appropriate response. As central components of this signal processing, G-protein dependent activation of phospholipase D (PLD) has been discussed (Neer 1995, Munnik 2001). PLD firstly discovered in tobacco cells, named p90, is an important linker between membrane and MTs (Marc *et al.* 1996, Gardiner *et al.* 2001). PLD is associated with MTs and therefore might transduce alterations in the plasma membrane to the cytoskeleton (Gardiner *et al.* 2001). For instance, mastoparan, xylanase, NaCl, and hyperosmotic stress could activate PLD protein to induce the reorganization of MTs in BY-2 cells and n-butanol, considered as a potent inhibitor of PLD, could separate cortical MTs from the plasma membrane and induce partially depolymerization of MTs **(Figure 4)** (Dhonukshe *et al.* 2003). Under hyperosmotic stress, the induced aggregations of tubulins, called "macrotubules", could be prevented by n-butanol treatment whereas external phosphatidic acid (PA) treatment could positively affect macrotubule formation which illustrates that PLD activity affect the reorganization of MTs through the PA signalling pathway (Komis *et al.* 2006). In addition, PLD could also play the role as signalling molecule through the manipulation of actin organization. For instance, PLDδ interacts with F-actin during the dynamic process of

membrane-cytoskeleton interaction (Motes *et al.* 2005, Pleskot *et al.* 2010). In fact, phospholipases C and D are activated as early as 15 seconds after cold treatment (Ruelland *et al.* 2002), and generate, by hydrolysis of membrane phospholipids, phosphatidic acids (PtdOH) as membrane-located secondary messenger. The overexpression of PLDδ in *A. thaliana* could enhance cold tolerance and increase the production of PA species (Li *et al.* 2004). Muzi *et al.* (2016) revealed the same phenomenon that different time of cold treatment could induce the activation of PLD and affect the levels of PA production on the membrane. PA is the active signalling molecule involved in many signal transduction pathways, such as pathogen attack, wound and ABA induced stomatal closure. PA is an early signal factor produced by the activation of PLD by the activation of G protein and calcium ions which can activate NADPH oxidases activity to activate the production of ROS under cold stress, and could also activate protein kinases and/or protein phosphatases involved in hormone signalling (Janda *et al.* 2013). For example, the subunit α of G-protein functions together with PLDα1 to mediate ABA inhibition of stomatal opening (Mishra *et al.* 2006) and further research verifies the PLDα1 and PA could lead to the arrangements of MTs and the calcium increment to induce ABA related stomatal closure (Jiang *et al.* 2014).

Heterotrimeric G-proteins, consisting of α-,β-,γ- three subunits, are conserved plasma membrane-bound proteins to perceive extracellular stimuli and transmit the downstream signals to ion channels, phospholipase, kinases/phosphatases and other effector proteins to control plant growth, cell proliferation, defence, hormonal and stress response (Chakraborty *et al.* 2015, Xu *et al.* 2015). In response to various stresses, the activated subunit Gα could activate many enzymes, like PLC and PLD to further modulate the expression of some stress related genes to improve the stress tolerance (Zhao 2015). For example, some G-protein activators, like mastoparan, ethanol and so on could also activate the PLD enzyme activity (Munnik *et al.* 1995). All these data demonstrate that G-protein is closely related with PLD activity. However, PLDs are a linker between MTs and membrane, thus G-protein might transfer the signal to the MTs via PLD to respond to various stresses, including cold stress.

1.2.5 Reactive oxygen species and nitric oxide

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), singlet oxygen ($^1O_2$) and hydroxyl radical ($HO^·$) are been traditionally seen as by-products of disequilibrated electron transfer reactions and symptoms or causes of cellular injury (Suzuki and Mittler 2006). However, in recent years, large amount of researches have denoted that ROS have emerged as central (and complex) signals, whose rapid accumulation and dynamic turnover orchestrates adaptive responses to stress as regulators of plant growth, development and defence pathways (Mittler *et al.* 2011, Awasthi *et al.* 2015). ROS are generated in different compartments in plant cells, such as chloroplasts, mitochondria, peroxisomes, other micro bodies (Suzuki *et al.* 2012). However, NADPH oxidases are also important proteins which locate at plasma membrane, containing an EF-calcium binding as well as phosphorylation domains, could generate O_2^- by the one electron reduction of O_2 in response to various abiotic stresses (Ogasawara *et al.* 2008). O_2^- could not easily diffuse through the plasma membrane because of the electricity of plasma membrane and it could be easily converted to H_2O_2 by superoxide dismutases (SODs) that could transfer across membranes passively or through water channels (Miller *et al.* 2010). Therefore, H_2O_2 is referred to as an ideal intercellular signalling molecule. In addition, blocking the activity of NADPH oxidase of plant cells by diphenylene iodonium could reduce the production of ROS during biotic or abiotic stresses (Pellinen *et al.* 1999, Dat *et al.* 2003). As mentioned above, ROS could, besides being toxic, also perform a signal transduction function. Mittler *et al.* (2011) revealed several possibilities or reasons that ROS could be referred to as a signalling molecule. Firstly, the dynamic variation of ROS levels in the cells controlled by rapid production and scavenging systems and the tight control over the subcellular localization of ROS signals. Secondly, ROS could act as rapid long distance auto-propagating system, where H_2O_2 is the paramount one. In *Arabidopsis* the moving speed could be up to 8.4cm/min (Miller *et al.* 2009). Last but not the least, ROS are so closely related to the cellular homeostasis and metabolism that

even small environmental changes could induce the production of ROS which make ROS a good signal to monitor changes in cellular metabolism. Even more, ROS, working as signalling molecules, are associated with other networks, such as the protein kinase networks, calcium signalling, CaM, the activation of G-proteins and phospholipid signalling **(Figure 5)** (Gupta *et al.* 2011, Mittler *et al.* 2011).

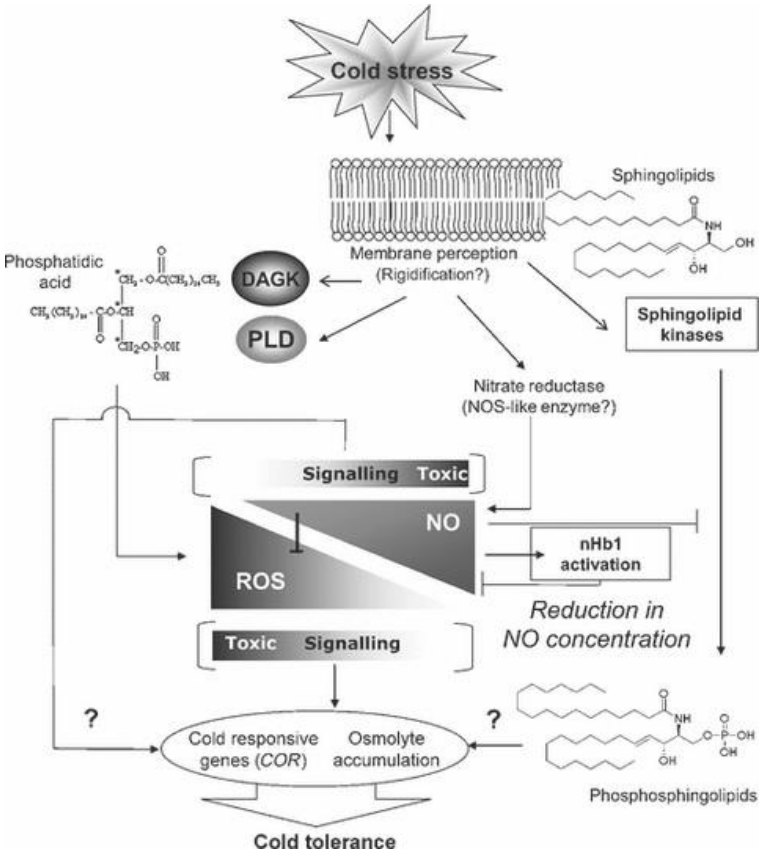

Figure 5. The model of ROS and NO signalling pathway involved in low temperature stress (Gupta *et al.* 2011). Firstly, in response to cold stress, the production of phosphatidic acid through the activation of diacylglycerol kinase (DAGK) and/or phospholipase D (PLD) could activate the production of ROS. In the meanwhile, NO is generated by nitrate reductase, with a possible contribution from a NO synthase (NOS)-like enzyme. However, at high concentration, both of them are toxic and low concentration they could act as signalling molecules. The ROS concentrations are likely to be directly suppressed by NO. However, the content of NO is reduced to a subtoxic concentration by haemoglobins (Hb).

In addition to calcium influx, fluidity dependent modulation of NADPH oxidase might stimulate the production of ROS in the apoplast. There are evidences for the regulation

of Ca^{2+} channels (reviewed in (McAinsh and Pittman 2009) and calcium activated MAPK cascades (reviewed in (Colcombet and Hirt 2008) by ROS. Similar to MTs, ROS could act both, upstream and downstream, since the activation of diacylglycerol kinase and/or phospholipase D (PLD) in response to decreased membrane fluidity will modulate the activity of NADPH oxidase which could induce the production of ROS under cold acclimation. Likewise, a cold sensitive mutant in *Arabidopsis* which is impaired in the mitochondrial electron transport chain showed decrease cold tolerance by high production of ROS affecting the *COR* gene expression (Lee *et al.* 2002).

Except the importance of ROS, recent researches have indicated nitric oxide (NO) is also a crucial signalling molecule as well as ROS involving in various abiotic stresses, such as drought, salt UV-B radiation and cold stresses (Garcia-Mata and Lamattina 2001, Shi *et al.* 2005, Zhao *et al.* 2007, Liu *et al.* 2010) to affect the plant growth and development. In addition, NO integrates with other signal molecules, such as calcium, PA as well as ROS, to complete the signal transduction in response to environmental changes. In plants, NO is generated by the nitrate reductase (NR), a NO synthase (NOS)-like enzyme (see review by (Palavan-Unsal and Arisan 2009). NO plays an important role under cold stress, since NO could act as antioxidant in various conditions (Beligni *et al.* 2002) which could reduce the abundance of activated oxygen species to protect plant from oxidative damages (Ruelland *et al.* 2002, Mittler *et al.* 2011). Furthermore, the external application of NO could enhance cold tolerance in many crops such as wheat, maize and tomato (Neill *et al.* 2003) and chilling induced NO accumulation can effectively protect against oxidative injury (Liu *et al.* 2010). In addition, some cold responsive genes such as members of the CBF family are regulated in a NO dependent manner indicating the crucial role of NO in cold regulation (Puyaubert and Baudouin 2014). In *Arabidopsis*, NO is not only important for non-acclimated plants' survival, but also acquired for cold acclimation to enhance cold tolerance, such as the NR-deficient plants are sensitive to cold stress and lack the ability of cold acclimation through affecting the expression of CBFs and cold responsive genes (Zhao *et al.* 2009, Cantrel *et al.* 2011). In conclusion, calcium, membrane rigidification,

ROS- together with NO-signalling takes place in cold stress. The high concentration of ROS could be suppressed by NO, while the concentration of NO induced by cold stress is controlled by haemoglobins (Hb) to non-toxic concentration to perform the signal function (Watts *et al.* 2001). One group also showed that NO could affect cytoskeleton, such as the dynamics of actin, to influence the vesicle trafficking and recycling of wall polysaccharides in the root apices of maize (Kasprowicz *et al.* 2009). However, for the relationship between MTs and NO in different biotic or abiotic stresses responses evidence is lacking.

1.2.6 Hormonal responses to cold stress

Plant hormones are important molecules determining plant growth and development and they are paramount of importance when plants suffer different biotic and abiotic stresses (Peleg and Blumwald 2011). Amongst phytohormones auxin, abscisic acid (ABA), ethylene (ETH), cytokinins (CKs), gibberellins (GAs), salicylic acid (SA) and jasmonic acid (JA) are involved in cold stress and functional cross-talk with sophisticated signalling cascades by either CBF-dependent or -independent pathways to regulate plant cold tolerance (Shi *et al.* 2015). ABA is the most well-established and is consider as a central regulator of cold stress signalling with emerging roles in CBF-dependent manner (Eremina *et al.* 2016). External treatment of plants with ABA could significantly improve cold tolerance (Rikin *et al.* 1980). A protein kinase involved in ABA signalling pathway, named open stomata 1 (OST1) could positively regulate the ICE-CBF cascade to modulate freezing tolerance (Ding *et al.* 2015). In addition, JA is mainly linked to wounding and pathogen defence. However, increased levels of JA in response to cold stress are found in rice, wheat and *Arabidopsis* (Kosova *et al.* 2012, Hu *et al.* 2013, Maruyama *et al.* 2014). Exogenous application of JA induces the expression of CBF transcription factors to improve cold tolerance (Hu *et al.* 2013). JA may also have suppressive effects on GAs to prioritize defence mechanisms over growth (Yang *et al.* 2012). The production of JA also might be related to the activity of PLD which could

mediate wound induction of JA in response to wounding and LOX2 is probably a downstream target activated by PLD to promote the production of JA (Wang *et al.* 2000). Guan *et al.* (2015) showed that external treatment of JA could induce the central MTs disassembly. This phenomenon might be related to PLD activity as well.

1.2.7 Role of CBF transcription factors under cold stress

As we discussed, the sensor to perceive cold stress and the signal transduction are pretty complex and sophisticated and still remain ambiguous. Many molecules participate in the upstream signal transduction, including plasma membrane, calcium ions, MTs, ROS, phospholipase D, G-protein and the phytohormones. However, downstream signals, such as the activation of gene expression and new protein synthesis, are required for cold tolerance as well. The expression of cold related transcription factors could control genes required for the synthesis of final metabolites, such as sugars, cryoproteins and antioxidants which are the major factors to determine and enhance cold tolerance. The well-established model of gene expression under cold stress is the rapid induction of some transcription factors, the important family of CBFs which could rapidly induce the expression of a set of cold responsive (COR) genes to enhance cold tolerance (Chinnusamy *et al.* 2007, John *et al.* 2016). However, other molecules could also affect the expression of COR genes under cold stress. For example, a role of MTs for cold signalling has been demonstrated by a study, where the activation of the *Brassica napus* BN115 promoter had been mapped by pharmacological manipulation (Sangwan *et al.* 2001). The activity of this promoter could be induced by cold-induced changes of membrane rigidity which were transduced by modulations of calcium influx upon microtubule disassembly. The WRKY, CBF transcription factor and B-box-type finger protein are also identified in different grapevine species and suggest they all could enhance cold tolerance (Takuhara *et al.* 2011, Wang *et al.* 2014). In addition, the regulation of COR genes depends on the regulation of histone acetyltransferases (HATs) (Pavangadkar *et al.* 2010). Moreover, a calcium independent protein kinase called *ZmCPK1* was identified in maize and showed negative regulation under cold stress

(Weckwerth *et al.* 2015). In rice, a *CHILLING-TOLERANCE DIVERGENCE1* (COLD1) was demonstrated to be associated with cold tolerance in superior cold tolerance cultivar "*japonica*". They identified this COLD1 as a plasma membrane localized protein which could activate G-protein α-subunit 1 and affect the calcium levels in the cell for sensing low temperature under cold stress (Ma *et al.* 2015). Furthermore, a CTB4a (cold tolerance at booting stage) was identified and cloned which interacted with AtpB (a beta subunit of ATP synthase) to prove cold tolerance in rice (Zhang *et al.* 2017). All the researches illustrate the complex downstream signal transduction under cold stress and although as of now the CBF transcription factors are the central transcriptional regulators.

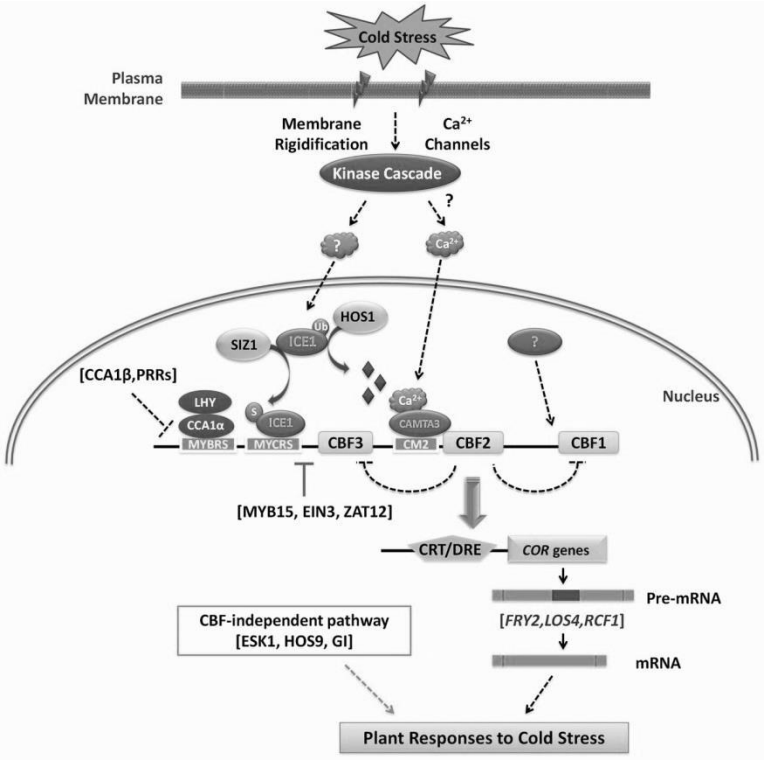

Figure 6. The schematic model summarizes how CBF signal pathway is involved in cold stress (Shi *et al.* 2015). Abbreviations: CBF, C-repeat binding factor; CAMTA3, CaM-binding transcription activator 3; ICE1, inducer of CBF expression 1; HOS, high expression of osmotically responsive genes; SIZ1, SAP and Miz 1; CCA1, circadian clock-associated 1; LHY, late elongated hypocotyl; PRR, pseudo response regulator; COR, cold responsive; FRY2, FIERY 2; LOS4, low expression of osmotically responsive gene 4; RCF1, regulator of CBF gene expression1; ESK1, eskimo 1; GI, GIGANTEA; EIN3, ethylene insensitive 3; CRT, C-repeat elements; DRE, dehydration-responsive

elements; MYBRS, MYB transcription factor recognition sequence; MYCRS, MYC transcription factor recognition sequence; CM2, conserved DNA motif2; Ub, ubiquitination; S, sumoylation.

