Upstream of gene expression - what is the role of microtubules in cold signalling?

Lixin Wang¹,³, Ehsan Sadeghnezhad²,³, Peter Nick³*

¹ College of Horticulture, Hebei Agricultural University, Baoding, 071001, Hebei, China

² Department of Plant Biology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

³ Molecular Cell Biology, Botanical Institute, Karlsruhe Institute of Technology, Fritz-Haber-Weg 4, D-76131 Karlsruhe, Germany

* To whom correspondence should be addressed. E-mail: peter.nick@kit.edu

# These authors contributed equally to this work.
Abstract

Cold stress is a major abiotic stress, restricting plant growth and development. Therefore, the gene expression in response to cold stress and during cold acclimation has been studied intensively, including the ICE-CBF-COR pathway, as well as the modulation of this cascade by secondary messengers, for instance MAPK cascades. In contrast, the early events of cold perception and cold adaption have obtained far less attention. This is partially due to the fact that cold is a physical signal, which requires the conceptual framework to be adjusted. In this review, we address the role of microtubules in cold sensing, and propose a model, whereby microtubules, while not being part of signalling itself, act as modulators of cold sensitivity. The purpose of this model is to derive implications for future experiments that will help to get a more complete understanding of cold adaptation.

Key words: microtubules, calcium, membrane, cold sensitivity, sensory adaptation, gene expression
Introduction

Adaptation to cold stress – significance and mechanisms. Damage imposed by low temperature represents one of the most serious constraints for agriculture. Especially in early spring, when mild weather has evoked a precocious development, even short cold episodes can have devastating consequences. For instance, three days of frost in April 2017, following a very mild March, almost annihilated the German apple harvest to a value of less than 500 000 tons, according to statistical data published by the German Federal Ministry of Nutrition and Agriculture (2017). Such extreme temperature fluctuations are expected to be accentuated in the future by global climate change. Thus, it is of vital importance to understand the biological consequences of cold damage, but also the mechanisms of cold adaptation.

Cold stress comes in two versions – subzero temperatures cause irreversible membrane damage due to the formation of ice crystals (a comprehensive and still valid review is given by Burke et al., 1976). While the mechanisms underlying this so-called freezing stress are well understood, there exists a second form of cold stress, where temperatures above zero will cause irreversible damage. Already in the 19th century, this chilling stress has been described as so called „Erkältung“ (Molisch, 1897). The relationship between chilling and freezing tolerance is asymmetric: all freezing tolerant plants are also chilling tolerant, but not vice versa. Despite being known for a long time, this type of chilling stress has remained somewhat enigmatic. For instance, chilling sensitive cucumbers are already killed by temperatures as mild as +10°C, which is far from any ice-induced membrane disruption. While the freezing tolerant species, such as winter wheat, can withstand temperatures as low as -10°C. This variation of chilling and freezing sensitivity indicates that a perturbation of physiological homeostasis, rather than a simple physical phenomenon, is responsible for the damage, a hypothesis put forward already by the comprehensive classical review by Lyons (1973).

Chilling and freezing tolerance, while clearly regulated by genetic factors, are not constant, but subject to change: Pretreatment with mild cold stress above the threshold for irreversible damage can boost tolerance to subsequent, more stringent, challenges that otherwise would lead to the death of the plant. This phenomenon, known as cold acclimation, or cold hardening, arose considerable interest, because it meant that it should become possible to improve cold tolerance by genetic approaches (for review see Guy 1990). Especially transcription factor cascades have been studied intensively in this context (reviewed in Thomashow, 1999, 2010) see also the most recent comprehensive review by Ding et al. (2019b), because they regulate the expression of cold responsive (COR) genes that encode enzymes for detoxification of reactive oxygen species, compatible osmolytes that prevent freezing-induced losses of turgescence, fatty-acid desaturases that sustain...
membrane fluidity, or anti-freeze proteins that suppress the formation of ice crystals (reviewed in Guy 1990; Thomashow, 1999). The first level of this transcription factor cascade is formed by the ICE1 protein, which in turn controls the expression of so-called C-box (Cold-Box) factors (CBFs), forming the second level of the cascade (reviewed in Thomashow, 2010). Since ICE1 is expressed constitutively, signalling seems to be conveyed by secondary messenger pathways upstream of this transcriptional regulator. In fact, the stability of ICE1 has recently been shown to be under control of several signalling pathways. As will be worked out in detail below, the partially discrepant conclusions on the role of these pathways can be resolved by adjusting a more rigorous conceptual framework for signalling (one of the motivations for the current review).

While transcriptional activation, as well as the secondary messengers controlling expression or activation of these transcription factors have been studied in considerable detail, the early signalling at the plasma membrane that deploys these secondary messengers, has remained more elusive. The induction of desaturases leading to an increase in unsaturated fatty acids (that will increase membrane fluidity, because of their kinked configuration) has been recognised as an important adaptive mechanism to chilling stress (for a comprehensive review see Nishida and Murata, 1996). Likewise, the stability of microtubules has been linked with cold tolerance (Jian et al., 1989; Rikin et al., 1980). However, it should be kept in mind that these events are part of the downstream response, not of upstream signalling. A dissection of early cold signalling upstream of secondary messengers has only rarely been attempted: Using a cold-responsive promoter-reporter construct, changes in membrane fluidity, calcium influx, and the cytoskeleton (both actin filaments, and microtubules) could be demonstrated as relevant factors using an inhibitor-based strategy (Orvar et al., 2000; Sangwan et al., 2001). Later it could be shown, for winter wheat, that microtubules have to be dynamic to effectively deploy the signalling culminating in cold hardening (Abdrakhamanova et al., 2003).