During the last two decades, the mechanism of cold acclimation is well understood by the researches about ICE1-CBF-COR transcriptional cascade **(Figure 6)** (Shi *et al.* 2015). CBF proteins belongs to AP2/ERF transcription factor family which could bind to the CRT/DRE(C-repeat/Dehydration Responsive Element) DNA regulatory element in the promoters of many COR genes (Stockinger *et al.* 1997). CBF is firstly discovered in *Arabidopsis* and widely studied in other species, like wheat, barley, rice, maize, grapevine and so on. The three members of CBF family, CBF1, CBF2 and CBF3 are rapidly induced after cold treatment (Medina *et al.* 2011) and the mutant of CBF1 and CBF3 could decrease freezing tolerance (Novillo *et al.* 2007). In addition, the overexpression of CBFs could enhance their freezing tolerance in canola (*Brassica napus* L.), tomato (*Solanum lycopersicum* L.) and poplar (*Populus balsamifera* subsp. *trichocarpa*) (Hsieh *et al.* 2002a, Savitch *et al.* 2005, Benedict *et al.* 2006). In grapevine, the CBF1-4 have also been isolated and other proteins associated with cold tolerance, such as H^+-pyrophosphatase and dehydrins (Venter *et al.* 2006, Xiao *et al.* 2008). *VaCBF1* was isolated in *V. amurensis* and it was suggested that it could enhance cold tolerance by increasing antioxidant activities and promoting the induction of downstream cold responsive genes (Dong *et al.* 2013). Hence, CBF genes operate during cold stress in diverse plant species. Moreover, the CBF genes are also regulated by upstream transcription factors, such as the basic-helix-loop-helix (bHLH) transcription factor, ICE1, three closely related CAMTA transcription factors, CAMTA1-3, R2R3-MYB family protein, MYB15 and C2H2 zinc finger transcription factor, ZAT12 (Vogel *et al.* 2005, Agarwal *et al.* 2006, Kim *et al.* 2013, Zhu 2016), and the first two are positive regulators of CBFs and the last two function adversely. The *ice1* mutant could affect the expression of CBF2 and CBF3, except CBF1 and the mutant *MYB15* perform conversely, furthermore in *ice1* mutant the expressed level of MYB15 was higher than in the wild type plant, demonstrating MYB15 is negatively regulated by ICE1 under cold stress (Chinnusamy *et al.* 2003, Agarwal *et al.* 2006). ICE

could also be activated by SIZ1 which mediates sumoylation through the SUMO E3 ligase to enhance its stability and inactivated by ubiquitin E3 ligase HOS1 to cause subsequent proteasomal degradation (Chinnusamy *et al.* 2007). Recent researches indicated that a protein kinase OST1 (open stomata 1) could phosphorylate ICE1 which resulted in activation of ICE1 and enhancement of the expression of CBF3 against cold stress. In addition, the phosphorylation of ICE1 inhibits the affinity of HOS1 to prevent ICE1 degradation (Ding *et al.* 2015). In summary, CBF transcription factors which are regulated complexly by other transcription factors indeed play crucial roles under cold acclimation. However recent research by (Park *et al.* 2015) demonstrated that only one third of the increment of cold tolerance is dependent on CBF regulation. The regulation is highly interconnected, other transcription factors are co-regulated with CBF regulons, such as HSFC1, ZAT12, ZF, ZAT10 and CZF1. In *Arabidopsis*, putative 2000 COR genes exist and 1200 of them are expressed by low temperature treatment, but only 170 of them are regulated by CBF transcription factors (Park *et al.* 2015). It demonstrates that there must other transcription factors independent of CBF to regulate the left large amount of COR genes which still remain elusive.

1.3 Scope of this study

Grapevine, an important fruit crop of global importance and high economic yield is cultivated over broad areas that strongly differ in climatic conditions. Viticulture is strongly limited by temperature - not only with respect to freezing during the winter, but also pertaining to chilling damage in young leaves and flowers during late spring. Although there seems to be genetic variation in freezing tolerance, because certain wild species of grapevine as well as some cultivars can tolerate even freezing conditions, it is still unclear how grapevine can recognize low temperature and acquire cold tolerance (Takuhara *et al.* 2011). Information about the events or molecular components that convey cold signalling from stimulus to perception, and then further from perception to early signal transduction culminating in cold adaptation is central to identify genetic factors that promote cold hardiness.

As I discussed above, numerous signalling factors are involved in the upstream signal perception and downstream signal transduction against cold stress. MTs are an important one, since in addition of its role in cell division and axial cell expansion, they also associate with other molecules to function as a sensory hub for the signal transduction, such as responding to cold tress or pathogen attack. Manipulation of the state of MTs could enhance plant cold tolerance such as the transient disassembly of MTs (Abdrakhamanova *et al.* 2003). Thus, in my Ph.D. work, I would detect the early changes of MTs under cold stress and look for which signals acting upstream of MTs, and how MTs act in providing adaptive responses (cold acclimation, gene regulation). These insights can be used in the future to design strategies targeted on improved cold tolerance, either by molecular-assisted breeding, or, alternatively to genetic changes, by chemical manipulation of early signalling events in order to improve cold tolerance of cultivars which are otherwise cold-sensitive in temperate climates.

Central questions were:

1. What are the functions of MTs and other related molecules involved in cold early signal transduction?

In our lab, a transgenic grapevine suspension cell line expressing a fluorescent tubulin marker was generated by Guan *et al.* (2015) in the background of the osmotolerant American grapevine species *V. rupestris*, where MTs are visible by GFP and she had used this cell line to identify remodelling of MTs in response to biotic and abiotic stresses as early signal event by using spinning-disc microscopy. Compared to the traditional methodology, like the immunofluorescence, this transgenic GFP-tubulin line could allow us for the first time to detect early and small nuances of MTs changes under cold stress. Thus, my strategy was to combine pharmacological and advanced microscopically approaches (e.g. spinning disc) with molecular studies to follow what would happen to MTs during cold stress and adaptation in grapevine *in vivo*. We

dissected the early events of cold signalling upstream of MTs, such as the relationship to plasma membrane, calcium ions, ROS, JA, the activity of PLD protein and G-protein. We used the classical pharmacological treatment like the inhibitors and activators to pre-treat the cells and observed the changes of MTs at different temperature (0℃ and 27℃, respectively). We showed that calcium influx, membrane rigidification, and activation of NADPH oxidase contribute to signalling, and that a G-protein in concert with phospholipase D conveys the signal towards MTs, whereas CaM seems to be not involved. However, we found that the cold induced activation of the jasmonate pathway is required for an efficient microtubule response. Based on these observations, we arrive at a model where the upstream signalling molecules are linked with MTs to trigger the cold acclimation.

2. Might MTs function together with the induction of the transcription factor, *VvCBF4*, to inquire cold acclimation?

As we discussed above, MTs play an essential role under cold acclimation, especially the transient disassembly of MTs, but the function of CBF transcription factors could also not be negligible, even though the CBF alone are not sufficient for the maximal induction of cold acclimation (Vashegyi *et al.* 2013). Thus, the disassembly of MTs might work together with CBFs to achieve the cold acclimation or in other words, the induction of *VvCBF4* might depend on the changes of MTs pathway to affect cold acclimation. In order to further verify this hypothesis, we also used the same transgenic cell line which was pretreated with some microtubule stabilizing or destabilizing compounds to observe cell mortality, microtubule changes and the expression of *VvCBF4* under cold stress. We illustrated that the disassembly of MTs, the increment of membrane fluidity, the influx of calcium ions and the induction of *VvCBF4* are essential for grapevine cold acclimation. The increased membrane fluidity could partially prevent the disassembly of MTs, but independent of CBF pathway to modulate cold acclimation. JA could promote the disassembly of MTs independently of the *VvCBF4* pathway to adjust cold acclimation. The central factor is calcium which plays imperative role in

cold acclimation through diverse functional branches, one depends on microtubule pathway and another depends on the CBF pathway.

2. Materials and methods

2.1 Plant material

A suspension cell culture of *V. rupestris* expressing the fluorescent tubulin marker *GFP-AtTUB6* (Guan *et al.* 2015) was used in this experiment. The cells were cultivated in liquid medium containing 4.3 $g \cdot L^{-1}$Murashige and Skoog salts (Duchefa, Haarlem, The Netherlands), 30 $g \cdot L^{-1}$ sucrose, 200 $mg \cdot L^{-1}$ KH_2PO_4, 100 $mg \cdot L^{-1}$ inositol, 1 $mg \cdot L^{-1}$ thiamine, and 0.2$mg \cdot L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.8, and 35 $mg \cdot L^{-1}$ hygromycin. Every 7 days, 5 ml of cells were transferred into 30 ml of fresh medium in 100 ml Erlenmeyer flasks. The suspension was incubated at 27 ℃ in the dark on a horizontal shaker (KS250 basic, IKA Labortechnik, Staufen, Germany) at 150 rpm.

2.2 Cold treatment

For the first part of observing MTs, the cells where either used in the exponential phase (3 days after inoculation), or in the stationary phase (5 days after inoculation). To administer cold stress (0 ℃), either the entire Erlenmeyer flask with the suspension, or aliquots of 2 ml in reaction tubes (Eppendorf, Hamburg) were placed in an ice water bath and shaken in the dark on a horizontal shaker, or a small chamber putted on the slide under microscope. The temperature cooling rate (27 ℃ to 0 ℃) for cells treated at day 3 after sub-cultivation was 6.75 $℃ \cdot min^{-1}$, and for cells treated at day 5 after sub-cultivation was 4.70 $℃ \cdot min^{-1}$. Samples of three days cells for central microtubule observation and five days cells for cortical microtubule observation or chemical treatment were collected at specified time points during the cold treatment.

For the cold acclimation part of experiments, the difference between above is that the cells were treated in ice water (0 ℃) or cold room (nearly 8 ℃) at day 4 after sub-cultivation for three days with or without chemical treatment and the cell mortality

was tested by Evans Blue method at different time points after cold treatment. In the whole experiment, the entire Erlenmeyer flask with the suspension cells was placed in an ice water bath on a horizontal shaker. In the meanwhile, the cortical MTs were observed by confocal spinning disc microscopy.

For the screening of cold tolerance of *Vitis vinifera ssp. Sylvestris* plants, all accessions of *Vitis vinifera ssp. Sylvestris* are from the 'Ketsch' peninsula at the Rhine River, in the Southern Germany and from different sites in the Upper Rhine Valley. These species are already collected and preserved as living specimens in the botanical garden of KIT (Duan *et al.* 2015). Thus, for the cold treatment of leaves, the fourth or fifth leaves counted from the apex of all the genotypes were collected. Then the individual leaf was put into a big petri dish and was transferred into a 4 ℃ chamber to perform the cold treatment. The leaves were harvested at different time points after the treatment and immediately frozen in liquid nitrogen, stored at -80 ℃ for RNA analysis.

2.3 Pharmacological treatments

Manipulation of Ca^{2+} influx at the plasma membrane. Ca^{2+} influx into tobacco protoplasts was enhanced by disruption of MTs and actin filaments (Mazars *et al.* 1997). Thus, the Ca^{2+} ionophore A23187 (Sigma-Aldrich, Germany), dissolved in DMSO, was used to promote Ca^{2+} in the absence of cold stress (at 27 ℃), at a final concentration of 10 μM (Monroy and Dhindsa 1995). In contrast, to prevent Ca^{2+} influx, either 8mM EGTA (Roth, Germany) as calcium chelator, dissolved in H_2O, or 0.15 mM $GdCl_3$ (Sigma-Aldrich, Germany) as inhibitor of calcium-influx channels were used. The concentration of EGTA and $GdCl_3$ was based on the results of previous studies in grapevine cells (Liu *et al.* 2013a). For the A23187 treatment, the cells were observed by microscopy directly after addition of the agent to the cells at 27 ℃. As negative control, cells were treated with the same concentration of the solvent DMSO. For the $GdCl_3$ experiments, the cells were first treated for half an hour with the respective compound at 27 ℃ before transfer of the cells to 0 ℃.

Manipulation of membrane fluidity. To modulate membrane fluidity, benzyl alcohol (BA), an amphiphilic molecule, can be used as a membrane "fluidizer" that affects lipid bilayer structures (Ebihara *et al.* 1979). In contrast, dimethylsulfoxide (DMSO), as polar aprotic solvent, is a well-documented membrane rigidifier (Örvar *et al.* 2000). BA and DMSO (Roth, Germany) were added directly to the suspension cells at the concentrations indicated in Figure 3. For DMSO treatment, the cells were observed directly after addition to the cells at 27 ℃. In case of the BA experiments, the cells were first treated for 1 hour with the indicated concentration at 27 ℃ before incubation at 0 ℃.

Manipulation of ROS and NO. Diphenyleneiodonium (DPI) inhibits the production of ROS by specifically binding to flavin-binding enzymes, particularly the NAD(P)H oxidase (Li and Trush 1998). Sodium nitroprusside (SNP) can be used as donor for the important stress-signalling molecule NO (Delledonne *et al.* 1998). The suspension cells were treated with different concentrations of DPI (Sigma-Aldrich, Germany), diluted from a 100 µM stock solution in DMSO, for 2min at 27 ℃, before transfer to 0 ℃. As negative control, cells were treated with the same concentration of the solvent DMSO. Aliquots collected from the cold-treated population at specific time points were directly analysed by microscopy. For the SPN treatment, the suspension cells were treated with 1 mM SNP (Sigma-Aldrich, Germany), dissolved in water at 27 ℃ for 1 h and then observed by microscopy. The concentrations of these treatments were derived from preparatory experiments with respect to the absence of toxicity symptoms.

Manipulation of CaM. Calmodulin (CaM) is a Ca^{2+} binding protein which has been implicated in cold-triggered microtubule disassembly (Fisher and Cyr 1993). Trifluoperazine (TFP) is a widely used antagonist of CaM (Feldkamp *et al.* 2010). Thus, the suspension cells were treated with 100 µM TFP (Sigma-Aldrich, Germany), dissolved in water, for 2 hat 27 ℃ and then transferred to 0 ℃ and observed by microscopy directly after sampling at specific time points.

Manipulation of the jasmonate pathway. The lipoxygenase inhibitor phenidone (1-phenylpyrazolidinone) can be used to block JA synthesis in grapevine cells (Ismail *et al.* 2012). Cells were treated with 4 mM of phenidone in an aqueous solution containing 0.1% polyoxy-ethylene-orbitan monolaurate (Tween-20) (both obtained from Sigma-Aldrich, Germany) for 2 h at 27 ℃, and then transferred to 0 ℃ and MTs observed by microscopy directly at sampling after specific time intervals. As negative control, cells were treated with the same concentration of the solvent. In contrast, 50 µM jasmonic acid (Sigma-Aldrich, Germany) diluted from a 500 mM stock in absolute ethanol were added directly to the suspension cells, then the cells were followed directly by microscopy at 27 ℃. As solvent control, 0.1% ethanol was used.

Manipulation of phospholipase D. Phospholipase D activity can be diverted by replacing water as acceptor of the trans-phophatidylation by small primary alcohols such as *n*-butanol. As a consequence, instead of the active signalling compound PA inactive phosphatidyl alcohols are generated (Munnik *et al.* 1995). Secondary butanol can be used as negative control. Cells were therefore treated with 1% (v/v) *n*-butanol and *sec*-butanol (Roth, Germany), respectively, either under cold stress (0 ℃), or at room temperature (27 ℃)

Manipulation of G-proteins. PLD is activated by a trimeric G-protein. Therefore, pertussis toxin (Sigma-Aldrich, Germany), a blocker for trimeric G-proteins (Warpeha *et al.* 1991), was used. Cells were pre-treated for 30 min with $10 ng \cdot mL^{-1}$ of pertussis toxin at 27 ℃, then transferred to 0 ℃ and observed by microscopy directly after sampling at specific time intervals. The G-protein activator AlF_4^- (Bigay *et al.* 1985)was freshly prepared as described in (Campanoni and Nick 2005), and then added directly to the suspension cells at 27 ℃, directly following the cellular response by microscopy at 27 ℃.

2.4 Cold acclimation treatment and Evans blue assay

Firstly, cells were treated in ice water (0 ℃) and cold room (nearly 8 ℃) for 72 hours,

respectively. Secondly, cells were pretreated in cold room (nearly 8℃) for 72 hours and then were transferred into ice water (0℃) for another 48 hours. Thirdly, the cells were pretreated with 10μM taxol or oryzalin (Sigma-Aldrich, Germany) for half an hour respectively which could stable and depolymerize the MTs, and then the cells were putted into ice water treatment. However, for the pronamide treatment the cells were pretreated with 25μM and 50μM pronamide (Fluka, Germany) for 2 hours to disassemble MTs at 27℃, then the medium containing this compound were washed away with a big mesh device and the cells were re-dissolved in new MS medium and cultivated at 27℃ for 3 hours for the recovery of MTs. Then the cells were treated with ice water (0℃) for another 72 hours. In the meanwhile, all the cells were collected every 24 hours to do the mortality test and the cortical MTs were observed. As a negative control, cells were treated with the same concentration of the solvent DMSO.

For the chemical treatment assay, the cell were pretreated with 2mM EGTA, 10mM BA, 200nM Latrunculin B (Sigma-Aldrich, Germany) and 50μM JA respectively for half an hour and then the cells were treated with ice water for 72 hours. The mortality of cells was tested every 24 hours. As negative control, cells were treated with the same concentration of the solvent DMSO or ethanol.

Mortality was assessed by the traditional method, named Evans blue assay (Gaff and Okong'o-Ogola 1971). 300μl cells were transferred into a small mesh-like device to get rid of the medium and then put the device into a custom-made staining chamber containing 2.5% (W/V) Evans Blue (Sigma-Aldrich, Germany). The frequency of the dead cells (blue color) was counted under bright-field illumination with an AxioImager Z.1/ApoTome microsocopy. For each experiment, at every time point, at least 500 cells were counted and at least four independent experiments were performed.

2.5 MDA assay

Malone dialdehyde (MDA), a product of lipid peroxidation, can be used as readout for oxidative damage using a colorimetric assay based on conversion of 2-thiobarbituric

acid (TBA) according to (Hodgson and Raison 1991) with minor changes in sample preparation as follows: Cells were collected in aliquots of 1 ml and spun down for 10 minutes at 10.000 g to remove the supernatant and record fresh weight of the cells. Immediately after recording fresh weight, cells were shock-frozen in liquid nitrogen, and homogenized with steel beads (Tissue Lyser, Qiagen/Retsch, Germany) in a standardized manner (twice 30 s at 25 Hz). Each sample was complemented with 1 ml of 10 mM sodium phosphate buffer (pH 7.4), homogenized, and briefly spun down. 200 microliters of the supernatant were added to a reaction mixture containing 100 µl of 8.1% (w/v) sodium dodecyl sulphate (SDS), 750 µl of 20% (w/v) acetic acid (pH 3.5), 750 µl of 0.8% (w/v) aqueous TBA, and 200 µl of Milli-Q water. An identical reaction mixture, where the supernatant from the sample was replaced by an equal volume of buffer was used as blank. Both reaction mixtures were then incubated at 98 ℃ for 1 h. After cooling to room temperature, the mixtures were centrifuged for 5 min, and absorbance measured at 535 nm (specific signal) and 600 nm (background). Lipid peroxidation could then be calculated as µM MDA from A535-A600 using an extinction coefficient of 155 $mM^{-1}{\cdot}cm^{-1}$.

2.6 Measurement of extracellular alkalinisation

Extracellular alkalinisation was measured by combining a pH meter (pH 12, Schott Handylab) with a pH electrode (LoT 403-M8-S7/120, Mettler Toledo). The suspension cells were pre-equilibrated on an horizontal shaker for around 60 min and then treated with either 0.15 mM $GdCl_3$, 8 mM EGTA, or 4 mM benzyl alcohol, respectively, while incubating the sample in ice water (0 ℃). In contrast, the pH responses to10 µM A23187, or 1% (v/v) DMSO, respectively, were measured at 27 ℃. All samples were kept in the dark on a horizontal shaker. Alkalinisation was followed over 1 hour, and peak values (corrected for the basal value established during pre-equilibration) reached at around 35 min after the onset of treatment were plotted relative to the value in the control (treatment with 0 ℃ without addition of any compounds). In addition to this ice-water control without any chemical treatment, a second control kept at room temperature (27 ℃) was included - in this second control no fluctuation of apoplastic pH was

observed.

2.7 Microscopy and image processing

The response of central and cortical microtubules was followed over time using the GFP-AtTuB6 marker (Guan *et al.* 2015) after application of cold stress (0 ℃) and the different chemical treatments in individual cells by spinning-disc confocal microscopy. Confocal images were recorded with an AxioObserver Z1 (Zeiss, Jena, Germany) using a 63 × LCI-NeofluarImmCorr DIC objective (NA 1.3), the 488 nm emission line of an Ar-Kr laser, and a spinning-disc device (YOKOGAWA CSU-X1 5000). At different time points after onset of the treatment, z-stacks were captured and processed using the ZEN software (Zeiss, Oberkochen) to generate orthogonal projections from the recorded stacks and to export them in TIFF format.

2.8 Quantification of microtubule integrity

The orthogonal projections obtained from the confocal z-stacks were used to quantify microtubule integrity according to (Schwarzerova *et al.* 2002) using the ImageJ image analysis software (http://imagej.nih.gov/ij/). In brief, mean density profiles of fluorescence were sampled for probing four lines of 8 pixel width, whereby the lines were oriented as rectangle around the nucleus) as schematically shown in **Figure 1C**. The averaging over the width of the probing line was important to suppress stochastic fluctuations of background. The resulting peaks representing the cross section of radial microtubules were approximately linear and symmetrical in consequence of this averaging. The weighted number of MTs could then be calculated using the algorithm described in Schwarzerová *et al.* (2002) with minor modification. The development of the method consisted in subtracting the minimal value of each profile as background. From this corrected density profile, this first derivative x'_i was calculated for each pixel position and divided by the absolute value of the first derivative:

$$f(x_i) = x'_i / ABS(x'_i)$$

This will yield a value of +1 for the rising flank of MTs, but -1 for the dropping flank. To filter out random fluctuations of intensity that were not MTs, the values for two subsequent positions were added up and divided by two: $z_i = ((f(x_i) + f(x_{i-1}))/2$ with i and i-1 being subsequent positions in the profile). This operation will only leave values that showed a steady change over at least two subsequent positions of the profile, whereas random fluctuations will get a value of 0. By adding $f(x_i)$ and $ABS(z_i)$, all pixels in a rising flank of a microtubule will get a value of +2, whereas the pixels in the sinking flank will get a value of 0. The sum over the profile divided by the length of the probing line will measure the proportion of pixels along the line that belong to a microtubule (based on the assumption of a more or less symmetrical peak). This value will decrease, when microtubules disintegrate (because then the line will experience a reduced likelihood to hit a microtubule) or when they become thinner. We define this value as "relative weighted number of MTs" to monitor and quantify the response of microtubules to the different treatments.

2.9 RNA extraction and cDNA synthesis

Total RNA from cells was extracted by Universal RNA Purification Kit (Roboklon, Germany) and that from the leaves was isolated by using the Spectrum^{TM} Plant Total RNA Kit (Sigma, Germany). A slight modification of the protocol was to employ the use of the DNase (Qiagen, Hilden, Germany) in order to remove the genomic DNA contamination from the samples. The concentration and quality of RNA were analyzed using the Nano drop machine (company) and 0.8% agarose gel. The cDNA synthesis is performed by the M-MuLV cDNA Synthesis Kit (New England Biolabs; Frankfurt am Main, Germany) according the manufacturer's protocol. The amount of RNA template was 1 µg.

2.10 Semi-quantitative PCR and real-time PCR

The reaction system and the PCR progress of semi-quantitative PCR was performed as

described previously (Duan *et al.* 2015). The annealing temperature is 58℃ for 25 seconds and 30 seconds synthesis at 68℃. The primer sequences of the house keeping gene (*VvActin*) and C-repeated binding transcription factor (*VvCBF4*) is showing here: *VvActin* (Sense, 5'-3' CTCTATATGCCAGTGGGCGTAC; Antisense, 5'-3' CTGAGGAGCTGCTCTTTGCAG); *VvCBF4* (Sense, 5'-3'CGAGGTAAGGGAGCCC AACAA; Antisense, 5'-3' GCCACGCAGAGTCCGCAAAAT). The PCR was performed using *Taq* polymerase from New England Biolabs (NEB, Frankfurt, Germany).

The quantitative real-time PCR was performed using 96-well plates, with a CFX96^{TM} real-time PCR cycler (Bio-RAD, USA) according to the procedure described by the manufacture for a reaction volume of 20µl. The *VvActin* gene was used as a house keeping reference gene. After the reaction finished running, the gene (*VvCBF4*) expressed level is calculated with the traditional $2^{-\Delta\Delta Ct}$ method (Ref).

3. Results

3.1 Chapter 1

3.1.1 Central and cortical microtubules disassemble under cold stress

In suspension cells, the microtubular marker line V. *rupestris* GFP-TuB6 displays two interphase arrays of MTs: radial MTs connect the nucleus through the transvacuolar strands with the periphery, whereas cortical MTs (**Figure 1A**, 0 min) are oriented in parallel bundles perpendicular to the long cell axis.

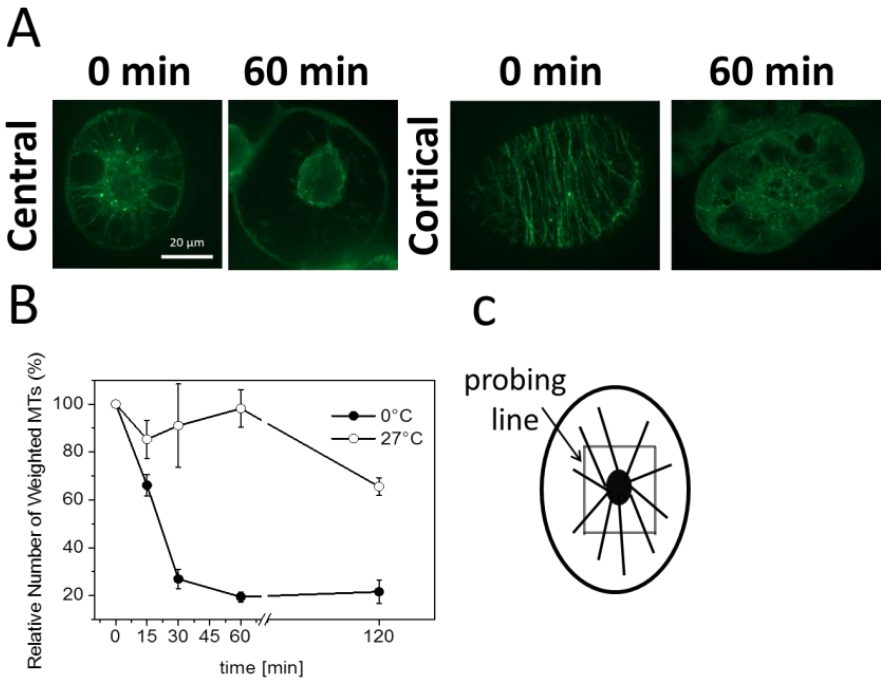

Figure 1. (A) Response of central and cortical MTs to cold stress (0 ℃) shown by time-lapse series recorded in the MTs marker line *V. rupestris* GFP-TuB6. 0 min shows the situation under normal temperature (27 ℃). 60 min represents the situation after 60 min of incubation in ice water. Observations are representative of at least four independent experimental series with a population of 50 individual cells for each treatment. Bars, 20 µm. (**B)** Quantitative analysis of the cold response of central MTs. Relative number of weighted MTs relate to the value observed in the control at time 0

min. Each value of the relative number of weighted MTs represents the average of 50 individual cells. (**C)** Schematic set-up of the probing lines used to quantify microtubule integrity.

For radial MTs, already after 15 minutes of cold treatment (0 ℃), the number of MTs decreased significantly, although most cells still retained a small number of MTs. After 30 minutes, most MTs had disassembled, and only remnants of MTs were occasionally seen. When the cold treatment exceeded 30 minutes, MTs disappeared nearly completely (**Figure 1A**). One hour was sufficient for a disassembly of transvacuolar MTs. We therefore used a cold treatment over 2 hours to ensure that central MTs were fully eliminated. The response of cortical MTs was qualitatively similar, but a little bit resistant - large part of MTs depolymerized within 1 hour under cold stress (0 ℃). However, in contrast to radial MTs, the cortical MTs became disordered during partial depolymerization over the 30 minutes (**Figure S1**).

In order to statistically verify the microtubular response to cold stress (0 ℃), density and integrity of microtubules were quantified by a parameter termed "relative number of weighted MTs " (for details, see Material and Methods) which allowed to measure the responses of radial MTs over time (**Figure 1B**). Without cold treatment, this parameter remained stable over the first 60 minutes of observation, and did not exhibit any significant changes. Only during the second hour, it would decrease slightly by around 30%. This indicated that potential effects of photo bleaching on the GFP labelled MTs were negligible. Moreover, from this time course it could be concluded that observation of a given cell over 1 hour should yield reliable and stable results. In contrast to the stable behaviour for the relative number of weighted MTs under control conditions, this parameter dropped rapidly under cold stress to approximately 20% of the initial value within 30 minutes, and then remained stable at a very low level over the remaining 90 minutes of the observation period. In preparatory experiments, the graphs generated from plotting "number of weighted MTs" were found to match closely the situation seen in the confocal images (**Figure 1A**).

3.1.2 Calcium influx into the cytoplasm is necessary to trigger cold-induced depolymerization of MTs

Calcium as an important secondary messenger for the signalling of different stresses has also been proposed to be responsible for microtubule disassembly. In order to verify this hypothesis, we either blocked calcium influx by 150 µM $GdCl_3$, or removed calcium ions from the medium by chelation with 8 mM EGTA for 30 minutes, before following the response of MTs to cold stress (**Figure 2A**). We observed that both treatments almost suppressed the elimination of MTs under cold stress (**Figure 2A**). This means that calcium influx from the exterior was necessary for cold-induced microtubule disassembly. To test, whether calcium influx would also be sufficient for MT disassembly, the cells were treated with 10 µM of the calcium ionophore A23187 at 27 ℃. This treatment failed to cause the disassembly of MTs (**Figure 2A, B**) suggesting that calcium influx is not sufficient, but has to interact with other cold-induced events to activate MT disassembly. To probe for a potential role of calmodulin, we used 100 µM of Trifluoperazine dihydrochloride (TFP), but this treatment did not inhibit the disassembly of central and cortical MTs under cold stress (**Figure S2**).

The activity of calcium influx channels in the membrane is linked with the co transport of protons. The resulting extracellular alkalinisation can therefore be used to conveniently monitor the activity of calcium influx, a strategy that has been widely used to follow plant defence. We compared this pH shift for cold treatment alone with that produced after pre-treatment with 150 µM $GdCl_3$ or 8 mM EGTA, respectively. The alkalinisation proceeded after a few minutes and reached a peak at 35 minutes after start of the cold treatment. These peak values were used as readout for the amplitude of the pH response. Both, chelation of calcium by EGTA, and inhibition of channel activity by $GdCl_3$, effectively inhibited alkalinisation, while A23187 promoted alkalinisation slightly (**Figure 2C**). The early activation of alkalinisation, along with the fact that alkalinisation was inhibited by EGTA and $GdCl_3$ in the same way as the cold response of central and cortical MTs was inhibited, lends further support to a role of calcium

influx as upstream signal in cold-induced MT disassembly.

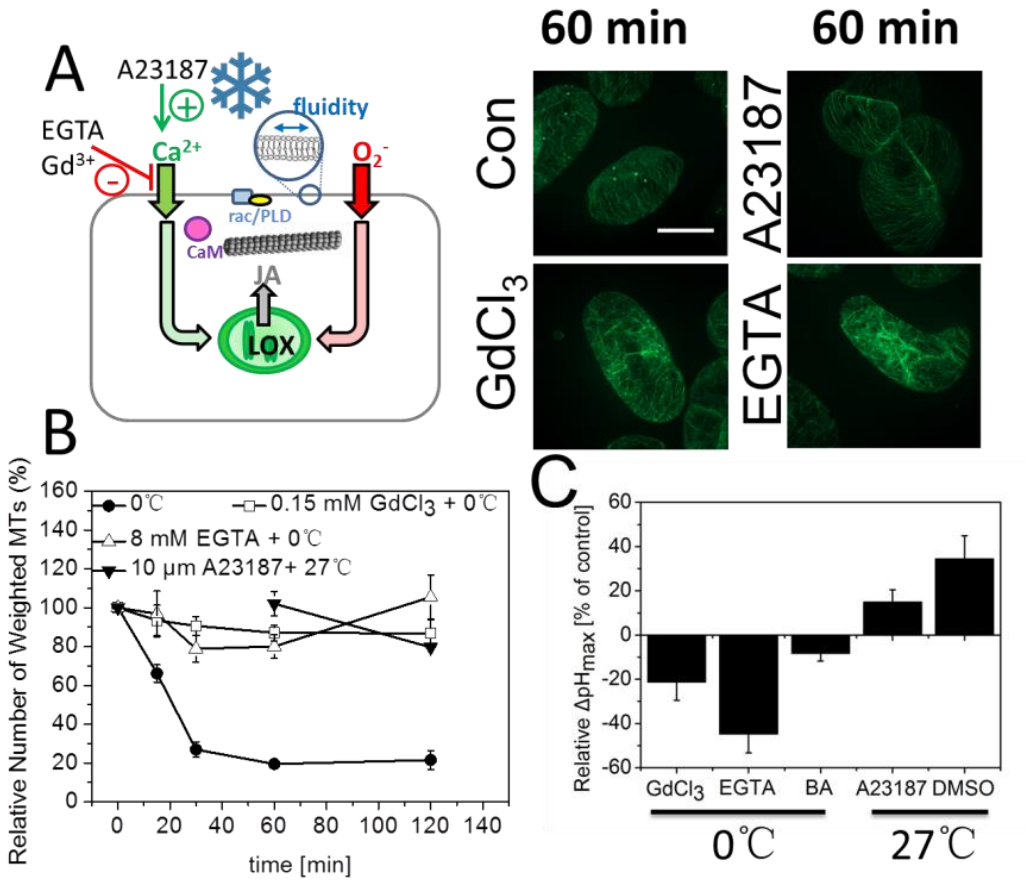

Figure 2. (**A)** Effect of $GdCl_3$, EGTA and the calcium ionophore A23187 on the microtubular response to cold stress (0 ℃) and normal temperature (27 ℃) in the *V. rupestris* GFP-TuB6 cell line. Representative images for the response of cortical MTs after 60 minutes of treatment to 0.15 mM $GdCl_3$, 8mM EGTA and 10 µM A23187 at 27 ℃ are shown here. Observations are representative of at least three independent experimental series for each treatment. Size bars, 20 µm. (**B)** Quantitative analysis of the central MTs response to cold stress (0 ℃) in presence of $GdCl_3$ and EGTA, and response to 10 µM A23187 under normal temperature (27 ℃). (**C)** Apoplastic alkalinisation in response to cold stress (0 ℃) in presence of $GdCl_3$, EGTA, and BA (Benzyl Alcohol), as well as the response to A23187 and DMSO under normal temperature (27 ℃). Data in (**B)** and (**C)** represent mean values and standard errors from three independent biological replicates.

3.1.3 Membrane rigidification is necessary and sufficient to trigger MT disassembly

Membrane fluidity is reduced in low temperature and this change has been discussed as a primary event of cold perception. Therefore, benzyl alcohol (BA) was used to inhibit membrane rigidification under cold stress, and DMSO to increase membrane rigidification at 27 ℃, to investigate the response of MTs. In presence of 4 mM BA, central and cortical MTs appeared normal, and cell proliferation was not affected. However, cold induced disassembly was suppressed mostly (**Figure 3B**). In contrast, treatment with 1% DMSO at 27 ℃ eliminated central MTs within 30 minutes (**Figure 3B**). In addition, cortical MTs became disordered and partially disappeared (**Figure 3A**). These data suggest that membrane rigidification was necessary for cold-induced disassembly of MTs. Further, rigidification induced by DMSO was able to mimic cold stress with respect to microtubule disassembly.

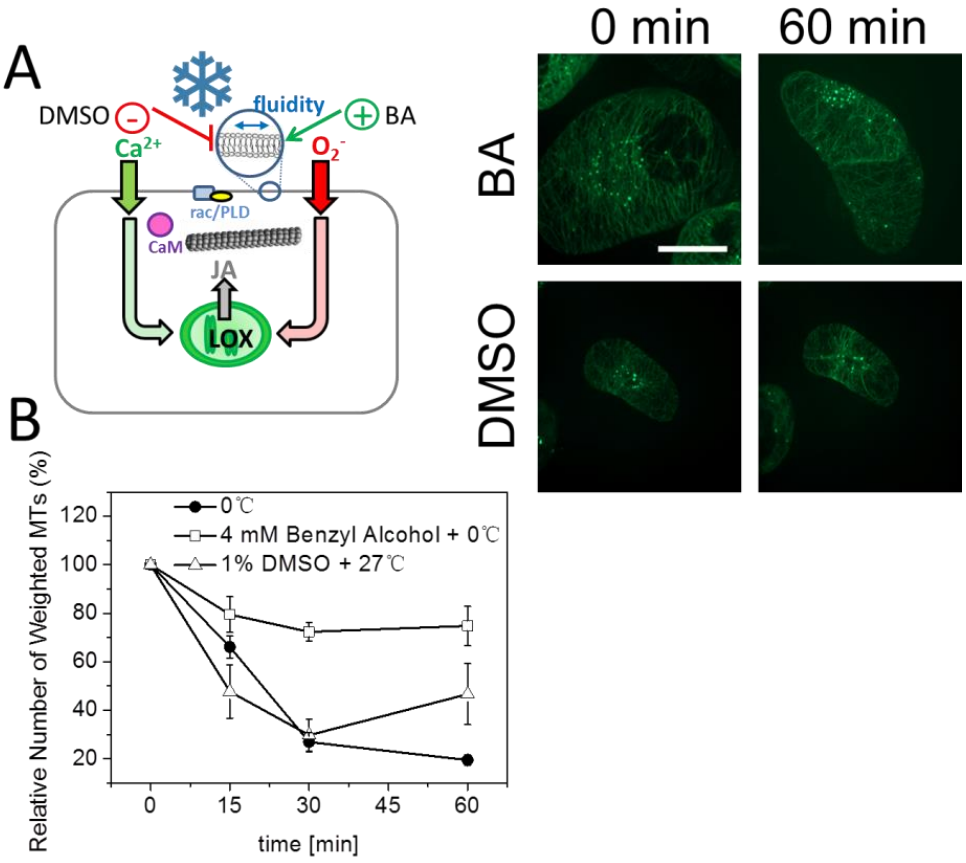

Figure 3. (**A)** Effect of BA and DMSO on the microtubular response to cold stress (0 ℃) and normal

temperature (27 ℃). The response of cortical MTs on 60 minutes to 4 mM Benzyl Alcohol and to 1% DMSO are shown. Observations are representative of at least three independent experimental series for each treatment. Size bars, 20 μm. (**B)** Quantitative analysis of the response of central MTs to BA and DMSO under cold stress (0 ℃) and normal temperature (27 ℃).