What is the role of microtubules in cold sensing? Why we need a clearer conceptual framework. The rapid disassembly of microtubules in the cold is one of the few molecular responses to low temperature that can be seen in vitro, and even has been used to purify tubulin by cycles of centrifugation steps shifting temperature between cold and warm (Shelanski et al., 1973). Therefore, the role of microtubules in cold tolerance has been investigated extensively, but with seemingly contradicting results. In some studies, cold hardiness was linked with a cold stability of microtubules, in others, stabilisation of microtubules impaired survival under freezing (reviewed in (Nick, 2008, 2013). However, these discrepancies are not located in the realm of phenomena, but in the realm of interpretation. Microtubules exert more than one function – they can be part of the downstream adaptive
response to cold stress, but they can also be part of the early sensory events that deploy cold signalling. As components of the response, microtubules are downstream targets of cold signalling, as components of the sensory system, microtubules are upstream regulators of cold signalling. It is highly misleading, when the two levels are mixed up, as it has happened repeatedly. In the current review, we want to resolve some of these apparent discrepancies on the connection between microtubules and cold adaptation. In order to do so, we first have to define a conceptual framework that will be used to interpret, sort, and explain the empirical record. For this purpose, four logical cases will be introduced – in the first two models, the perceptive system remains constant, while in the last two models, the perceptive system changes as result of the signalling. In each of these two set-ups, two variations have to be considered, depending whether the trigger is of chemical, or of physical nature.

**Model 1: perception by ligand-receptor interaction.** The classical paradigm for signalling (Fig. 1A) is based on a concept, where the perceptive step is brought about by interaction of a stimulus with a receptor (in most cases at the plasma membrane), leading to a conformational change that deploys signal transduction, usually by secondary messengers. This signal transduction will convey the signal into the nucleus, where gene expression is modulated (often, but not exclusively, by transcriptional activation or repression).

Formally, this type of interaction follows a Michaelis-Menten model, such that the amplitude of signalling depends only on the concentration of the ligand (dose-dependency). Whether the ligand arrives in a single sweep at high concentration, or whether low concentrations of the ligand reach the receptor over time, does not play a role here. Signalling follows the Bunsen-Roscoe Law of Signal Reciprocity (Bunsen and Roscoe, 1855).

**Model 2: perception by susceptor-receptor interaction.** The ligand-receptor model can be used as first approximation to describe the response to chemical signals. However, some of the most relevant signals for a plant are of physical nature. This includes gravity, mechanical stimulation, but also heat and cold. Since signal transduction is usually of chemical nature (examples would be calcium, phosphorylation cascades, or cAMP), perception must involve some „translation“ of the world of physics into the world of chemistry. Following the nomenclature introduced by Björkman (1989) to describe the role of amyloplasts for gravity sensing, we will refer to this „translation device“ as susceptor (Fig. 1B). The difference from a receptor can be easily illustrated using the amyloplast case: the amyloplast itself is not sensing anything, it is a simple statolith and could be replaced by any heavy inorganic particle. It is simply „translating“ the gravity vector into a pressure upon a mechanosensitive ion channel, which will then produce a calcium influx as chemical readout that can then be used for signal
transduction. Compared to ligand-induced sensing, the perceptive structure is bipartite (susceptor and the actual sensor). Nevertheless, this perception will otherwise fulfill the criterion of reciprocity, which has actually been demonstrated for gravitropism (Johnsson et al., 1995). Again, it is the dose that matters, the timing of stimulation is not relevant.

**Model 3: perception followed by sensory adaptation of the receptor.** In the previous two models, the perceptive system remained constant, such that sensing was independent of time, but exclusively dependent on dose. While some signalling phenomena can be described, at least in first approximation, by such time-independent models, most cases of biological signalling show a clear dependence on time, such that the Bunsen-Roscoe Law of Reciprocity is violated. In these cases, the activity of the perceptive system depends on the signalling history of this perceptive system. As a rule, the number of receptors, or their affinity with the ligand, or their ability to deploy a signal, are downmodulated in response to ligand binding (Fig. 1C). This phenomenon is termed *sensory adaptation* and prevents that the continuous presence of a potential stimulus will lead to a continuous cellular response (which would be not only meaningless, but in case of stress signalling, even deleterious). The response amplitude is usually not depending on the absolute magnitude of the stimulus, but on its relative change, a rule that is known as Weber-Fechner Law (Fechner, 1889). The threshold for sensing will be, more or less, a fixed percentage relative to the strength of the preceding stimulus. The same stimulus that would activate signalling following a weak stimulus, would not be able to cross the threshold, if administered after a strong stimulus.

Unfortunately, the term adaptation is used in different ways, leading to inconsistencies and misunderstandings. We will follow here the terminology and conceptual framework developed by Galland (1991) to describe the perception of aneural organisms. The term *sensory adaptation* refers to modulated *sensitivity* following a stimulus and will become manifest as a time-dependent shift of the respective dose-response curve along the axis of stimulus dosage. For instance, a reduction in abundance or affinity of a receptor would require more ligand to achieve the same output. In the pure case, the shape of the dose-response curve would remain unchanged, but it would be shifted towards higher doses of the input. It is also possible that a step of signal transduction is modulated after a stimulus has been administered. In that case, the dose-response curve would not be shifted, but it would change in amplitude. Here, it is the *responsiveness* of the system that would be modulated. To separate this case from *sensory adaptation*, the term *habituation* has been coined and will also be used here. The Weber-Fechner law is clearly linked with *sensory adaptation*, not with *habituation*. These two phenomena (*sensory adaptation* based on changed *sensitivity* versus *habituation* based on changed...
responsiveness) are not mutually exclusive. In the real world they can, and often do, occur together.

**Model 4: perception followed by sensory adaptation of the susceptor.** In addition to a receptor for chemical signals, it is also conceivable that a *susceptor*, required for the perception of physical signals, is not constant, but modified depending on the preceding signalling (Fig. 1D). This will result in a change of *sensitivity*, similar as a reduction in the number or activity of the actual sensor would lead to *sensory adaptation*. While for the other three models, numerous examples have been described, both for animal and plant signalling, the case of sensory adaptation of the susceptor seems to be, at first, a merely theoretical option. However, we want to show in the current review that this model is useful to describe and understand the role of microtubules in cold sensing.