We also tested the effect of membrane rigidification on cold induced extracellular alkalinisation (**Figure 2C**). We found that 1% DMSO significantly promoted alkalinisation by about 40% and, thus, was more efficient than the calcium ionophore A23187. Conversely, BA reduced the cold response of pH slightly (although it was clearly not able to eliminate or at least strongly quench this response). These data suggest that membrane rigidification can modulate the cold-induced influx of Ca^{2+} into the cytoplasm. However, although this modulation of calcium influx was relatively minor, the effect of membrane rigidification on microtubules was drastic, indicating that membrane rigidity must control microtubules through a second pathway that is independent of calcium influx.

3.1.4 NADPH oxidase activity is required for cold induced MT disassembly

Reactive oxygen species (ROS) are not only toxic by-products of aerobic metabolism, but, similar to calcium have also been recognized as important signal in adaptive stress responses. This signalling function is linked with the activity of the membrane-located NADPH oxidase RboH (Respiratory burst oxidase Homologue). We therefore probed for a potential role of NADPH oxidase by using the specific inhibitor diphenyleneiodonium (DPI), and investigated the effect on cold-induced MTs disassembly.

In presence of DPI, cells incubated at 0 ℃ kept MTs intact over the entire observation time of 1 hour (for longer treatment, DPI caused progressive mortality), no matter, whether central or cortical microtubules were observed (**Figure 4A**). This was in

marked contrast to cold treatment in the absence of the inhibitor, where the weighted MT number decreased rapidly (**Figure 4B**), demonstrating that inhibition of NADPH oxidase activity effectively prevented cold-induced disassembly of microtubules.

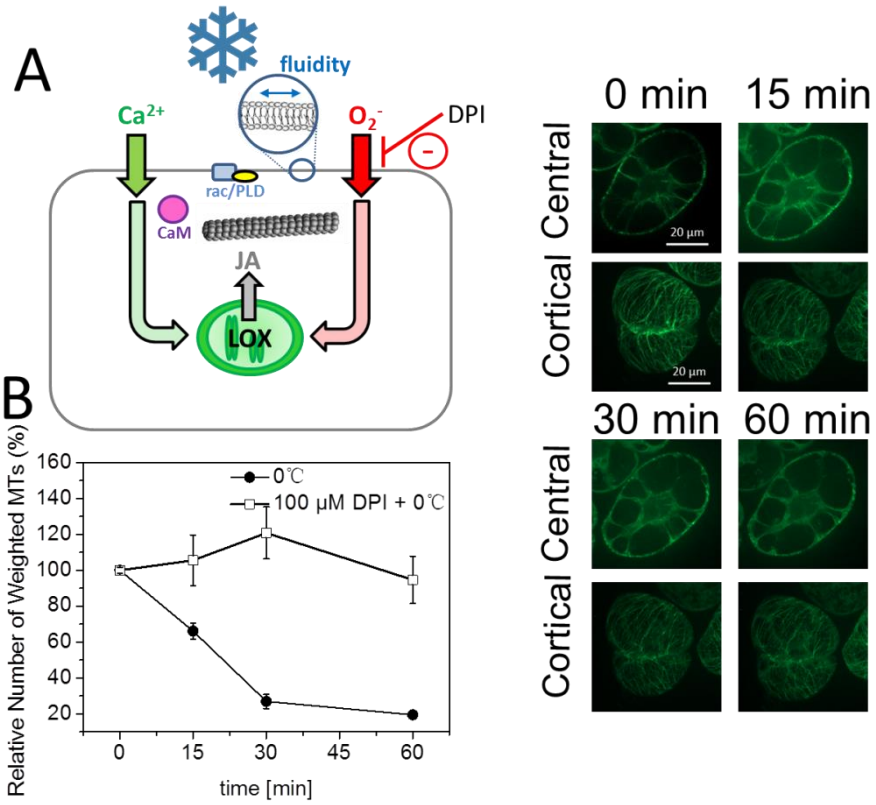

Figure 4. (A) Response of microtubules to cold stress (0 ℃) to in presence of 100 µM diphenyliodonium (DPI) at different time points after the onset of cold stress. Size bars, 20 µm. **(B)** Quantitative analysis of the cold response of central MTs in presence of 100 µM DPI under cold stress (0 ℃).

Membrane lipids are primary targets of ROS, generating oxidized reaction products of fatty acids including malone dialdehyde (MDA). The level of MDA can therefore be used as a marker to monitor lipid peroxidation. However, over 2 hours of cold treatment, we could not observe any significant changes of MDA concentration (**Figure S3**). Since DPI has been discussed to inhibit also the activity of nitric oxide synthase (NOS), we were also interested to see, whether nitric oxide might be able to induce microtubule disassembly. We therefore used the NO donor sodium nitroprusside (1 mM), but did not observe any response of neither radial, nor cortical microtubules, even for prolonged

observation over 2 hours (**Figure 8**). This indicates that exogenous NO cannot induce the disassembly of microtubules and, if it plays a role in the cold response at all, must act downstream of microtubules, or in a pathway not related to microtubules.

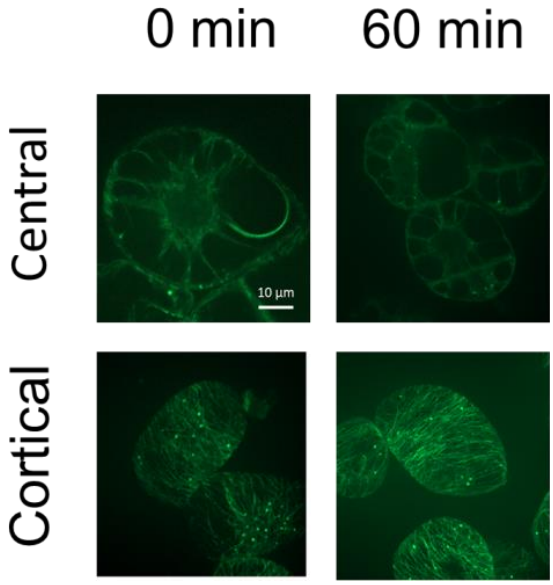

Figure 8. Effect of 1 mM SNP (sodium nitroprusside) on the cold response of radial and cortical microtubules in V. *rupestris* GFP-TuB6 cell line. Observations are representative of at least three independent experimental series. Size bars, 10 µm.

3.1.5 Jasmonic acid participates in cold-induced microtubule disassembly

Jasmonic acid is involved in the signal pathways of different biotic and abiotic stresses and therefore was investigated with respect to the microtubular response to cold. When the cells were treated with 50 µM JA in the absence of cold stress, central and cortical MTs disassembled quickly within 30 min, although most cells still retained a small number of residual MTs (**Figure S4**). This demonstrated that JA could mimic cold stress in triggering the response of MTs. In the next step, we asked, whether jasmonates are also necessary for cold-induced MT disassembly. We therefore used 4 mM of the lipoxygenase inhibitor phenidone to inhibit the production of JA under cold stress and tested the changes of MTs. Central and cortical MTs were slightly affected under cold stress in presence of phenidone (**Figure 5A**), resulting in a reduction of the weighted

number of MTs by 40% over 1 hour (**Figure 5B**). However, this decrease was significantly mitigated as compared to the control without phenidone. This means that exogenous JA is sufficient to induce the disassembly of MTs in the absence of cold stress, and that JA is necessary to convey efficient disassembly of MTs under cold stress, but that it is not the exclusive signal.

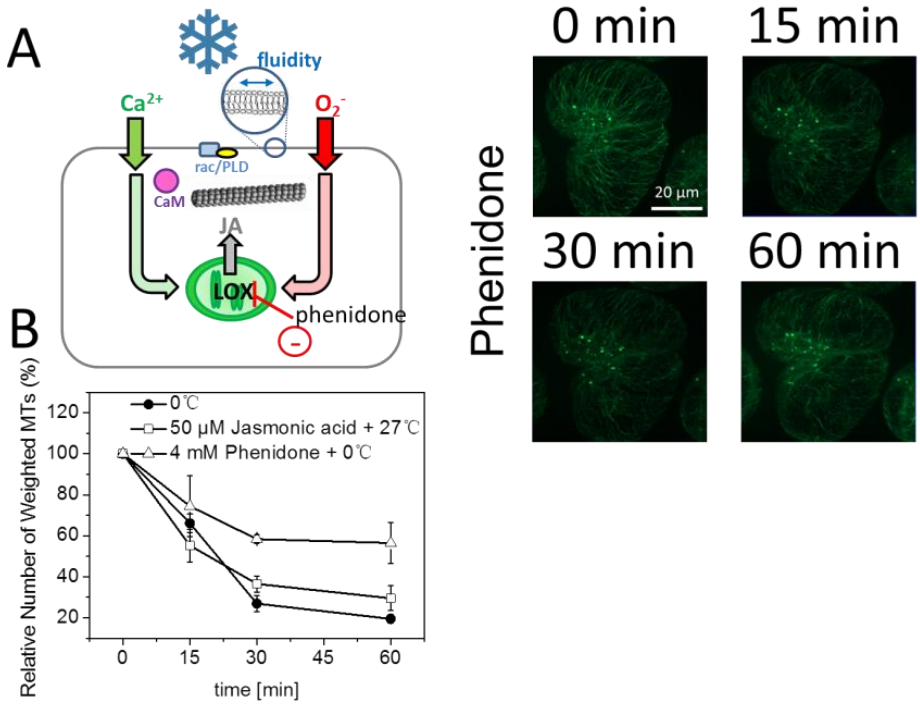

Figure 5. (A) Effect of Phenidone on the microtubular response to cold stress (0 ℃) followed over time in a representative cell file. Observations are representative of at least three independent experimental series for each treatment. Size bars, 20 µm. **(B)** Quantitative analysis of the cold response of central MTs in presence of Phenidone and Jasmonic acid along with the responses under normal temperature (27 ℃).

3.1.6 Phospholipase D is involved in cold stress signalling and modulates the state of microtubules

Phospholipase D (PLD) has been known for more than a decade to control the association of cortical MTs with the membrane and to modulate microtubule

disassembly. This enzyme represents an important signalling hub, where signalling through the enzymatic product, the phosphatidic acids (PA), diverges from a different signal that is independent of PAs. Phospholipase D can be activated by different alcohols, for instance butanols, and this activation can be used to steer the two diverging pathways differentially: On the one hand, *n*-butanol can activate PLD and act at the same time as acceptor for the resulting PA, such that the PA-dependent signalling will be interrupted. In contrast, *sec*-butanol can only activate PLD, but does not bind to PA, such that the PA-independent branch of signalling can proceed. We observe that 1% of *n*-butanol can, in the absence of cold stress, induce a partial disassembly of central and cortical microtubules by about 40% within 30 minutes (**Figure 6**). In contrast, 1% of *sec*-butanol can suppress the majority of cold-induced MT disassembly (**Figure 6**). These data suggest that interruption of PA-dependent signalling partially mimic cold stress with respect to microtubule disassembly, while activation of PA-independent PLD signalling can partially suppress cold-induced MT disassembly.

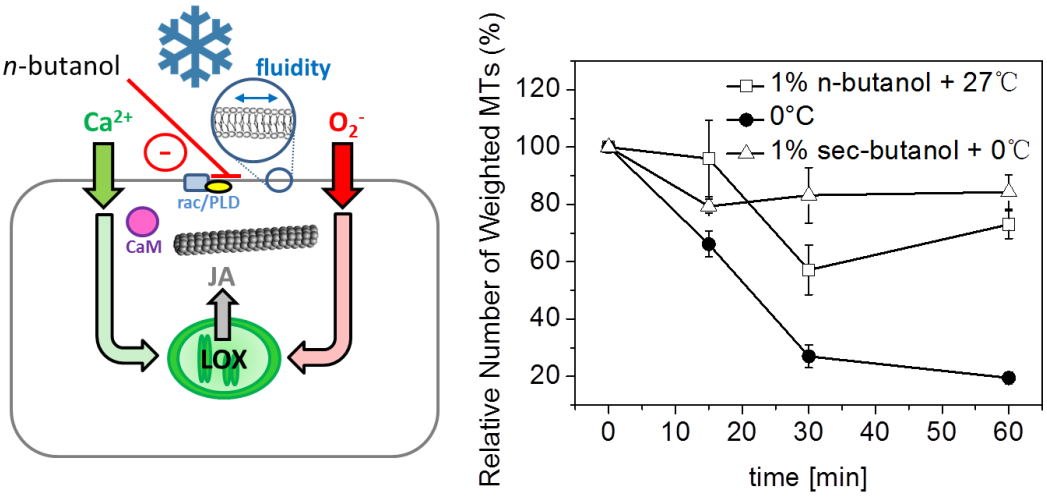

Figure 6. Quantitative analysis of the cold response of central MTs in presence of *sec*-butanol and *n*-butanol compared to their response under normal temperature (27 ℃). Each value represents the average of 10 individual cells.

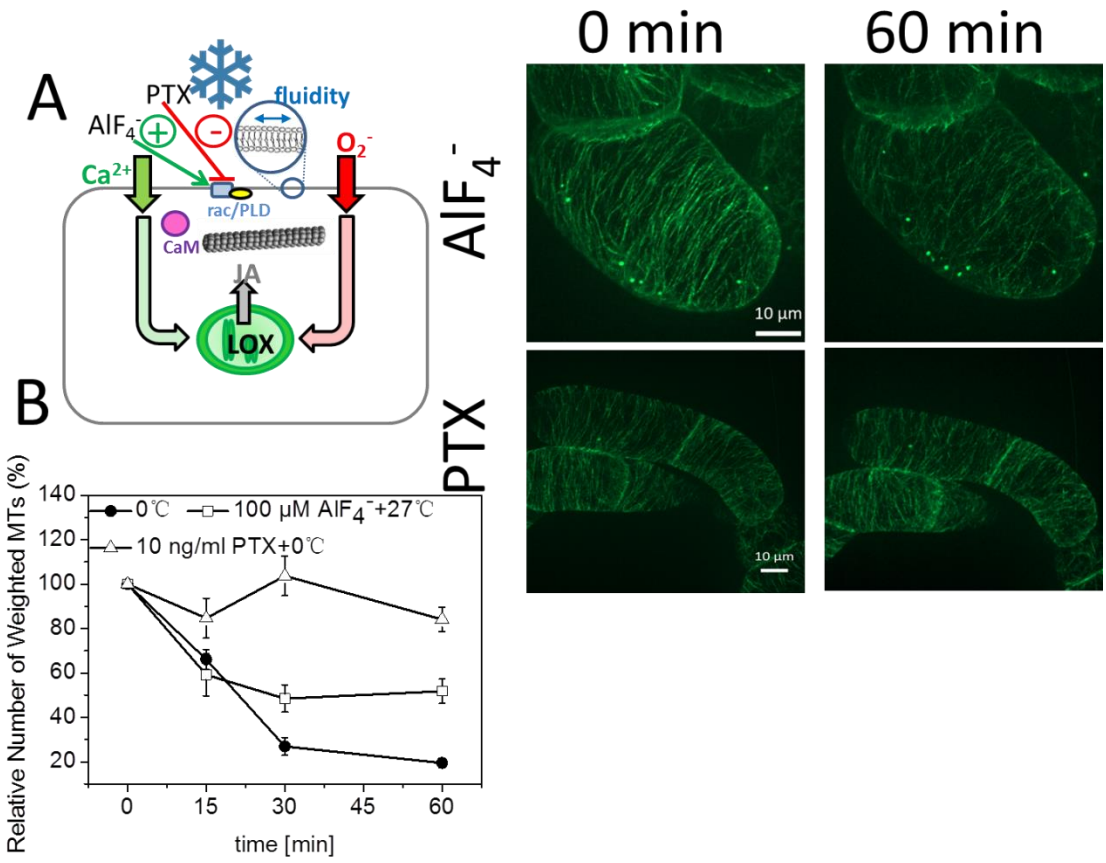

Figure 7. (**A)** Effect of 10 ng·mL^{-1} PTX (Pertussis Toxin) and 100 µM AlF_4^- (Aluminium Tetrafluoride) on the microtubular response to cold stress (0 ℃) and normal temperature (27 ℃). Two representative cell files are shown for 60 min of treatment compared to the situation prior to addition of the compound. Observations are representative of at least three independent experimental series. Size bars, 10 µm. (**B)** Quantitative analysis of the response of central MTs response to cold in presence of PTX and AlF_4^- and under normal temperature (27 ℃).

Since PLD is activated through a trimeric G-protein, we tested the effect of pertussis toxin (PTX), a specific G-protein inhibitor. After pre-treatment with 10ng/ml PTX for 30 min at 27 ℃, the cells were transferred to ice water. We observed that PTX efficiently suppressed cold induced disassembly - all central and cortical MTs persisted as if they would not have been challenged by cold (**Figure 7A, B**). In the next step, we used Aluminium Tetrafluoride, a G-protein activator. A treatment with 100 µM of this compound caused MTs disassembly in the absence of cold stress within 30 min (**Figure 7A, B**), reaching almost the same degree of disassembly as incubation at 0 ℃, and thus

partially mimicking the effect of cold stress. These data suggest that cold-induced activation of a trimeric G-protein is required for cold-induced microtubule disassembly. At the same time, pharmacologically induced activation of this G-protein is sufficient to activate microtubule disassembly in the absence of cold.

3.2 Chapter 2

3.2.1 Cold shock could induce cell death and the expression of *VvCBF4*, but chilling pretreatment can mitigate cold shock-induced cell death.