**Sorting complexity by timing: a simplified framework to understand the role of microtubules in cold-stress signalling**

With the development of molecular biology, the mechanism of cold acclimation or tolerance has been widely studied, especially in the model plants *Arabidopsis* and *rice*, leading to a long list of molecular players that interact in a complex, often redundant, and partially antagonistic manner (Ding et al., 2018; Li et al., 2017; Liu et al., 2019; Liu et al., 2018; Liu et al., 2017; Zhang et al., 2017; Zhao et al., 2017). The most recent and comprehensive review of these findings is given in Ding et al. (2019b). However, a part of this complexity might be due to the representation, and not necessarily to the phenomenon itself: The data in *Arabidopsis* mostly refer to freezing stress, because *Arabidopsis* is fairly chilling tolerant, while most data in *rice* refer to chilling stress, as *rice* is fairly susceptible to chilling damage. Moreover, the time scales of the numerous responses differ, which is often ignored in the rather static representations derived from genetic experiments. It is important, though, to separate events involved in primary cold signalling from those that, at a later stage, modulate gene expression, and, according to the terminology described above would rather fall into the realm of habituation. Interestingly, microtubules, while mostly being acknowledged as upstream factor of signalling together with changes of membrane fluidity, are usually treated in a rather laconic way – for instance, the otherwise very comprehensive Tansley review by Ding et al. (2019b) spends less than half a page on the cytoskeleton, mostly quoting data from two decades ago. It is one intention of the current review to address this gap of knowledge, at least by some conceptual input. To prepare this, the following section will outline a simplified framework of cold signalling, by making extensive use of Occam’s Razor and by sorting early (Fig. 2A) from later (Fig. 2B) events.
Cold perception. To sense low temperature as physical input, requires a susceptibility step (Fig. 1B). The drop in membrane fluidity is generally thought to represent the actual signal (reviewed in Los and Murata, 2004). In fact, desaturases that increase the proportion of unsaturated fatty acids that, due to their kinked configuration occupy larger cross-areas and therefore increase fluidity, have been shown to be relevant for cold adaptation in Arabidopsis (Martiniere et al., 2011). This does not mean, however, that they participate in cold signalling – the time scale of gene expression and product accumulation is much longer than the fast events that trigger cold signalling.

How a drop of membrane fluidity should culminate in a mechanic force that can be used as perceptive event, is rarely asked, but far from trivial to understand. The fluid-mosaic model by Singer and Nicolson (1972) puts emphasis on the heterogeneity of biomembranes, where lateral diffusion is impeded by “cytoskeletal corrals” but also local agglomerations of proteins, or other macromolecules. When temperature drops, the drop in fluidity should not occur homogenously, but in specific patches, creating local asymmetries that would lead to a force along the borderline of fluid and less fluid patches. However, these forces are expected to be minute and should barely exceed those of thermal noise. Moreover, they should statistically be levelled out. However, if these minute forces were collected along a highly anisotropic probing structure, able to integrate and transmit compression forces, this should lead to a net force that might be perceived by a mechanosensitive structure. The best candidate for this highly anisotropic structure that can transmit compression forces, are the microtubules, because they are endowed with a high rigidity (with a Young’s Modulus similar to glass) and able to efficiently transmit vibrations, especially in short time scales (Koch et al., 2017). They would, thus, be seen as part of the susceptible structure, together with heterogeneous rigidification of the membrane (Fig. 2A, ○).

The third element of the plant cold-susceptor seem to be plasma membrane located calcium channels (Fig. 2A, ○). Using transgenic plants expressing aequorin, sharp peaks of cytosolic calcium could be observed within seconds after transfer to a cold shock (Knight et al., 1991). Similar rapid responses were seen after touching the plant, indicative of mechanosensitive calcium channels. Which channel is responsible for this rapid cold response, is not known, and hard to determine at the current state, given the huge number of candidates: Among the five known families of Ca²⁺-permeable channels in Arabidopsis (reviewed in Kudla et al., 2018), only one family belonged to mechanosensitive channels (MCAs – two members, reviewed in Kurusu et al., 2013), while others are gated by cyclic nucleotides (CNGCs – 20 members, Zelman et al., 2012), glutamate (GLRs – 20 members, Lacombe et al., 2001; Price et al., 2012), or hyperosmotic stress (OSCAs – 15 members, Yuan et al.,
In addition, two pore channels (TPCs) form a further group (Morgan and Galione, 2014).

**Signal amplification.** The primary input for cold signalling are probably the minute mechanic forces caused by membrane rigidification. Even if integrated by the above-mentioned microtubule-based susceptor system, efficient signal amplification is needed to reach the clear calcium peaks observed within seconds after the transition to low temperature (Knight et al., 1991). The recently discovered transmembrane protein COLD1 (Ma et al., 2015) might play a key role in this signal amplification (Fig. 2A, ②). This protein had been discovered through map-based cloning of chilling tolerance in rice and found to be necessary for cold induced calcium influx. This transmembrane protein is physically linked to RGA1, the Gα protein of higher plants. Whether COLD1 might be a calcium channel by its own virtue, is still debated. The authors have tested this by expressing COLD1 in *Xenopus* oocytes to follow the resulting currents after cold treatment. They see inward currents, if co-expressing COLD1 and its interaction partner RGA1, the only known G-protein α subunit found in plants. However, there is no significant difference from the water control, if COLD1 alone is expressed. Since endogenous *Xenopus* calcium channels are activated by phosphatidic acids, the products of phospholipase C (Bourinet et al., 1992), and since also a C-terminally truncated loss-of-function version of COLD1 was producing the current, if co-expressed with RGA1, the current is quite unlikely to be caused by a putative channel activity of COLD1 itself, but rather through the stimulation of endogenous *Xenopus* channels by the co-expressed RGA1. In other words: the published evidence can be explained by a scenario, where COLD1 acts as facilitator of calcium channels, but does not need to be a channel by itself. Activation of Gα triggers phospholipase D activity (Munnik et al., 1995), and, in fact, the accumulation of phosphatidic acids (partially from PLD, partially from the concert activity of Diacylglycerol Kinase and PLC, which is also activated by G-proteins) belongs to the earliest cold responses that can be detected after the onset of cold stress (Ruelland et al., 2002). Interestingly, PLD requires high concentrations of calcium (in the mM range) to be fully active (reviewed in (Wang, 2005), providing a tight barrier to signalling as long as the calcium channel remains closed. The product of phospholipase D, phosphatidic acid, can stimulate the NADPH oxidase Respiratory burst oxidase Homologue (RboH), a central input for plant stress signalling, through recruiting the small GTPase Rac for RboH (Wong et al., 2007). The apoplastic reactive oxygen species (ROS), produced by RboH further amplify the opening of calcium channels, an evolutionarily conserved positive feedback loop (reviewed in Mori and Schroeder, 2004). To sum up, the initial minute opening of calcium channels, facilitated by COLD1-dependent signalling and transduced by phosphatidic-acid mediated stimulation of RboH, will self-amplify, through
apoplastic ROS, into the strong and clear calcium peak observed in response to cold stress (Fig. 2A, ②).

**Signal transduction to the nucleus.** As result of the self-amplifying loop described above, three signalling outputs are generated: (i), a sharp increase in cytosolic calcium, (ii), a sharp increase in apoplastic ROS that can enter the cell through aquaporins, probably as hydrogen peroxide as to be concluded from scavenging by exogenous catalase (Chang et al., 2011), and (iii), increased levels of phosphatidic acid. As worked out in the following, our simplified model assigns the primary signal to the ROS, while the calcium peak will be linked with habituation (Fig. 2B). The perceptive events at the membrane must deploy a signal that has to reach the nucleus, where the steady-state protein levels of the transcriptional master switch Inducer of CBF expression 1 (ICE1) has to increase to activate a transcriptional cascade. This master switch is constitutively synthetized, but also continuously degraded in the proteasome, such that in absence of cold, the steady-state levels of ICE1 are low. They can be increased, however, when the recruitment of ICE1 for the proteasome is inhibited. As to be expected, more than one signal travels this path from membrane to nucleus, whereby the discussion has focussed on two MAPK cascades: The one cascade culminating in activation of MPK6 from around 5 min, and MPK3 from around 15 min after the onset of cold stress (Zhao et al., 2017) will lead to phosphorylation of ICE1 at three sites (Ser94, Thr366, Ser403), recruiting this master switch to the proteasome. The other cascade, culminating in activation of MPK4 from around 30 min (i.e. much later), blocks the activity of MPK3/6 and therefore (indirectly) promotes the stability of ICE1. We sort both of these cascades rather into the realm of habituation (Fig. 2B), because they are of inhibitory nature. Before any inhibition can be effective, something must be activated as a first step – MAPK cascades cannot be this first step, therefore. The only candidate for the primary positive signal, we could locate from screening the literature of the last decade, is the kinase Open Stomata 1 (OST1). This kinase can phosphorylate ICE1 at a Ser278 (i.e. at a site different from those targeted by MPK3 and MPK6), and this modification prevents that ICE1 is recruited for proteolysis (Ding et al., 2018, (Fig. 2A, ④). How is OST1 activated? It has to be released from a complex by the activity of a class 2C protein phosphatase, abscisic acid insensitive 2 (abi2), what happens, for instance, when ABA binds to its receptor PYR. This mechanism would not work as early response, because the accumulation of ABA in response to cold is a slow process with a time frame of hours. However, abi2 is also activated by hydrogen peroxide, a mechanism that is fast and completed within few minutes (Meinhard et al., 2002 (Fig. 2A, ③). The ROS output from the above described signal amplification loop should therefore lead to a rapid release of active OST1 from the membrane and in consequence, an elevated activation of ICE1.
Transcriptional activation in the nucleus. When the master switch, ICE1, accumulates, because its degradation is inhibited by OST1, ICE1 can bind to the promoters of Cold Box Factors (CBFs) and activate their transcription (Chinnusamy et al., 2003), a process that can be detected from around 15 min after the onset of cold stress (Thomashow, 2010, Fig. 2A, ©). The CBFs, in turn, will activate downstream COR (Cold Responsive) genes, a process initiating in the range of 2 h and leading to a significant modulation in the steady-state transcript levels of around 1000 genes that are up regulated, while another 1000 genes are down regulated (Fig. 2A, ©).

Habituation of cold signalling. The constitutive activation of cold-stress signalling would bind numerous resources, and also lead to cellular damage. It is vital that stress signals, once they have been deployed, are attenuated. In fact, most of the numerous signalling events discovered in response to cold stress, are functionally linked with the habituation of cold-stress signalling:

The calcium signal generated as early step of primary cold signalling, can be read out by a plethora of binding proteins that allow for ample cross-talk between signal chains and certainly also for further amplification (for recent reviews refer to Guo et al., 2018; Kudla et al., 2018). Examples are calmodulin (CaM), CaM-like proteins, calcium-dependent protein kinases (CDPKs), calcineurin B-like proteins (CBL), but also the newly discovered IQ-domain proteins (Burstenbinder et al., 2017).

For cold habituation, the CDPKs seem to be central, because these proteins are specific to plants and can activate MAPK signalling (Sangwan et al., 2002; Xie et al., 2014). Some members are cold inducible (Martin and Busconi, 2001), and overexpression of one of these cold-inducible members, OsCDPK13, conferred elevated cold tolerance (Abbasi et al., 2004). Thus, a straightforward mechanism for habituation would be the activation of CDPK signalling by calcium / calmodulin (Fig. 2B, ©). This would then lead to MAPK signalling activating the activity of MPK3 and/or MPK6 (Fig. 2B, ©), which phosphorylate ICE1 in the destabilising sites (Ser94, Thr366, Ser403), such that it would be recruited for the proteasome.

Fine-tuning of MAPK signalling seems to be produced by a different target of calcium-calmodulin: the Calcium/Calmodulin Regulated Receptor Like Kinase 1 (CRLK1), which is located at the inner side of the plasma membrane (tethered by a N-terminal transmembrane domain), and endowed with two calmodulin-binding domains (Yang et al., 2010a; Yang et al., 2010b), interacts with MEKK1 (Fig. 2B, ©), which results in stimulation of MPK4 (an antagonist of MPK3/6 signalling), leading to the stabilisation of ICE1. CRLK1 also downregulates the activity of MPK3/6 signalling itself (Zhao et al., 2017): in a mutant, where CLRK activity is knocked down, MPK3/6 signalling is deregulated already prior to the onset of cold stress and further increased as compared to the wild type. Although CRLK1 itself is not part of the early...
cold signalling, it seems to function as a factor fine-tuning habituation, by restoring the amplitude of transcriptional activation.