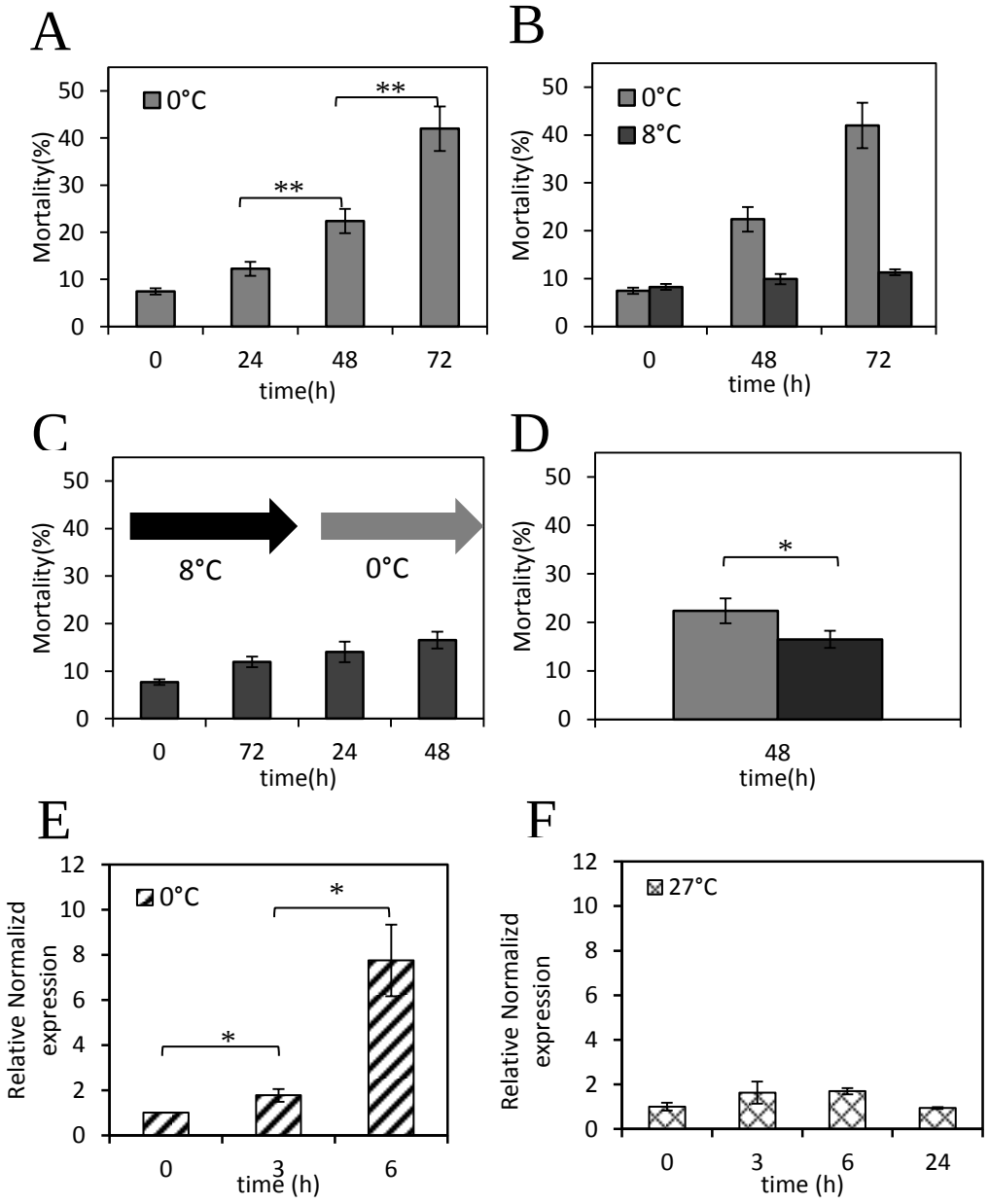

Figure 1. Time course of cell mortality and the expression level of *VvCBF4* in response to cold stress. The relative frequency of dead cells after treatment with ice water (0℃), defined as cold shock **(A)** and 8℃, defined as chilling **(B)**, respectively; and cold acclimation treatment (firstly chilling treatment for 72h, then cold shock treatment for another 48h) **(C)**, was followed over the

time scoring samples of at least 1500 cells for each data point. **(D)** Comparison of the cell death from two 48h time points with cold shock treatments in **(A)** and **(C)**. * indicate differences that are statistically significant on the $P < 0.05$ level and ** indicate $P < 0.01$ level. Data represent mean values from at least three independent experimental series, error bars represent standard error.

The expression level of *VvCBF4* in response to cold stress (cold shock treatment), **(E)** as compared to 27℃ treatment **(F)** which was considered as a negative control. * indicate differences that are statistically significant on the $P < 0.05$ level. Mean values and standard errors from three independent experimental series are shown, error bars represent standard error.

Cell mortality is the direct consequence of plants in response to cold stress. As shown in **figure 1A**, the cell mortality grew up gradually after 72h ice water (0℃) treatment and here we defined this treatment as cold shock. In the first 24h treatment, the cells were still healthy and afterwards the cell mortality rose to 25% in 48h treatment and finally arrived at nearly 40% after 72h treatment. However, when the cells were treated at 8℃ for 72h (here we defined this mild and positive cold treatment as chilling), the cells remained stable and healthy, and showed no significant increase in cell death **(Figure 1B)**. In the meanwhile, in order to test whether the transient disassembly of MTs is related to cold acclimation, we observed the changes of cortical MTs during these two treatments. As shown in **figure 2,** all the cortical MTs were totally eliminated after cold shock treatment for 24h and retained this status for 72 hours. Nevertheless, the cortical MTs were largely disassembled at chilling treatment for 72h while some microtubule filaments were still maintained and could be observed clearly.

Furthermore, to test whether the cold acclimation existed for the grapevine system, we did the chilling pretreatment for 72h to prepare and/or acclimatize the cells to the cold treatment. During this phase we observed no significant increase in cell death **(Figure 1C),** which was consistent with the results from **Figure 1B**. Then we treated these cold-hardy cells with other 48h cold shock treatment. The cell mortality began to rise gradually and finally reached up to 20% at 48h time point **(Figure 1C)**. However, in order to compare this time point (48h) to the same time point (48h) in **figure1 A**, we

analyzed whether the significant difference existed or not between them. The results showed that the mortality was lower at the cold acclimation system and the difference was significant **(Figure 1D)**. In case of the changes of cortical MTs, all the cortical MTs were totally gone in anther 48h cold shock treatments **(Figure 2)**.

The CBF transcription factors are known to play a critical role under cold acclimation. In order to test whether the grapevine genotype response to low temperature is also regulated by this transcription factor; the expression level of *VvCBF4* was studied under cold shock treatment. As shown in **figure 1E**, the induction of *VvCBF4* increased to approximately 2 fold after 3h treatment compared with that in 0h time point and kept increasing to nearly 8 fold after 6h treatment. However, in the negative control, the induction level of *VvCBF4* showed no significantly increase at 27 ℃ treatment for 24 h **(Figure 1F)**.

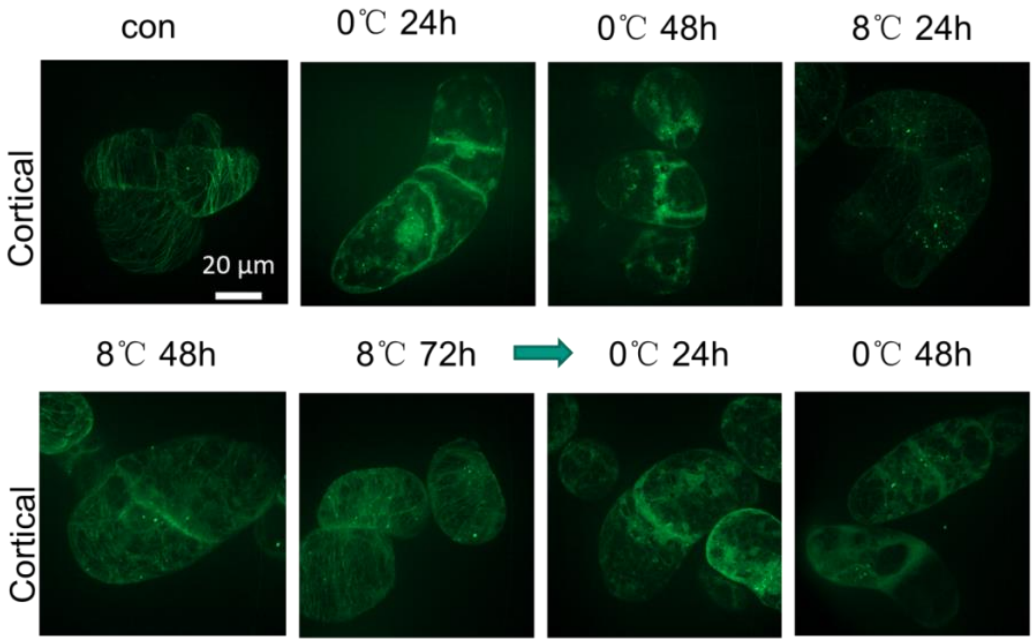

Figure 2. Response of cortical MTs to cold shock (0 ℃) and chilling (8 ℃) treatments shown by time-lapse series recorded in the MTs marker line *V. rupestris* GFP-TuB6. con shows the situation under normal temperature (27 ℃). Observations are representative of at least four independent experimental series with a population of 40 individual cells for each treatment. Bars, 20 μm.

3.2.2 Taxol treatment could mimic cold acclimation, but oryzalin treatment does not and both of them do not induce the expression of *VvCBF4*

Taxol is a microtubule stabilization drug and oryzalin functions oppositely. To test whether through manipulating the MTs by these two drugs could affect cold acclimation, the cell mortality and changes of MTs were tested during these two drug treatments. As shown in **figure 3B,** with taxol treatment for 72h at 0℃, the cell mortality increased slightly and finally reached 20% at 72h time point. Comparing this to the control (only 0℃) at 72h time point, the decrement of cell mortality was significant. However, oryzalin treatment could not retrieve the cell mortality (**Figure 3A**).

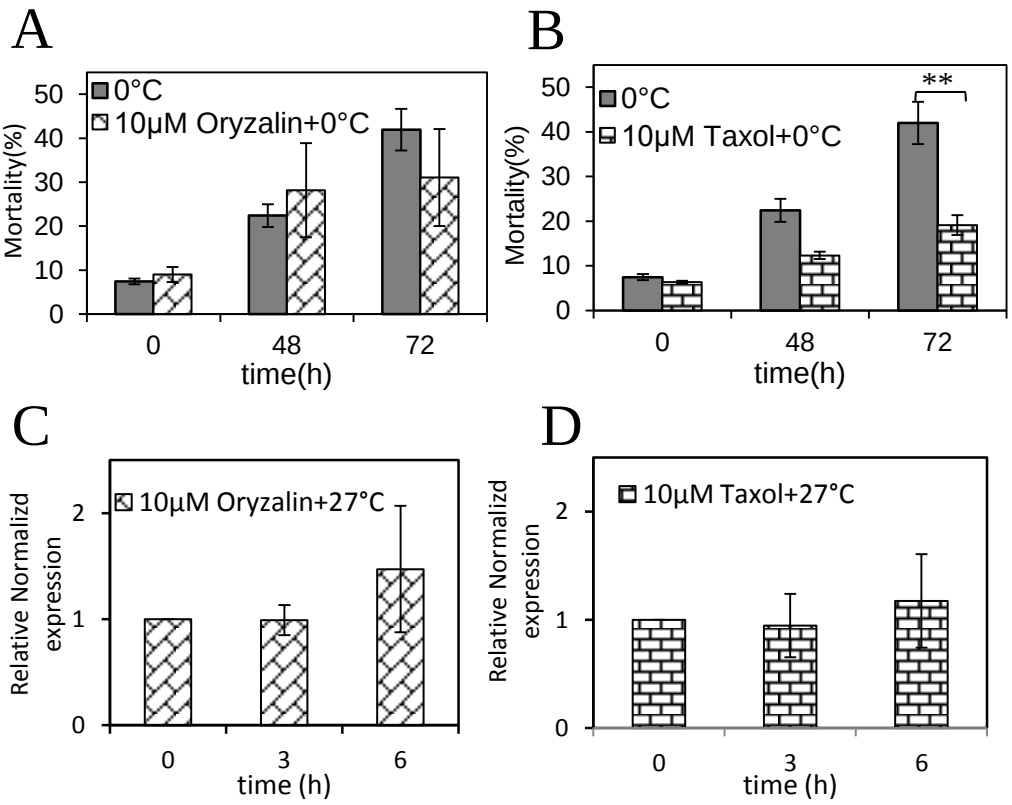

Figure 3. Time course of cell mortality and the expression level of *VvCBF4* in response to taxol and oryzalin treatments, respectively. The relative frequency of dead cells after pre-treatment with 10μM oryzalin **(A)** and 10μM taxol **(B)** for half an hour at 27℃ and then treated with cold shock for 72h, respectively. ** indicate differences that are statistically significant on the P <0.01 level. Data represent mean values from at least three independent experimental series, error bars represent

standard error.

The expression level of *VvCBF4* in response to 10μM oryzalin **(C)** and 10μM taxol **(D)**, respectively**,** at 27℃ treatment. Mean values and standard errors from three independent experimental series are shown.

For the changes of cortical MTs, pre-treated cells with 10μM taxol could stabilize MTs for half an hour at 27℃ and then the cells were treated with cold shock for 3 days. As shown in **figure 4,** the MTs showed no difference after half an hour taxol treatment at 27℃ (**Figure 4 con**). Then further cold shock treatment for 24h, showed that some cortical MTs still existed and this phenomenon was absolutely different with that only under 0℃ treatment in which case they had totally disassembled (**Figure 2**). We also noticed that all the MTs were totally disassembled after 48h and 72h cold shock treatment (**Figure 4**). However, for oryzalin treatment, all the cortical MTs depolymerized after 1h 27℃ treatment and maintained the same status after 72h cold shock treatment (**Figure 4**).

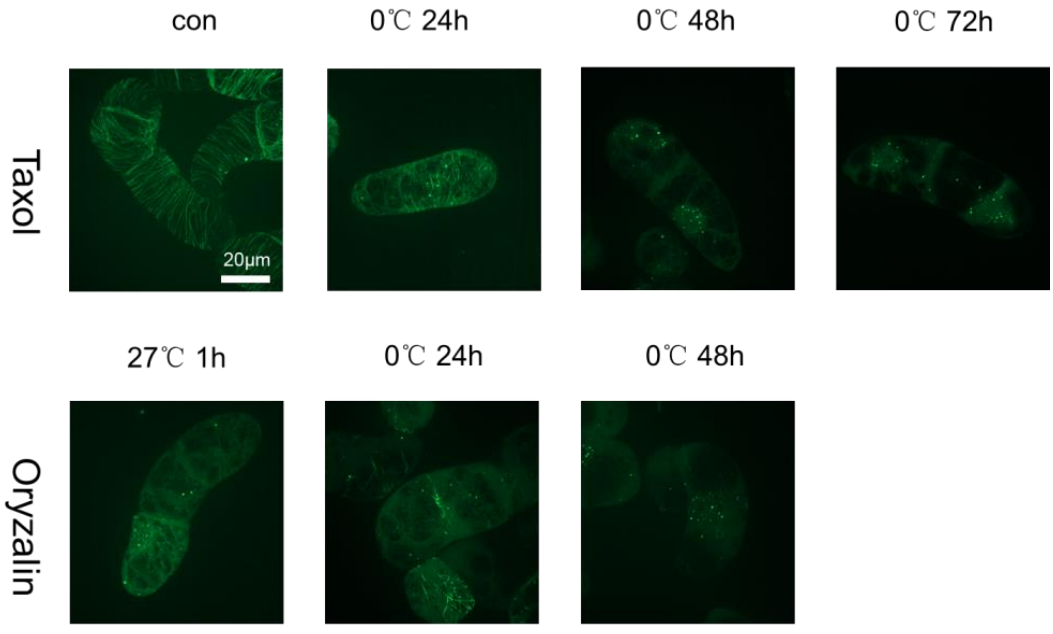

Figure 4. Response of cortical MTs to 10μM taxol and 10μM oryzalin with cold shock shown by time-lapse series recorded in the MTs marker line *V. rupestris* GFP-TuB6. Con shows the situation under normal temperature (27℃). Observations are representative of at least four independent

experimental series with a population of 50 individual cells for each treatment. Bars, 20 μm.

A role of MTs for cold signaling and acclimation has also been discussed in the first part of results, to test whether the induction of *VvCBF4* by cold stress depended on the induced changes of the MTs, the microtubule stabilizing drug (taxol) and destabilizing drug (oryzalin and pronamide) was used on cells at 27 ℃. As shown in **figure 3C and D** and **figure 5B**, the induction of *VvCBF4* did not increase by these three drug treatment at 27 ℃, meaning the activation of *VvCBF4* did not depend on the changes of microtubule pathway.

3.2.3 Pronamide can mimic cold acclimation with respect to microtubule elimination, but does not induce the expression of *VvCBF4*

Oryzalin is really toxic to cells and it is difficult to be washed away. Thus, Pronamide which is an herbicide was considered as another ideal compound to disassemble MTs. It is also easy to be washed away which provided an opportunity to manipulate the transient depolymerization of MTs at 27 ℃ treatment and furthermore to check whether this process could improve the cold acclimation or not. As shown in **figure 5C,** with 2h pronamide treatment at 27 ℃, nearly all the cortical MTs disappeared. Then we used a big mesh device to wash away the compound and incubated the cells at 27 ℃ for another 3h and after this treatment, most of the MTs recovered, but eventually all the cortical MTs were eliminated at cold shock treatment after another 48 hours and 72 hours.

With different concentration of pronamide treatment at 0 ℃, the cell mortality also went up quickly. At 48h time point the cell mortality displayed no significant difference in comparison with the solvent control (DMSO) treatment and ice water treatment. However, after 72h cold shock treatment with the present of 50μM pronamide, it dropped to nearly 30% which is significant to the results of solvent control (DMSO) and

ice water treatment (**Figure 5A**).