Also the Cold Response Protein Kinase 1 (CRPK1) is localised at the inner face of the plasma membrane and can phosphorylate 14-3-3 proteins (Liu et al., 2017, Fig. 2B, ③). These interact physically with CBF3 (Fig. 2B, ③) as shown in vitro by pull-down assays, which promotes proteolytic degradation of their target, and, thus, negatively regulates the expression of cold-induced genes. The activation of CRPK1 is in the range of 1-3 hours, and therefore is clearly linked to silencing stress responses, once they had been initiated by early signalling.

However, habituation is also targeted to OST1 itself: This kinase can be sequestered to the membrane by a cold induced newly discovered clade-E growth-regulating 2 (EGR2) phosphatase that is anchored by a myristoyl moiety (Ding et al., 2019a, Fig. 2B, ④). The expression of EGR2 becomes detectable from 3 hours and will therefore repress OST1 signalling in the long term. This repression of OST1 activity is independent of ABA, i.e. also independent of ab12. With prolonged cold treatment, the induction of EGR2 is considerably increased, which will lead to increasing levels of un-myristoylated product softening the tethering of OST1. Thus, the initial downmodulation of OST1 signalling will cease with progressive cold treatment, which might already be part of the cold acclimation process.

The fact that several pathways are involved in habituation shows the importance of this phenomenon for cold tolerance. All of these pathways respond to signals produced by primary signalling (for instance calcium), but are activated more slowly (in the range of hours), partially, because gene expression is involved (as in the case of EGR2, Ding et al., 2019a).

**Microtubules act downstream and upstream of cold sensing – lessons from grapevine cells**

As mentioned above, the role of the cytoskeleton has been mainly addressed indirectly, for instance, by measuring activation of cold-responsive promoters after pharmacological interference with actin or microtubules (Orvar et al., 2000; Sangwan et al., 2001). The possibility to track microtubules in living cells by using GFP-fusions of plant tubulin opens the option to follow the microtubular response directly. Using this strategy, we were able to probe for the contribution of different signalling events to cold-induced disassembly of microtubules in grapevine cells (Wang and Nick, 2017). We could show that membrane rigidification through DMSO was sufficient to trigger the microtubule response, while membrane fluidisation through benzyl alcohol was able to suppress the microtubule response to cold. We further could demonstrate that calcium influx is necessary and sufficient for cold-induced microtubule disassembly, but that this calcium effect does not require
calmodulin. Likewise, the activity of Respiratory burst oxidase homologue (RboH) was found to be required, which would be expected from the model of a self-amplification loop (Fig. 2A, ②). We also could show that activation of PLD (blocked by n-butanol) is necessary, and activation of RGA, a Gα protein (activated by aluminium tetrafluoride, and inhibited by pertussis toxin) is necessary and sufficient for cold-induced microtubule disassembly, which can be easily explained by the amplifying activity of the COLD1-Gα complex (Fig. 2A, ②). These findings place microtubules downstream of the perceptive process, and the pharmacological signature of this process is consistent with the model worked out above, even for non-intuitive details, such as the dependence on RboH (Fig. 2A).

At the same time, microtubules appear to act upstream of cold perception in certain experiments. For instance, freezing tolerance could be modulated by compounds acting on microtubules in root tips of rye (Kerr and Carter, 1990), as well as in mesophyll cells of spinach (Bartolo and Carter, 1991). Interestingly, taxol, a compound stabilising microtubules, was found to constrain the development of freezing tolerance. This would indicate that a certain microtubule dynamicity is required for efficient activation of freezing tolerance. Congruent with this implication, rapid, but transient disassembly of microtubules during cold acclimation correlated with the degree of cold hardening in different varieties of winter wheat that differed with respect to freezing tolerance (Abdrakhamanova et al., 2003). In the same system, freezing tolerance could be induced in the absence of acclimation by transient elimination of microtubules using a pulse-chase treatment with pronamide. The freezing tolerance was accompanied by a progressive cold stability of microtubules, which was therefore discussed as possible mechanism of cold acclimation. The most straightforward way to explain these data would be a model, where microtubules constrain the calcium channel responsible for cold perception. Congruent with this model, cold-induced calcium influx in tobacco protoplasts was found to be negatively regulated by taxol, but promoted by pharmacological elimination of microtubules (Mazars et al., 1997), which is consistent with a model proposed the cytoskeleton in concert the membrane modulates mechanosensitive calcium channels (Örvar et al., 2000).

The few studies that address the role of microtubules in cold acclimation were conducted in seedlings (Abdrakhamanova et al., 2003; Bartolo and Carter, 1991; Kerr and Carter, 1990), where the cellular events are difficult to follow. On the other hand, some studies addressed the role of microtubules in the context of cellular events linked with cold sensing, evaluated the cell behaviour at room temperature and 4°C after treatment with pharmacological compounds acting on the cytoskeleton, the membrane, or signalling events, and measured the solute leakage as readout for cell death (Örvar et al., 2000; Sangwan et al., 2001). While these
studies demonstrated a role of actin filaments and microtubules in concert with membrane rigidification for cold tolerance, leading to a model, where cold induced activation of calcium channels is modulated by the cytoskeleton (Örvar et al., 2000), it is not so clear, to what extent this experimental system reflected cold acclimation. The operational definition of cold acclimation would require a set-up, where the system is first pretreated at cool, but not lethal temperatures before probing cold hardiness by a cold shock of otherwise lethal temperature. Thus, to address the function of microtubules during cold acclimation requires a more complex experimental design.

To close this gap, we used the grapevine cellular model described above to simultaneously follow cold hardening and microtubule responses in a cellular system accessible to live-cell imaging (Wang et al., 2019). In fact, it was possible to induce cold acclimation by prolonged chilling at 8°C, which rendered these cells more tolerant to a subsequent cold shock at 0°C. The chilling treatment could be replaced by either incubation with taxol, or by transient elimination of microtubules with pronamide. However, none of these treatments rendered microtubules cold stable, although the physiological effect (cold hardiness) was induced. This showed clearly that the acquired cold stability of microtubules seen in the winter wheat system (Abdrakhamanova et al., 2003) is not a conditio sine qua non for cold hardiness, but rather a parallel phenomenon.