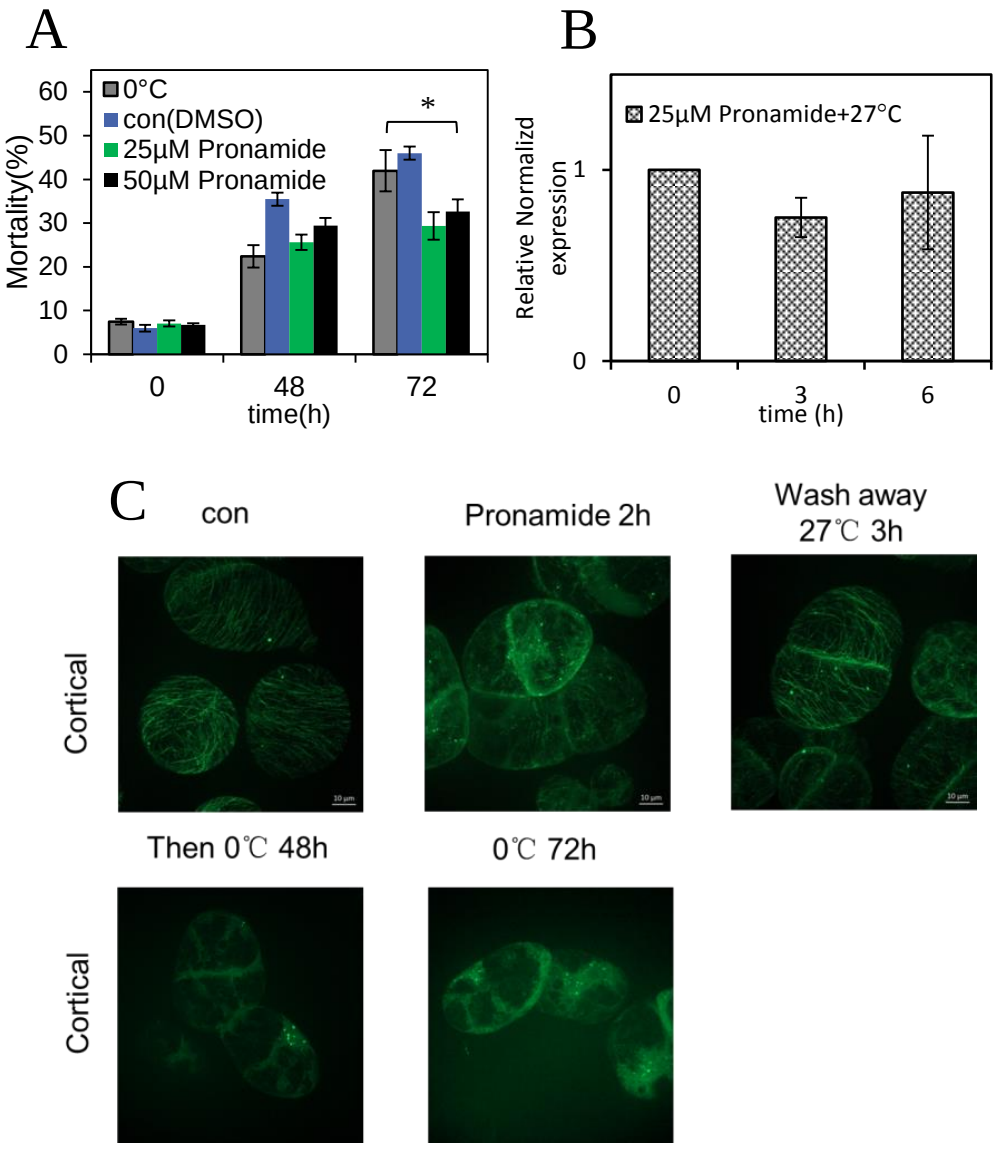

Figure 5. Time course of cell mortality and the expression level of *VvCBF4* in response to different concentration of pronamide treatment. **(A)** The relative frequency of dead cells after treatment with 25μM and 50μM pronamide, and the solvent control is DMSO at cold shock treatment. * indicate differences that are statistically significant on the P <0.05 level. Data represent mean values from at least four independent experimental series, error bars represent standard errors. **(B)** The expression level of *VvCBF4* in response to 25μM pronamide at 27°C treatment. Mean values and standard errors from three independent experimental series are shown. **(C)** Response of cortical MTs to 25μM pronamide with different temperature treatments shown by time-lapse series recorded in the MTs marker line *V. rupestris* GFP-TuB6. con shows the situation under normal temperature (27℃). Observations are representative of at least four independent experimental series with a population of

30 individual cells for each treatment. Bars, 10 μm.

3.2.4 Chelating with the Ca^{2+} could not rescue the mortality, but promoted the membrane fluidity that could mitigate cell death, and the activation of *VvCBF4* depends on the calcium dependent pathway.

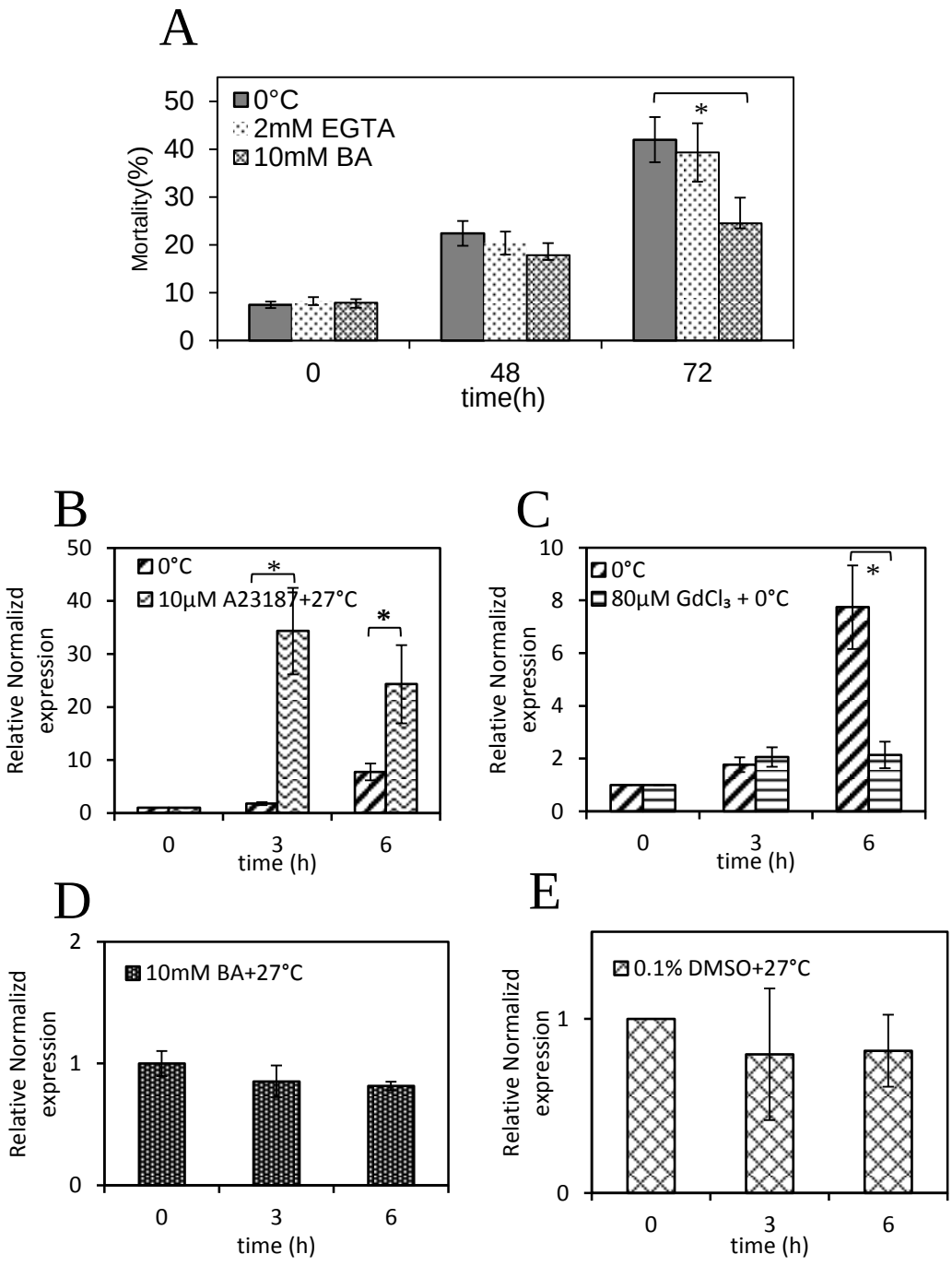

Figure 6. Time course of cell mortality and the expression level of *VvCBF4* in response to EGTA, calcium ionophore A23187, $GdCl_3$, Benzyl alcohol and DMSO treatment. **(A)**The relative frequency of dead cells after pre-treatment with 2mM EGTA and 10mM Benzyl alcohol for half an hour at 27℃ and then with cold shock treatment for 72h. * indicate differences that are statistically significant on the P <0.05 level. Data represent mean values from at least four independent experimental series, error bars represent standard errors.

The expression level of *VvCBF4* in response to 10μM A23187 **(B)**, 10mM Benzyl alcohol **(D)** and 0.1% DMSO **(E)** treatment at 27℃, and 80μM $GdCl_3$ **(C)** at 0℃, respectively. * indicate differences that are statistically significant on the P <0.05 level. Mean values and standard errors from three independent experimental series are shown.

Calcium influxes and membrane rigidification are indispensable during the process of cold acclimation. Thus, in order to test whether the influx of Ca^{2+} and membrane rigidification are sufficient for cold acclimation, we performed the experiments by pretreating the cells with 2mM EGTA and 10mM benzyl alcohol (BA). EGTA and BA help to chelate the calcium ions and to prevent the plasma membrane rigidification, respectively. As shown in **figure 6A**, with EGTA treatment, the cell mortality that increased up to 40% after 72h cold shock treatment, was as same as the changes in control treatment, indicating that blocking calcium influxes could not rescue the cell mortality. However, after BA treatment the cell mortality also increased to 20% within 48h cold shock treatment, which was consistent with the changes of control. However, the cell mortality increased to 27% after another 24h cold shock treatment which significantly eased the cell mortality comparing to the control or EGTA treatment. These results demonstrated the increment of membrane fluidity could decrease the cell mortality in response to cold stress, but blocking calcium influx did not lead to better cell viability.

A transient influx of Ca^{2+} into the cytoplasm is among the earliest events of cold acclimation. To test whether the influx of Ca^{2+} is sufficient for the induction of the *VvCBF4* transcription factor, the calcium ionophore A23187 which allows calcium to

enter the cytoplasm in the absence of an external stimulus, was applied. As shown in **figure 6B and C**, the induction of *VvCBF4* went up sharply to around 35-fold with the calcium ionophore A23187 at 3h 27 ℃ treatment. Whereas with $GdCl_3$ treatment which could block the calcium channel on the plasma membrane, the expression level of *VvCBF4* was reduced to 75% after 6h 0 ℃ treatment. This was significantly different compared to 0 ℃ treatment at the same time point.

As discussed above, the changes in the plasma membrane fluidity indeed played an important role under cold acclimation. Thus, in order to check whether the induction of *VvCBF4* by cold stress required the changes of plasma membrane; cells were treated with DMSO which could induce membrane rigidification and benzyl alcohol which could increase membrane fluidity at 27 ℃. As shown in **figure 6D and E**, the induction of *VvCBF4* was not influenced by both drug treatments.

3.2.5 Depolymerization of actin could not mitigate cell death, but external JA could, and the activation of *VvCBF4* is dispensable of JA pathway.

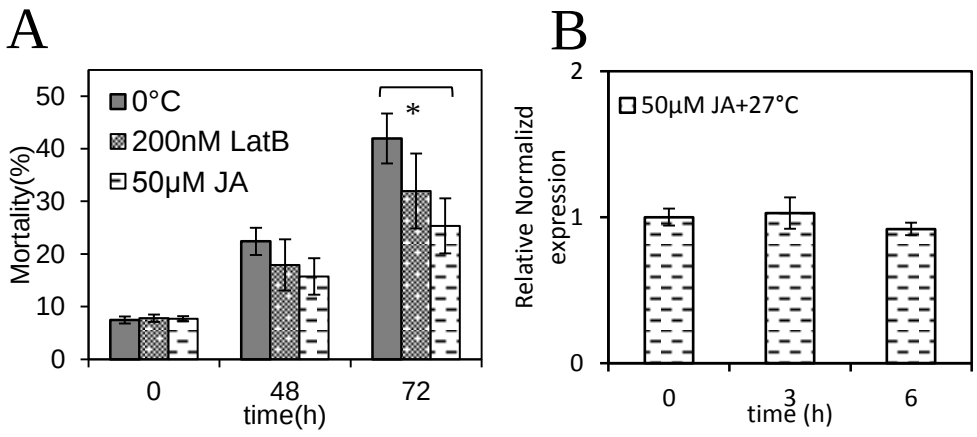

Figure 7. Time course of cell mortality and the expression level of *VvCBF4* in response to Latrunculin B (LatB) and Jasmonic acid (JA) treatment. The relative frequency of dead cells after pre-treatment with 200nM LatB and 50μM JA **(A)** for half an hour at 27 ℃ and then with treatment of cold shock for 72h. * indicate differences that are statistically significant on the P <0.05 level.

Data represent mean values from at least four independent experimental series, error bars represent standard errors.

The expression level of *VvCBF4* in response to 50μM JA **(B)** treatment at 27℃. Mean values and standard errors from three independent experimental series are shown.

As the disassembly of MTs played a role in cold acclimation, we found it interesting to understand if actin (plant cytoskeleton) had similar function to MTs. Thus, the Latrunculin B which could depolymerize the actin filaments was applied. As shown in **figure 7A**, after this treatment, it could not retrieve the cell mortality, which also increased gradually and reached up to 35% after 72h cold shock treatment. This treatment showed no significant difference comparing to the changes of control treatment. However, the external application of jasmonic acid treatment induced the cell mortality after 48h 0℃ treatment to 20%. But after 72h 0℃ treatment, the cell mortality decelerated to 26% which illustrated significant difference in contrast to the control treatment at the same time point.

Thus, we observed that the jasmonic acid was also involved in cold acclimation, however, in our system, as shown in **figure 7B**, the external performance of JA could not induce the expression of *VvCBF4*.

4. Discussion

4.1 Chapter 1: The relationship between microtubules and other related molecules involved in cold early signalling transduction

In the current work, we have examined the response of microtubules to cold stress in transgenic grapevine cells where microtubules are visible by a GFP-tagged tubulin. Motivation for this work was to get insight into early cellular responses to cold stress, since MTs as cold sensitive intracellular structure have been hypothesized to function as 'thermometer' for low temperature (Abdrakhamanova *et al.* 2003; reviewed in Nick 2013).

Microtubules are endowed with innate dynamics, whereby α/β tubulin heterodimers polymerize and depolymerize continuously. This dynamic instability shows a distinct polarity, with assembly dominating at the plus end disassembly at the minus end. Modulated by associated proteins, the dynamic equilibrium between assembly at the plus end and disassembly at the minus end can be shifted to rapid decay (so called microtubule catastrophe) in a non-linear fashion. Using life-cell imaging based on GFP-tagged tubulin, cortical MTs of *Arabidopsis* have been shown to treadmill through the cell cortex with both plus- and minus-ends contributing to dynamics (Shaw *et al.* 2003). Modulation of dynamic instability in response to environmental stress factors is a common phenomenon in animal cells (for review see Walczak 2000), for instance the cold tolerance of Antarctic fishes correlates with a reduced cold sensitivity of microtubules (Detrich *et al.* 2000, Walczak 2000, Nick 2013). In plants, cold stability of microtubules can be regulated by stress-induced hormones, such as abscisic acid (Rikin *et al.* 1983, Sakiyama and Shibaoka 1990), but also by cold-induced factors that differ from abscisic acid (Wang and Nick 2001). Since cold sensitive microtubules can be

rendered cold stable by cleavage of the C-terminus-tubulin (Bokros *et al.* 1996), binding of associated proteins at this domain is a prime candidate for cold induced elimination of MTs. Molecular candidates for such proteins are MAP65 that can increase cold stability of animal MTs *in vitro* (Mao *et al.* 2005), or calmodulin that, dependent on the concentration of calcium has been reported to decrease microtubule stability in lysed protoplasts of carrot (Fisher *et al.* 1996).

On account of their cold sensitivity, microtubules have long been discussed as factors determining cold tolerance. Initially, a correlation between cold stability of microtubules and cold hardiness has been suggested (Jian *et al.* 1989, Kerr and Carter 1990). However, stabilization of microtubules by taxol was reported to decrease freezing tolerance (e.g. (Bartolo and Carter 1991), which was obviously not congruent with a model, where cold hardiness was explained in terms of microtubule stability. These apparent discrepancies can be resolved, when the timing of events is considered: Whereas a high microtubular dynamics is beneficial during the early, sensory phase of the response to low temperature, the subsequent adaptive events eventually culminate in the formation of stable microtubule bundles (Nick 2013). The initial disassembly of microtubules seems to be essential: In tobacco cells, tubulin accumulated in interphase nuclei during the response to prolonged chilling that progressively eliminated cortical microtubules, and new microtubules regenerated from this pool within minutes upon rewarming of the cells (Schwarzerova *et al.* 2006). A link between microtubule disassembly and active signalling is also suggested by the functional analysis of a low-temperature induced receptor kinase in rice (Liu *et al.* 2013b). In winter wheat, a transient, initial microtubules disassembly is necessary and sufficient to trigger cold acclimation (Abdrakhamanova *et al.* 2003). This acclimation will then, in a second step, lead to a completely cold-tolerant array of microtubules.

Based on the published records, we interpret the fact that already within 30 min of cold treatment, most MTs had disassembled, as sensory event. We think that this rapid disassembly of microtubules acts to sense and amplify the signals generated by the

cold-dependent reduction of membrane fluidity. In the current paper, we have now tried to dissect the events acting upstream of this sensory event:

Under cold stress, changes of membrane fluidity modulating level of cytosolic Ca^{2+} are widely accepted as the primary signal for cold perception. However, their relationship to the cold response of microtubules has remained unclear. A role of microtubules for cold signalling has been demonstrated by a study, where the activation of the *Brassica napus* BN115 promoter had been mapped by pharmacological manipulation. Here, oryzalin, a compound that eliminates microtubules by sequestering tubulin heterodimers could mimic the effect of low temperature, whereas taxol could suppress the activation of this promoter by cold (Sangwan *et al.* 2001). Gadolinium ions and BA could prevent the induction of this reporter by low temperature, and, conversely, the calcium ionophore A23187, or DMSO could activate the reporter in the absence of cold treatment (Sangwan *et al.* 2001). Our findings that the membrane rigidifier DMSO cause depolymerization of microtubules under normal temperature, whereas the calcium channel blocker $GdCl_3$, the calcium chelator EGTA, or the membrane fluidizer BA could block the disassembly of microtubules under cold stress are consistent with a model, where microtubules transduce the condition of calcium and membrane as upstream events that are modulated under cold stress. This pattern is exactly congruent with the sequence found for the cold activation of the *Brassica napus* BN115 (Sangwan *et al.* 2001) and supports a model where cold-induced changes of membrane rigidity are transduced by modulations of calcium influx upon microtubule disassembly. Rapid increases of cytosolic calcium in response to cold stress have been shown repeatedly, for instance by using transgenic plants expressing aequorin as a reporter which allowed to follow calcium signatures through monitoring changes of bioluminescence (Knight *et al.* 1991). The most straightforward mechanism would be the activation of calmodulin. Cortical microtubules have been shown to be decorated with calmodulin depending on the concentration of calcium (Fisher and Cyr 1993), and calmodulin can induce their disassembly (Fisher *et al.* 1996). However, when we tested trifluoperazine (TFP), a widely used inhibitor of calmodulin (Feldkamp *et al.* 2010), we

did not observe any inhibition of the microtubular cold response. This not only indicates a different second messenger, such as calcium dependent protein kinases found to confer cold tolerance in rice (Saijo *et al.* 2000), it also indicates that the upstream signalling cannot be reduced to influx of calcium activating calmodulin, but must involve additional events.