As marker for cold acclimation, the expression of Cold Box Factor 4 (CBF4) was measured – steady-state transcript levels of CBF4 in response to cold stress were found to be strongly induced in the cold-tolerant species Vitis amurensis from North China, but not in the cold-susceptible species Vitis coignetia from subtropical China as to be expected from a marker for cold hardiness. Also, in the cellular system, the expression of CBF4 could be induced by cold stress. Interestingly, this induction was seen earlier and to significantly higher amplitudes under chilling at 8°C as compared to cold shock at 0°C. Using this molecular readout, the role of calcium influx and microtubules was assessed by respective inhibitors (Wang et al., 2019). While the expression of CBF4 was found to be under tight control of calcium influx, it was fairly independent of both microtubules, as well as of membrane fluidity.

The previous model proposed that microtubules constrain a calcium channel, such that cold-induced disassembly of microtubules would deploy calcium influx and, thus, cold signalling culminating in cold acclimation (Örvar et al., 2000; Nick, 2008, 2013). However, this model failed to explain the following empirical conclusions:

- The expression of CBF4 was tightly linked with calcium influx
- The expression of CBF4 was well correlated with cold hardiness
- The expression of CBF4 was independent of microtubules
- Transient elimination of microtubules induced cold hardening
- Stabilisation of microtubules by taxol induced cold hardening
- Treatments that induced cold hardening, did not induce stability of microtubules

The task of the following paragraph will be to integrate these apparently discrepant and partially even paradox findings into the conceptual framework (Fig. 1). As already demonstrated for the simplified model of cold signalling and habituation (Fig. 2), it is worth to consider the temporal sequence of these events. A second component of this framework will be that microtubules act as susceptors to amplify the signalling in response to cold stress.

**Microtubules do not signal anything, but they modulate the sensitivity of signalling.** Microtubules might amplify signal transduction in a manner similar to the COLD1-PLD-ROS-calcium amplification loop described above (Fig. 2, ②). If this would be true, disassembly of microtubules by factors different from cold should deploy cold responses, such as activation of CBF4. This was not observed (Wang et al., 2019). On the other hand, influx of calcium was necessary for cold-induced induction of CBF4 transcripts and also sufficient to produce this induction in the absence of cold, indicative of a very tight coupling between calcium influx and induction of CBF4. Although microtubules definitely do not transduce the cold signal, they play a role in cold hardening: both, treatment with the microtubule-stabiliser taxol, as well as a transient elimination of microtubules by pronamid were able to induce cold hardening to subsequent cold stress (Wang et al., 2019). What appears to represent a paradox, can be resolved, when microtubules are not placed downstream, but upstream of cold perception. If microtubules are determinants of cold sensitivity, the effect of their manipulation would not be seen in the absence of, but only after the application of, cold stress. Thus, pharmacological modulation of microtubules cannot replace cold with respect to signalling, but it can replace chilling with respect to cold hardening. Microtubules do so by increasing the sensitivity of cold perception, such that even a stimulus that otherwise would not be able to deploy cold signalling in an optimal manner, can trigger a strong and efficient response, culminating in successful adaptation.

This concept has several implications that are testable: (1) The signalling in response to deleterious cold stress (in case of grapevine cells, a cold shock at 0°C) should be less efficient compared to the signalling in response to mild cold stress (in case of grapevine cells, chilling at 8°C). This implication has already been experimentally validated (Wang et al., 2019): while the steady-state transcript levels of CBF4 were induced more than ten-fold within 3 h after mild cold stress (8°C), twice of treatment time was needed to induce a comparable induction under deleterious cold stress.
(0°C). (2) Stabilisation of microtubules (taxol treatment), or mild and transient elimination of very dynamic microtubules (pronamide treatment) can increase the sensitivity of cold perception, such that also a suboptimal signal (0°C) can efficiently activate cold adaptation. Again, this implication has been experimentally validated (Wang et al., 2019). Here, it would be interesting to see, whether cold hardening is correlated with a stronger response of CBF4.

Again, timing matters: while cold hardening at 8°C required long time scales (>1 d) to develop, direct manipulation of microtubules was improving cold hardiness with much shorter time scales (>1 h). Thus, microtubules are probably modulated in consequence of cold signalling. This modulation leads to a change of subsequent cold signalling, rendering it more efficient. As discussed in the following paragraph, microtubules seem to act as susceptors.

**Microtubules as cold susceptors.** As described in a previous chapter, the physical input for cold sensing is a drop in membrane fluidity. This will cause subtle asymmetries in the mechanic stress acting upon the membrane. To activate mechanosensitive ion channels, forces in the range of 1 mN m⁻¹ are required (Sachs and Morris, 1998), which corresponds to around a forth of the force that will break a plant membrane (Kell and Glaser, 1993). Thus, considerable signal amplification is required to sense cold. Microtubules with their high rigidity – their Young Modulus is comparable to that of glass (Gittes et al., 1993) – are good candidates for such a cold susceptor (Fig. 3, ⊃). Stabilisation of these microtubules as long-term response to chilling should therefore improve the focussing of the minute forces originating from local drops of membrane fluidity, and thus render cold sensing more efficient (Fig. 3, ⊅). This corresponds to the model shown in Fig. 1D, where sensory activity will feed back to the perceptive system by adaptation of the susceptor. The effect of chilling can be pharmacologically mimicked by stabilisation of microtubules by taxol.

However, it should be kept in mind that the susceptor in cold sensing is not only comprising microtubules, but also the membrane as second partner. It is the rigidification of the membrane that generates the primary input which is just collected, amplified and transduced by microtubules. Thus, there might also be a feedback of cold sensing on the membrane. In fact, there is sufficient evidence for such a feedback, even on several levels:

The tethering of the central cold signal component OST1 (Fig. 2B, ⊅) to the membrane is downmodulated upon prolonged cold treatment, because myristoylation of the tether EGR2 phosphatase decreases, such that OST1 is more readily deployed in response to additional cold stress (integration of clade-E growth-regulating 2 (EGR2) phosphatase. Thus, the amplitude of cold signalling would increase after prolonged cold stress. However, this would not be sensory
adaptation, but represent a case of cold habituation (*in sensu* Galland, 1991), since it is acting on the level of signal transduction, not on the level of perception.