In fact, we are able to identify some of these additional upstream factors:

Reactive oxygen species (ROS) are central signals in the response to various stress factors. Originally, just considered as toxic products produced in consequence of stress-induced damage, they are meanwhile seen as important signals mediating the transduction of stress adaptation (Mittler *et al.* 2011). Although numerous reports describe, how ROS are generated in response to cold stress, a role of ROS as signals has only been rarely addressed. Using inhibitors, hydrogen peroxide as well as nitric oxide has been shown to be required for the cold response of myo-inositol phosphate in alfalfa (Tan *et al.* 2013). Similar to this report, our results suggest a role of ROS for cold-induced signalling. Inhibition of the NADPH oxidase by DPI could effectively prohibit the depolymerization of central and cortical microtubules. Since the superoxide anion will trigger lipid peroxidation leading to accumulation of the end product malondialdehyde (MDA), we probed for the accumulation of MDA using a colorimetric assay based on conversion of 2-thiobarbituric acid (Hodgson and Raison 1991) but did not see significant changes after 2 hours of cold treatment, indicating that the temperature sensitivity of the lipid peroxidation limits the accumulation of MDA, such that this widely used assay for ROS detection is not suited for this purpose. In contrast to the situation in alfalfa (Tan *et al.* 2013), we were not able to detect a role of NO, since generation of exogenous NO by nitroprusside could not induce the disassembly of microtubules. This does not preclude that NO maybe one of the products generated under cold stress possibly targeted to other responses such as myo-inositol phosphate synthase, but it precludes that it is acting upstream of microtubules. Calcium influx can activate the NADPH oxidase via a calcium-dependent protein kinase pathway, can

activate (Dubiella *et al.* 2013), and apoplastic oxidative burst, in turn, can stimulate calcium influx (Rentel and Knight 2004), constituting a self-amplifying signalling loop, which might be responsible for cold-induced MT disassembly.

Both, calcium and ROS are diffusible signals, microtubules as their target, are endowed with a strong directionality and spatial order. There must be a point, where the diffusible primary signals must translate into structural information. We hypothesize that this point might be linked with phospholipase D, an important membrane associated protein identified as central regulator for the interaction of cytoskeleton and membrane (reviewed in (Testerink and Munnik 2011)). For instance, p90, a specific PLD isoform from tobacco, had been identified as membrane linker of plant microtubules (Gardiner *et al.* 2001), and other specific isoforms are physically and functionally linked with actin suggesting phospholipases of the D type as signalling hub controlling the interaction between plasma membrane and cytoskeleton (Nick 2013). Whereas the original working model focused on the PLD product phosphatidic acid (PA) as active signal, the PLD have progressively emerged as point, where different signalling chains diverge, some of which are independent of PA. Central for this debate is the use of *n*-butanol, a compound that activates PLD, but then consumes the resulting PA. As negative control for the specificity of this inhibitor, *sec*-butanol has been used by several authors, because it can activate PLD, but not acts as trans-phosphatidylation substrate and thus will block the production of PA (Munnik *et al.* 1995, Gardiner *et al.* 2001, Chang *et al.* 2015). However, in some cases, *sec*-butanol does not act as inactive analogue, but can elicit signalling by itself (Peters *et al.* 2007, Liu *et al.* 2013a), indicating that here the activation of PLD leads to a downstream signal that is independent of PA. We observe now that 1% of *n*-butanol can induce MT disassembly in the absence of cold stress, but this disassembly remains partial. Even more strikingly, 1% of *sec*-butanol can suppress the majority of cold-induced MT disassembly. Both findings indicate that PA is not the relevant signal here. Interestingly, when the same cell line, *V. rupestris*, was probed for the link between PLD activation and actin, it turned out that, here, *n*-butanol was very efficiently suppressing elicitor-triggered actin

bundling, whereas sec-butanol did not show any effect (Chang *et al.* 2015), consistent with a mechanism, where PLD regulates actin-membrane interaction via a PA-dependent pathway, whereas microtubule-membrane interaction is controlled by a PA-independent pathway.

The activation of PLD requires calcium and is stimulated by reciprocal stimulation with a trimeric G-protein (reviewed in (Wang 2005)). We therefore probed for the effect of inhibition pertussis toxin (PTX) as inhibitor of trimeric G-proteins (Warpeha *et al.* 1991) and aluminium tetrafluoride, which constitutively activates by chemical mimikry of the GTP -phosphate of GTP within the G subunit (Bigay *et al.* 1985). We observed that PTX efficiently suppressed cold induced disassembly, whereas aluminium tetrafluoride could cause MT disassembly in the absence of cold stress. These data demonstrate that activation of a trimeric G-protein is necessary and sufficient for microtubule disassembly. In contrast to the partial inhibition observed for *n*-butanol, PTX produces a mostly inhibition of cold-induced disassembly, consistent with a working model, where the G-protein acts upstream of the branch point mediated by PLD. A branching of signalling at the trimeric G-protein would be conceivable, though - for instance, the alpha subunit of heterotrimeric G proteins was shown to activate GTP hydrolysis of sheep-brain tubulin *in vitro* (Roychowdhury *et al.* 1999), and might thus control microtubule polymerization at the plus-ends and hence influence MT stability. However, we do not require such a PLD-independent branch to explain our observations, and therefore assume that the signal from activation of the trimeric G-protein is fully conveyed by PLD.

We obtained, however, some evidence for divergent signalling downstream of PLD, when we investigated the role of jasmonic acid. Jasmonic acid and its derivatives respond to various biotic and abiotic stresses, such as wounding, drought, salt stress, and is thought to act as important downstream compound activating different stress responses (Balbi and Devoto 2008). Modulations of jasmonate and jasmonate-isoleucine levels have also been reported as responses to cold stress, for

instance in winter wheat (Kosova *et al.* 2012). However, to the best of our knowledge, a role of JA as upstream signal of cold stress has not been reported so far. We find that phenidone, a growth regulator that can downregulate jasmonate levels by inhibition of lipoxygenases (Bruinsma *et al.* 2010) can partially suppress the depolymerization of microtubules (to around half of the level induced by cold stress), whereas, conversely, exogenous JA could mimic cold stress with respect to causing MTs disassembly. This shows that, while JA is sufficient to induce the MTs response, cold-induced JA only account for around half of the response, placing JA in a parallel pathway enhancing the signal conveyed by calcium influx, NADPH oxidase, and a trimeric G-protein. The fact that a similar partial inhibition of cold-induced MTs disassembly is observed, when phospholipase D is blocked by *n*-butanol, points to a functional link between the two events. In fact, phospholipase D has been shown to activate jasmonate synthesis in the wound response (Wang *et al.* 2000) leading to a working model, where jasmonate is placed downstream of cold-dependent activation of phospholipase D.

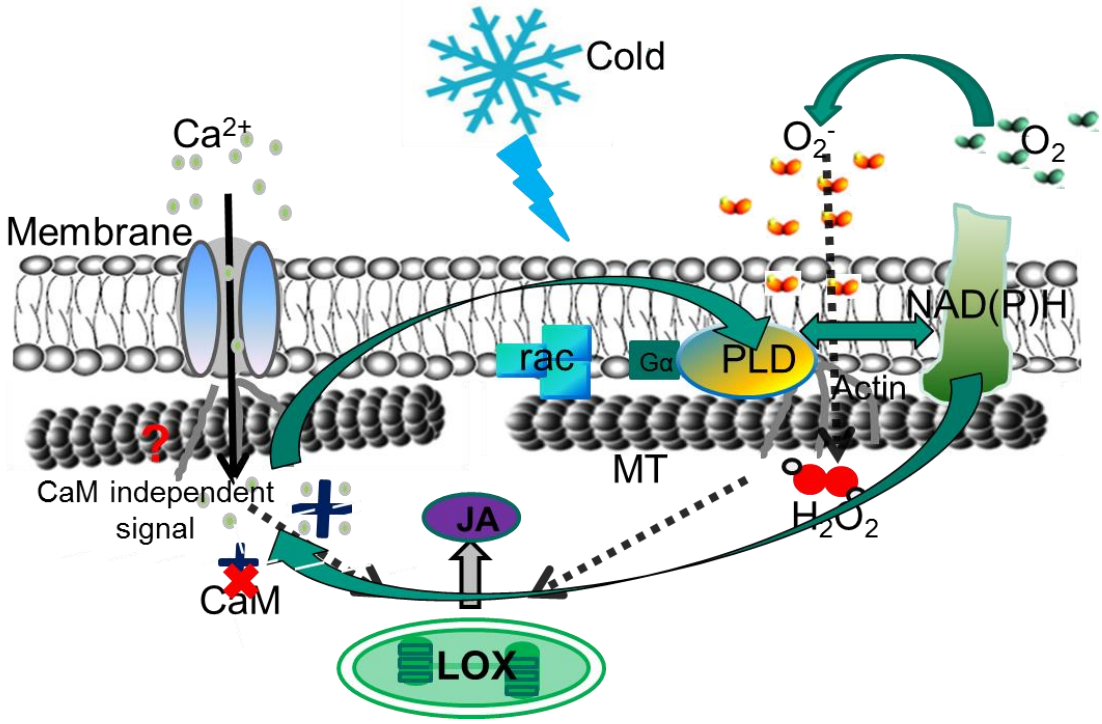

Figure 9. Working model of the early signal transduction pathway responses to cold stress. Calcium influx, membrane rigidification, and activation of a NADPH oxidase and G-protein in concert with a phospholipase D convey the signal towards microtubules as factors in signal susception and

amplification, whereas calmodulin seems to be not involved. Moreover, activation of the jasmonate pathway in response to cold is required for an efficient microtubule response. Abbreviations: Ca^{2+}, Calcium; rac, GTPase; PLD, Phospholipase D; O_2, Oxygen; O_2^-, Superoxide anion; H_2O_2, Hydrogen peroxide; MT, Microtubule; CaM, Calmodulin; LOX, Lipoxygenase; JA, Jasmonic acid.

We arrive at a model (**Figure 9**), where cold-induced membrane rigidification stimulates calcium influx and NADPH-oxidase mediated oxidative burst. These two early events seem to be connected by mutual amplification resulting in activation of a trimeric G-protein that in turn activates phospholipase D. Here, the pathway branches into a PA-dependent signal (possibly targeted to actin filaments), and a PA-independent signal (targeted to microtubules). In parallel, activation of PLD activates the jasmonate pathway, which serves to sustain and stabilize the microtubule response. Microtubule disassembly would thus function as final step of a 'thermometer' triggering cold adaptation (reviewed in Nick 2008). The downstream effects of this thermometer might be cold-induced voltage-dependent calcium channels resulting in an increased influx of calcium (Mazars *et al.* 1997) leading to further self-amplification. However, in consequence of cold adaptation, microtubules acquire cold stability (Pihakaski-Maunsbach and Puhakainen 1995, Abdrakhamanova *et al.* 2003), which in turn, should constrain the activity of cold-activated calcium channels and thus prevent disregulated signalling. This mechanism would downregulate sensitivity upon prolonged stimulation, which represents a key element of biological sensing.

4.2 Chapter 2: Microtubules function together with the induction of the transcription factor, *VvCBF4*, to inquire cold acclimation

As the above works, extensive attention has been paid to examine the potential factors, such as calcium influx, membrane rigidification, and activation of a NADPH oxidase and G-protein in concert with a phospholipase D, related with MTs as the early cellular response to cold stress. In our current work we also looked for the later cellular response of MTs and the expression of cold responsive genes to cold stress, since the transient disassembly of MTs, the accumulation of tubulins in the nucleus may facilitate the activation of cold responsive genes that play key roles under cold stress (Abdrakhamanova *et al.* 2003, Schwarzerova *et al.* 2006, Chinnusamy *et al.* 2007) reviewed in Nick 2013).

Cold stress could trigger various physiological and biochemical changes at cellular conditions, such as membrane damage, cytoplasmic viscosity, enzyme activities and eventually result in cell death by the formation of crystal ice or cytoplasmic dehydration, and oxidative stress (Lissarre *et al.* 2010, Ruelland and Zachowski 2010). Furthermore, for the entire plant species, the reduced leaf expansion, wilting, growth rate of plant (dwarf phenotype) and chlorosis, necrosis (death of tissue) and immaturity of reproductive development are the imperative symptoms of cold stress (Achard *et al.* 2008, Huang *et al.* 2009, Yadav 2010). For instance, the spring cereals have an erect growth habit and photoinhibition of photosynthesis in response to cold stress (Dahal *et al.* 2012). However, cold acclimation is an imperative and beneficial method for plants to improve cold tolerance and this is firstly discovered and widely studied in *Arabidopsis* (Stockinger *et al.* 1997). In nature, plants suffer from various environmental conditions that result in the activation of different signalling pathways involved in changes of gene expression patterns and protein products in response to cold

acclimation (Gusta and Wisniewski 2013), and also the importance of MTs and calcium as we described above. Under cold stress, plant freezing tolerance is usually quantified by the LT50 value (the freezing temperature at which 50% of a plant population dies) (Kurepin *et al.* 2013) and the parameter of electrolyte leakage. However, the electrolyte leakage is a little bit complex and affected by other factors, thus cell mortality which is the direct result of plants response to cold stress is taken into account. In our system, the cell mortality increased gradually to nearly 40% at 72h cold shock treatment but nearly did not change with chilling treatment for 72h, and chilling pretreatment could significantly reduce cell mortality (**Figure 1A, B, C and D**). These results demonstrate the cold acclimation (induced by non-damaging temperature) existed in this grapevine system and this cell line could be a good material to study cold acclimation. In addition, in tobacco BY-2 cells, the cold stress induced chromatin changes are accompanied by a general decrease of cell viability which is considered as programmed cell death activated by cold stress (Koukalova *et al.* 1997). However, whether the cold induced cell mortality increment in our system is related to programmed cell death or not needs further demonstration. Even though, the key transcription factor CBF family may play an imperative function under this system. Since for the downstream activation of cold acclimation, the changes of gene expression and protein accumulation occur to improve plant cold tolerance, for example, the transcription factors CBF/DREB1 (C-repeat binding factor/dehydration responsive element binding1) are prevailing and of central importance through regulating cold related genes involved in cold acclimation. Many of them have been characterized and have conserved function. Overexpression of this transcription factor could obviously enhance cold tolerance in *Arabidopsis*, cotton, canola, rice, tomato, barley, grapevine etc. (Stockinger *et al.* 1997, Thomashow *et al.* 2001, Hsieh *et al.* 2002b, Dubouzet *et al.* 2003, Savitch *et al.* 2005, Siddiqua and Nassuth 2011, Ma *et al.* 2014, Marozsan-Toth *et al.* 2015). *VvCBF4* was isolated by Xiao *et al.* (2008) in *V. vinifera* and expressed in young and mature leaves denoting it maybe more important for the over-wintering of grapes than other *CBFs*. Our research has shown that the induction of *VvCBF4* came to around 8 fold after 6h of cold shock treatment compared to the control (**Figure 1E**), suggesting that the rapid response of

VvCBF4 to cold stress might be important for cold tolerance. And this has also been widely studied in many plants including in grapevine. In *V. amurensis*, the expression of *VaCBF1* increased in response to cold stress and the overexpression mutant could enhance cold tolerance in a transgenic tobacco line (Dong *et al.* 2013). While in the overexpressed grapevine line cv. 'Freedom', the *VvCBF4* could help to enhance freezing tolerance with reduced growth phenotype and reduced freezing-induced electrolyte leakage (Tillett *et al.* 2012). Furthermore, overexpression of CBFs could repress the plant growth and affect the onset of leaf senescence and dormancy (Wisniewski *et al.* 2011). In addition, with the application of the CRISPR/Cas9 technology, the *cbf* triple mutants showed extremely sensitive to freezing stress (Zhao and Zhu 2016). All these results indicate the importance of CBF family under cold stress and in our system the high expression of *VvCBF4* might contribute to the reduction of cell mortality and this needs to be verified by further testing the expressing level of *VvCBF4* at chilling treatment.

However, the CBF transcription factors alone are not sufficient for the maximal induction of cold acclimation, the transient disassembly of MTs contributes to the cold acclimation as well (Abdrakhamanova *et al*. 2003, Vashegyi *et al.* 2013). Initially, a correlation between cold stability of MTs and cold hardiness has been suggested (Jian *et al.* 1989, Kerr and Carter 1990). For example, in cotton, a chilling-sensitive species, anti-microtubular drugs significantly accelerated and enhanced chilling injury, whereas a pre-treatment with ABA prevented chilling injury and counteracted the sensitizing effect of anti-microtubular drugs (Rikin *et al.* 1980). In winter wheat, a transient and initial microtubule disassembly was necessary and sufficient to trigger cold acclimation and cold acclimation could be induced by pronamide treatment (Abdrakhamanova *et al.* 2003). These findings are consistent with our results where the pretreatment of pronamide could reduce the cell mortality, probably due to the transient disassembly of MTs **(Figure 5C)**, but total disassembly of MTs by oryzalin treatment **(Figure 3A and 4)** as well as depolymerization of actin filaments **(Figure 7A)** did not affect cell mortality. However, treatment of taxol, a stabilizer of MTs, could retrieve the cell mortality and

could not prevent gradual disassembly of MTs in 24h ice water treatment **(Figure 3B and 4).** This is a contradiction to previous research in which stabilization of MTs by taxol was reported to decrease freezing tolerance (e.g. Bartolo and Carter 1991). The explanations for this contradiction might be related to the important function of gradual disassembly of MTs. Because we found cells firstly treated with chilling (**Figure 1C and 2)** could also reduce the cell mortality accompanied by the gradual disassembly of MTs which is consistent with the results of taxol treatment. In summary these two common results demonstrate that the disassembly of MTs are sufficient for cold acclimation, and the disassembly of MTs might, together with the induction of *VvCBF4*, to possess functions in cold acclimation, although the induction of *VvCBF4* did not dependent on the changes of MTs, as all microtubule drugs could not induce the expression of *VvCBF4*. But the induction of *VvCBF4* could not be neglected and it must depend on another divergent pathway to regulate cold acclimation. However, this acclimation is also different of that found by Abdrakhamanova *et al.* (2003), showing the cold acclimation will then, in a second step, lead to a completely cold-tolerant array of MTs. Maybe the reason is that the time is not long enough to wait for the return of the cold tolerant MTs or MTs differ in different species in response to cold stress. For example, MTs are extremely cold-sensitive in chilling sensitive species, whereas they persist at low temperatures in chilling-tolerant species (Jian *et al.* 1989).

The function of several phytohormones is required for cold acclimation including ABA, GA, JA and others. Various studies show that MeJA could alleviate chilling injury by the induction of production of cryoprotective agents, ABA, lower activity of LOXs, and antioxidants (Wang and Buta 1994, Cao *et al.* 2009, Zhao *et al.* 2013). We also applied external JA under cold stress and could rescue cell mortality demonstrating that exogenous JA could alleviate cold shock injury (**Figure 7A)**. In addition, recent researches found the JA and brassinosteroid (BR) modulated freezing tolerance in *Arabidopsis* through ICE-CBF/DREB1 dependent or independent pathways. JAZ1 and JAZ4 which could repress the synthesis of JA, physically interacting with ICE1 and ICE2 to inhibit the expression level of CBFs, demonstrating jasmonate could positively

enhance cold acclimation-induced freezing tolerance in *Arabidoposis*. Similarly, *brassinazole-resistant 1* (BZR1) could interact with the promoter region of CBF1 and CBF2 to induce their expression levels and finally displayed enhanced freezing tolerance (Hu *et al.* 2013, Li *et al.* 2017). Furthermore, the up regulation of *MaCBF1, MaCBF2 and MaCOR1* in *Musa accuminata* by MeJA in response to cold stress indicates the crosstalk between JA and CBF signalling pathway (Zhao *et al.* 2013). However in our system, the exogenous application of JA could not induce the expression of *VvCBF4* (**Figure 7B**), but could induce the depolymerization of MTs (**Figure S4**), indicating that this cold acclimation depends on the function of JA through MTs pathway, and not the *VvCBF4* pathway. Even though we could also not exclude the CBF pathway, because the CBF family contains various members which are responding differently to cold stress, some are highly responsive others only weakly or entirely unresponsive (Campoli *et al.* 2009). For example, CBF2 has distinct function than CBF1 and CBF3 in cold acclimation where CBF2 negatively regulates a set of cold-responsive genes (Novillo *et al.* 2007). These researches demonstrate that different CBF members depend on different signalling pathways. In order to verify this question, traditional pharmacological components to block certain signalling pathway were applied to see the transcriptional changes of CBFs under cold acclimation. However, as we discussed above, we could arrive at a conclusion the expression or induction of *VvCBF4* was dispensable for microtubule and JA pathways. Until now, the widely approbative hypothesis of the signal transduction under cold acclimation is surmised by these following three aspects: firstly the induction of membrane rigidification and the rearrangement of cytoskeleton; secondly the influxes of calcium and the last is the induction of cold related genes, such as the key transcription factors of the CBF family (Zhu 2016).