Nevertheless, there exists also a true sensory adaptation in consequence of cold acclimation: As part of cold adaptation, the content of unsaturated fatty acids can be induced, such that the fluidity of the membrane is maintained even for lower temperatures (reviewed in Nishida and Murata, 1996). This would fall under the concept of sensory adaptation (on the level of a susceptor component, Fig. 1D). A second target are the integral membrane proteins constituting the primary amplification loop – including the calcium channel, the modulator COLD1, and the NADPH oxidase RboH (Fig. 2A, Θ) – these proteins are subject to the high dynamics of plant plasma membranes that are known to turn over in a few hours (Phillips et al., 1988). This integration of new membrane material is negatively regulated by dynamic microtubules (Liu et al., 2013), i.e. a subpopulation differing from the stable microtubules that convey membrane tensions upon the calcium channel (Fig. 3, Θ). If this dynamic population is eliminated, for instance by mild and transient treatment with pronamide, the integration of signalling components into the membrane is promoted, while the stable microtubules acting as force transmitters are still able to perform their function. This model can explain, why both, taxol and pronamide can mimic the effect of chilling with respect of cold acclimation – a phenomenon that otherwise would remain paradox.

It should be stated clearly and explicitly that this new model overthrows the previous idea that microtubules participate in sensory transduction by releasing the gate of calcium channels as proposed by Örvar et al. (2000), and also by previous work of our own lab (Abdrakhamanova *et al.*, 2003, Nick 2008, Nick 2013). If experimental evidence is not consistent with the implications drawn from a model, the model has to be changed. The major difference with respect to the role of microtubules is that microtubules are not part of signal transduction, but participate in defining the sensitivity of the perception process. This leads to the interesting question, where signalling begins and were it ends. The answer is that there is no clear line separating sensing and sensitivity, because sensing feeds back upon sensitivity. While the conceptual separation of perception, signal transduction, and signal response has been useful to reduce the complexity of biological signalling into elements that can be experimentally tested, we should never forget that this subdivision is not part of the biological system, but of our reductionist approach to study this biological system.

If one keeps in mind that one deals with a reductionist model, the cold acclimation process can be described as a sequence composed of the following elements: microtubules act as susceptors amplifying the effect of the membrane rigidification responsible for calcium influx as perceptive step. Calcium influx, amplified by the
self-referring COLD1-PLD-ROS-calcium loop would act as signal amplification, the resulting release of OST1 to the nucleus would correspond to signal transduction in the classical sense, the downstream transcriptional activation by the ICE-CBF-COR cascades would represent the interface between signal transduction and signal response. The activation of habituation (Fig. 2B) can be formally seen as part of the signal response. However, it differs from other adaptive responses, because it feeds back on the signalling machinery itself. The long-term changes in the machinery driving susceptibility (microtubules, membrane composition), perception (calcium influx channels), and signal amplification (COLD1, PLD, RboH) occur in parallel to habituation, i.e. as part of signal response. It should be terminologically separated from mere cold adaptation (for instance as accumulation of sugars) as cold acclimation in sensu stricto with the argument that it will modulate future signalling. Cold acclimation would, thus, be a kind of “memory” resulting from successfully coped stress experience.

Towards a molecular and functional model of the microtubule cold susceptibility. The microtubule susceptor model implies that microtubular dynamics and/or organisation are modulated depending on the history of cold signaling. Cold-inducible microtubule-associated proteins would be prime candidates for such a feedback from signaling upon cold sensitivity of the microtubule susceptor. The microtubule crosslinker MAP65 might be interesting in this respect, because the isotype MAP65-2 was found to stabilise microtubules against cold (Li et al., 2009). A similar role might be played by the protein WAVE-DAMPENED-LIKE 5 (Sun et al., 2015). Interestingly, some members of the MAP65 family have been found to decrease microtubule stiffness (Portran et al., 2013), which directly would impact on force transmission in response to membrane rigidification and might be the microtubular correlate to the adaptive re-fluidisation of the membrane by increasing the abundance of unsaturated fatty acids.

It should be kept in mind, however, that the feedback of cold signaling on the microtubule susceptor might also act independently of gene expression. For instance, activation of a specific MAPK by oxidative burst has been proposed as mechanism for ROS-triggered microtubule disassembly (Livanos et al., 2014a; Livanos et al., 2014b), which would create a functional link between cold-induced activation of RboH as early event in cold signaling (Fig. 2A, ⬀) and modulation of the susceptible structure, microtubules. A second candidate would be phospholipase D, which not only had originally been identified as microtubule-associated protein tethering cortical microtubules to the plasma membrane (Marc et al., 1996), but also can interact with MAP65 (Zhang et al., 2012) and renders microtubules stable against salt-induced elimination (Angelini et al., 2018). Also signaling events downstream of the primary signals (calcium and oxidative burst) might modulate the microtubule susceptor,
such as the type 2C phosphatases (involved in the release of OST1 from the membrane, see Fig. 2A, 3) that have been found to destabilise microtubules under drought (Bhaskara et al., 2017). A further candidate might be the plant specific SAMKs that are activated by microtubule elimination and calcium influx Sangwan et al., (2002).

What are the functional consequences of cold-signaling dependent modulations of microtubule stability or organisation? They would be twofold, depending on the functional subpopulation of cortical microtubules: Reduction of microtubule flexural rigidity (as brought about by MAP65-2) would prevent microtubular breakage under freezing stress, when the membrane is rapidly rigidifying, while bundling of microtubules is expected to improve force transmission towards the calcium channels as actual sensory structures. Disassembly of dynamic microtubules at the plasma membrane would support integration of sensory components into the plasma membrane from exocytotic vesicles (candidates might be RboH, but also the facilitator COLD1, or the calcium channels themselves). As a result, the efficiency of subsequent cold sensing would be increased in consequence of preceding cold sensing.