CBFs are widely studied on large amount of species, including grapevines and this *Vitis* CBF4 transcription factor indeed locates at the nucleus (Xiao, et al. 2008) and it expressed rapidly for a couple of hours responding to cold stress. Therefore, there must be some mechanisms existing how the signal is transduced from cold sensing at the

membrane to the nucleus. Recently one typical research finds a plasma membrane protein kinase, named CRPK1 which could phosphorylate 14-3-3 proteins, triggering its nuclear shuttling under cold stress to negatively regulate the cold tolerance in *Arabidopsis* (Liu *et al.* 2017). Another possibility for the nuclear translocation could be induced by the depolymerization of MTs which could guide the molecules to enter the nucleus to activate the key transcription factor because depolymerized MTs will accumulate in the nucleus simultaneously. We already demonstrate that the induction of *VvCBF4* is dispensable of microtubule pathway, but this does not exclude a function of MTs in the early signal transduction pathway.

The rapid influx of calcium into the cytoplasm ascribed to the procedure of cold acclimation, not only depends on its second messenger function, but is also related to the accumulation of calcium in the cytosol. It could activate the ICE-CBF-COR cascade pathway to enhance plant tolerance (Zarka *et al.* 2003, Chinnusamy *et al.* 2007). For example, Ca^{2+}-dependent protein kinases could induce the transient influx of calcium to the cytosol which could regulate COR gene expression (Saijo *et al.* 2000). In addition, the changes in composition of plasma membranes contributes to the plants survival from freezing stress (Steponkus 1984), especially the changes of the state of phospholipids. For instance, in cyanobacteria, the membrane fluidity increased under low temperature through the induced expression of specific fatty acid desaturases which are activated by two-component histidine kinases (Los and Murata 2002). Our results indicated chelating the calcium by EGTA could increase cell mortality, but promoting cell membrane fluidity could rescue cell viability by BA treatment (**Figure 6A**). Furthermore, the induction of *VvCBF4* was high with calcium ionophore A23187 treatment, and lower with $GdCl_3$ treatment compared to that with only cold treatment (**Figure 6B and C**). Both BA and DMSO treatment could not induce the expression of this transcription factor though (**Figure 6D and E**). This is congruent with previous research that the cold activation of the *Brassica napus* BN115 promoter could be induced by the calcium ionophore A23187 in the absence of cold treatment and inhibited by gadolinium ions under low temperature (Sangwan *et al.* 2001).

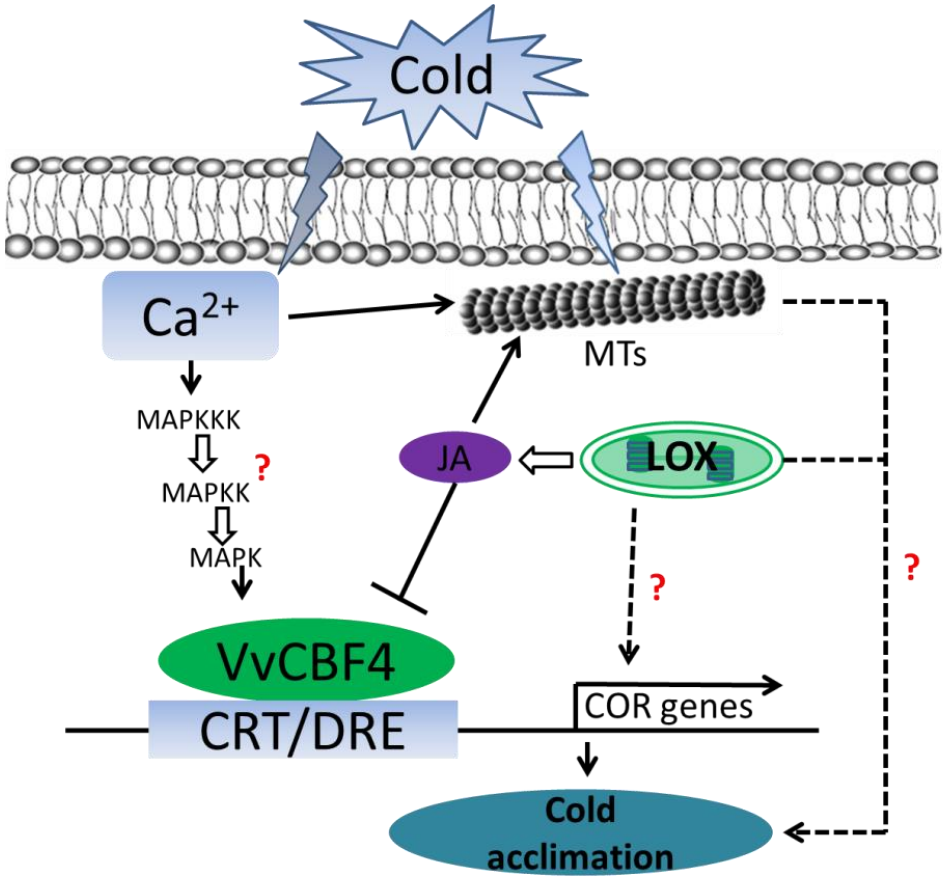

Figure 8. The diagram of potential molecules involved together with *VvCBF4* to play important function under cold acclimation. Cold stress could trigger the influx of calcium and the disassembly of MTs to modulate the induction of *VvCBF4* by different signalling pathways to arrive at cold acclimation. Broken arrows indicate unknown regulation, solid arrows indicate activation, whereas lines ending with a bar show negative regulation. Abbreviations: Ca^{2+}, calcium ion; MTs, microtubules; MAPK, mitogen-activated protein kinase; JA, jasmonic acid; LOX, Lipoxygenase; *VvCBF4*, C-repeat binding factor (an AP2-type transcription factor) in *V. vinifera*; CRT, C-repeat elements; DRE, dehydration-responsive elements.

Finally we arrived at a model **(Figure 8)** in which the disassembly of MTs, the increment of membrane fluidity, the influx of calcium ions and the induction of *VvCBF4* are essential for grapevine cold acclimation. The increasing membrane fluidity could partially prevent the disassembly of MTs, but independent of CBF pathway to modulate cold acclimation. JA could promote the disassembly of MTs independently of the *VvCBF4* pathway to adjust cold acclimation. The central factor is calcium ions which play an imperative role in cold acclimation through diverse functional branches, one

dependent on MTs and another one dependent on CBFs.

5. Outlook

Does the accumulation of tubulins in the nucleus under cold stress trigger the activation of CBFs transcription factors?

The above two models in which the cold-induced MT disassembly was seen as a part of cold-induced cellular damage, has over the years shifted into a model, where MT disassembly is interpreted as sensory process. At some point, this sensory event has to translate into changes of gene expression, requiring activation of transcription factors such as the dehydration-responsive element-binding protein 1s (DREB1s)/ C-repeat-binding factors (Ito *et al.* 2006) by the calcium pathway and finally induce cold acclimation. But whether MTs modulate chromatin structure by mechanic tensegrity required for this activation, or how does the MTs accumulated in the nucleus trigger this activation? needs to be addressed in forthcoming work.

Could we use the *VvCBF4* transcription factor to define genetic resources for cold tolerance breeding?

Based on our research, we have shown the sensory function of MTs and the imperative role of *VvCBF4* in cold acclimation. This knowledge would be beneficial for us to do the screening selection by taking advantage of the transcription factor CBF genes to hunt for some cold tolerant subpopulations of *V. sylvestris* which could be used for genetic breeding to achieve more cold tolerance grapevine species.

The European wild grape *V. vinifera* L. ssp. *sylvestris* Hegi currently faces the danger of extinction. Hence in order to save these grapevine treasures, my supervisor, Prof. Dr. Peter Nick devised a project to collect all the European wild grapes (for simplicity

termed *V. sylvestris*) to further maintain a complete copy of the genetic variations of these species in Germany. These species are now well maintained at the KIT botanical garden, Karlsruhe (Nick 2012). These wild grapevine species are important because they play a crucial role in plant breeding and evolutionary biology and could be valuable genetic resources for breeding. For instance, the screening of the population with respect to susceptibility to downy mildew of grapevines (*Plasmopara viticola*) revealed that the subpopulation of genotypes with high stilbene inducibility was significantly less susceptible as compared with low stilbene genotypes (Duan *et al.* 2015). Thus, maybe these old-wild precious genotypes of *V. sylvestris* could provide as important materials for genetic breeding to acquire cold tolerant species.

The CBF transcription factors are widely studied in various species and the common consent is that the higher-level expression or overexpression of these transcription factors could significantly enhance cold tolerance (Hsieh *et al.* 2002a, Savitch *et al.* 2005, Benedict *et al.* 2006) including grapevines. Therefore, the transcription factor, *VvCBF4*, could be considered as a major cold tolerance marker gene to further screen the cold tolerant genotypes. Based on this hypothesis, we tested the expression pattern of *VvCBF4* on all *V. sylvestris* genotypes and the results showed different expression level of *VvCBF4* on the *V. sylvestris* genotypes. This could be clustered into four groups: Type Ⅰ, consistent increased expression of *VvCBF4* in 3h cold treatment including 30 cultivars; Type Ⅱ, the expression of *VvCBF4* culminated in 1h cold treatment included 26 cultivars; Type Ⅲ, no increased expression of *VvCBF4* in 3h cold treatment including 10 cultivars; Type Ⅳ, down regulated expression of *VvCBF4* in 3h cold treatment including 4 cultivars. We shall not interpret deeper into these results without further confirmation by performing more repeats and establishing gene expression patterns using quantitative real time PCR. I have presented some of these figures for some cultivars in the appendix part (see **Figure S5**). Even though, the results indeed shown that the activation of *VvCBF4* was different on various *V. sylvestris* genotypes and maybe this is due to the differential activation of the promoter of *VvCBF4*. Thus it is necessary to detect this hypothesis by a promoter-reporter assay (Holl *et al.* 2013) in

the future work. Recently, one report has analyzed the activation of grape CBF1 and CBF4 on artificial promoters containing variations of the CRT sequence, and shown that the different activation of CBF1 on *V. riparia* and *V. vinifera*. was caused by one amino acid change. These results indicated that the different elements of promoter elements resulted in the quantitative difference in activation of *VrCBF1* and *VrCBF4* (Nassuth *et al.* 2014). In addition, according to this technology, the activity of the promoter of transcription factor *MYB14* which induces the stilbene synthesis related genes expression differed greatly in *Hö29* and the cultivated variety *Augster Weiss*. The former showed strong inducibility of stilbene synthase and the later showed weak stilbene production (Duan *et al.* 2016). All these evidences come to a hypothesis that the different expression of *VvCBF4* in our research may be due to the different activation of its promoter domains on different *V. sylvestris* genotypes. Thus, for the future research, it could be beneficial to clone out a small number of promoters of *VvCBF4* from some typical *V. sylvestris* genotypes where the expression patterns are significantly different. These could then be further analyzed by the promoter-reporter assay.

6. Appendix

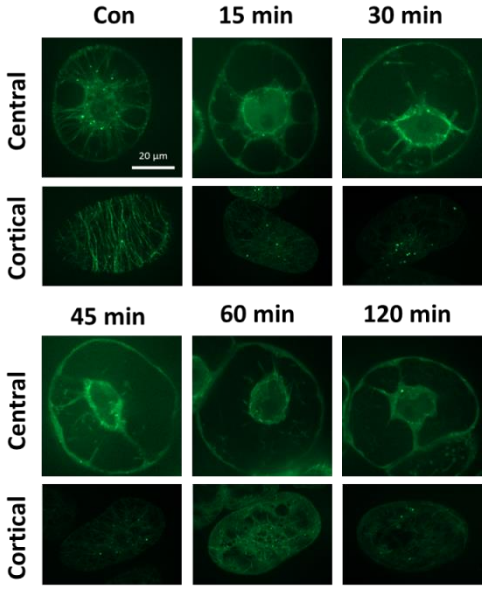

Figure S1. Representative images showing central and cortical MTs in cells subjected to different periods of cold stress (0 ℃) in the MTs marker line *V. rupestris* GFP-TuB6. Size bars, 20 µm.

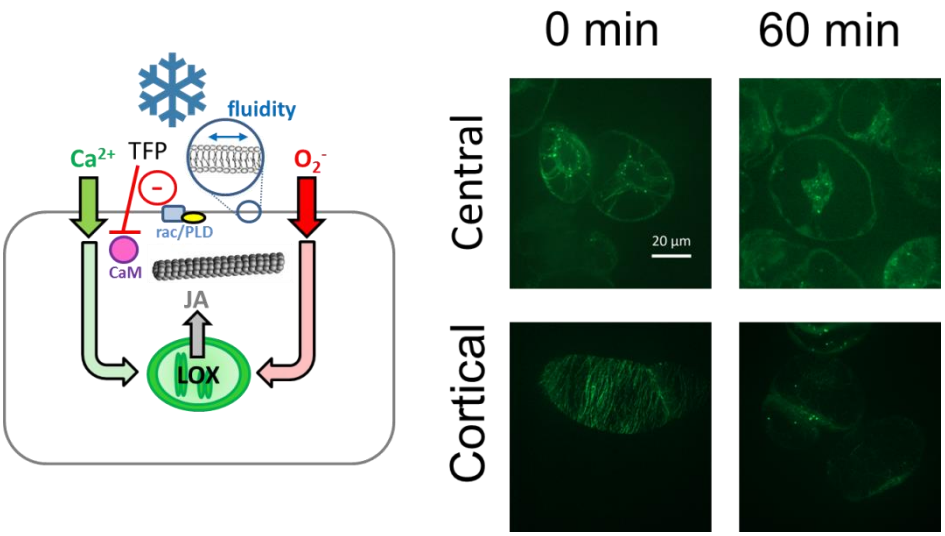

Figure S2. Effect of 100 µM TFP (Trifluoperazin dihydrochloride) on the cold response of radial and cortical microtubules. Observations are representative of at least three independent experimental series. Size bars, 20 µm.

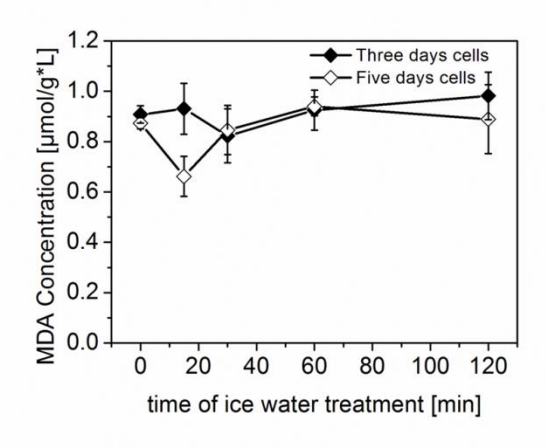

Figure S3. Time course of MDA accumulation *in vivo* in *V. rupestris* cells expressing the GFP-AtTuB6 marker response to cold stress treatments (0 ℃).

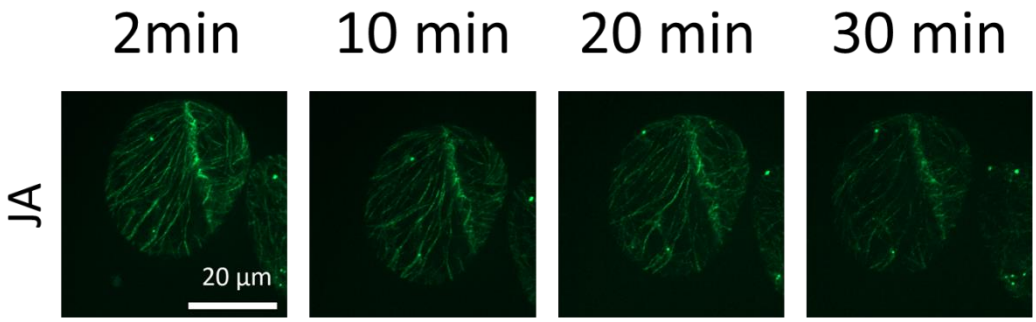

Figure S4. Effect of Jasmonic Acid on microtubules in normal temperature (27 ℃). Size bars, 20 μm.

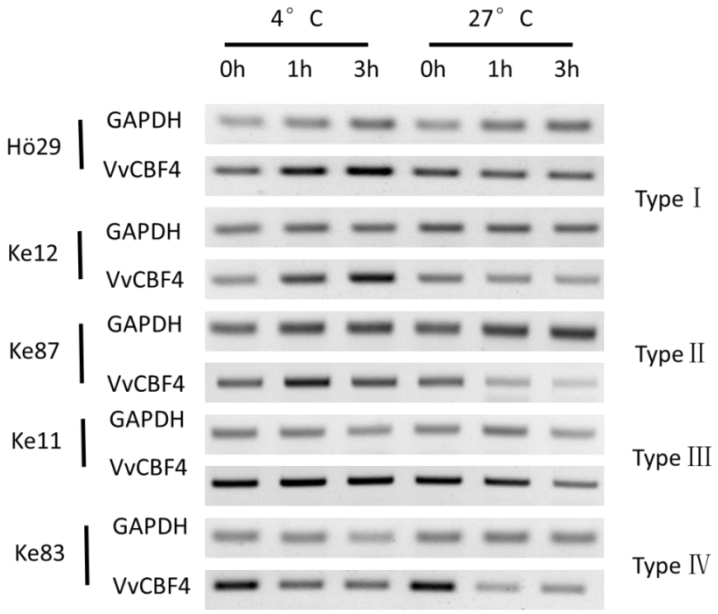

Figure S5. The different expression level of *VvCBF4* on the V. *sylvestris* genotypes under 4℃ and 27℃ treatment. Type Ⅰ, consistent increasing expression of *VvCBF4* in 3h cold treatment including Hö29 and Ke12; Type Ⅱ, the expression of *VvCBF4* culminated in 1h cold treatment included Ke87; Type Ⅲ, no increasing expression of *VvCBF4* in 3h cold treatment including Ke11; Type Ⅳ, down regulating expression of *VvCBF4* in 3h cold treatment including Ke83.

7. Reference

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8. Curriculum Vitae

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