Open questions for future work. While cold hardening or cold acclimation have been known for several decades by now, they are still far from understood. One reason for this sobering situation might again be the co-existence of different phenomena that are difficult to be sorted without more stringent conceptual frameworks: When a plant is subjected to chilling stress, it will respond by adaptation, albeit this adaptation will not be fully developed, because the conditions represent only a mild stress. The fact that this plant will cope better with a subsequent stringent cold shock may therefore be the mere consequence of this partial adaptation. This type of cold acclimation is, therefore, acting on the level of downstream responses. The bundling and cold stabilisation of microtubules observed as response of prolonged chilling in some (but not in all) systems (Abdrakhmanova et al., 2003; Pihakaski-Maunsbach and Puhakainen, 1995) is to be positioned here. However, the microtubular function treated in this review, is of a different nature: it is directly linked with the sensitivity of the perceptive process. Cold acclimation renders the perception and transduction of subsequent cold stress more efficient – as exemplarily shown by the example of CBF4 in grapevine cells (Wang et al., 2019). While naive cells required 6 h of stringent cold stress to accumulation of CBF4 transcripts, cold-acclimated cells were able to do so already after half of the time, indicative of a roughly twofold sensitivity of the perceptive process.

The phenomenon that a mild stress will amplify the adaptive response to a subsequent stringent stress, is known as priming (Hilker et al., 2016). Unfortunately,
in recent years, the term priming has been often used inappropriately, due to confusion with partial adaptation caused by the pretreatment. To describe the cold induced changes of microtubule-dependent susceptibility, the term cold priming is appropriate, though. The working model developed in this review stimulates a couple of new questions: Will it become possible to visualise and/or discriminate the two functional subpopulations of microtubules (force-transmitting microtubules, exocytosis-regulating microtubules) in living cells? What is the molecular nature of the calcium channel that is the target of microtubule-dependent force transmission? How can the sensitivity of cold perception be conceptualised in a manner that allows to test the model quantitatively? What is the molecular nature of microtubular modification in response to cold priming? How is the functional diversification of microtubules controlled in space and time?

The advances in molecular methodology allow to address questions that seemed unreachable some years ago. However, molecules will answer questions only, if combined with precise questions. The purpose of this review was not to give answers, but to develop such questions.

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References


Figure Legends

Figure 1. Conceptual framework to discuss the role of microtubules in cold signalling. The logical matrix is ordered according to the dependence of perception on time, and on the nature of the stimulus. In chemical signalling (A, C), a ligand (L) is binding to a receptor (R), which will deploy signalling culminating in a response (in most cases changes of gene expression). In the time-independent model of chemical signalling (A), the perception remains constant and signalling is only dependent on the dose (product of ligand concentration and exposure time), following the Bunsen-Roscoe Law. In the time-dependent model of chemical signalling (C), the abundance or the affinity of the receptor is modulated by a feedback from signalling (sensory adaptation). This feedback can be negative or positive. The activity of signalling is therefore dependent on the history of preceding signalling (following the Weber-Fechner Law). This may coexist with a feedforward from signalling upon the response (habituation), which is not followed further in this review. The two models for physical stimuli (B, D) follow the same scheme with the only difference that the ligand is replaced by a susceptor (S), which transforms the physical input into an activation of the receptor (which can be a ion channel).

Figure 2. Simplified model of cold signalling (A) and cold habituation (B) sorted by timing. (A) The cold susceptor system includes PM rigidity, MTs and calcium channels (①), which in concert with the facilitator COLD1 (②) would deploy activation of a Gα-protein (RGA), such that PLD would be triggered to produce PA, culminating in the stimulation of RboH, and apoplastic oxidative burst that would positively feed back to calcium influx. ③ abi2 is activated by hydrogen peroxide, causing the release the OST1 from PM to ④ phosphorylate and activate ICE1, such that CBFs are activated (⑤), which in turn will activate COR genes (⑥) to induce cold adaptation. (B) Habituation of cold signalling initiates with activation of CDPK by Ca²⁺/CaM (①), inducing the activity of MPK3/6 (②), such that ICE1 is phosphorylated and degraded. In parallel, CRLK1 is activated by Ca²⁺/CaM as well (②) followed by activation of MPK4 and negative regulation of the MPK3/6 pathway. As third mechanism of habituation, CRPK1 can phosphorylate the 14-3-3 protein (②), leading to nuclear import of the 14-3-3 protein and interaction with CBF3 that in consequence will be degraded by proteolysis (⑤). Under prolonged cold stress, the expression of EGR2 is stimulated, which is not followed by an equal response of myristolation, such that the membrane association of OST1 with the plasma membrane is down-modulated (⑥). Abbreviations: PM: plasma membrane, MTs : microtubules, Ca²⁺/CaM : calcium/ calmodulin, COLD1: Chilling Tolerance Divergence 1, Gα: α-subunit of trimeric G-protein, PLD: Phospholipase D, PA : phosphatidic acid, RboH: Respiratory burst oxidase Homologue, O₂: Oxygen.
Figure 3. Working model on the role of microtubules for cold acclimation. Microtubules as cold susceptors focus and transmit compression forces originating from membrane rigidification (①). This will deploy cold signaling through mechanosensitive calcium channels associated with the signal-amplification loop detailed in Figure 2A (②). As consequence of cold signaling, the cold-suscepting function of stable microtubules will be increased leading to more efficient susception (③). In parallel, molecular components of cold signaling and signal amplification will be synthetized and transported to the plasma membrane by vesicle flow leading to more efficient perception and amplification of the cold signal. The integration of these vesicles into the membrane is controlled by a dynamic population of microtubules (④). This model ascribes different (and antagonistic) roles to dynamically different subpopulations of microtubules.
Figure 1

(A) Chemical stimulus

(time independent)

Chemical stimulus → Signal transduction → Response

(B) Physical stimulus

(time independent)

Physical stimulus → Signal transduction → Response

(C) Chemical stimulus

(time dependent)

Chemical stimulus → Signal transduction → Response

sensory adaptation

(d) Physical stimulus

(time dependent)

Physical stimulus → Signal transduction → Response

sensory adaptation

habituation

Figure 1
Figure 2