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DARWIN REVIEW

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

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Abstract

Cold stress is a major abiotic stress, restricting plant growth and development. Therefore, 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 mitogen-activated protein kinase (MAPK) cascades. In contrast, the early events of cold perception and cold adaption have received 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 provide a more complete understanding of cold adaptation.

Keywords: Calcium, cold sensitivity, gene expression, membrane, microtubules, sensory adaptation.

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 led to precocious development, even short cold episodes can have devastating consequences. For instance, 3 d of frost in April 2017, following a very mild March, almost annihilated the German apple harvest to a value of <500 000 t, 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 was 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 removed from any ice-induced membrane disruption. On the other hand, 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 already put forward in 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, aroused considerable interest, because it meant that it should become possible to improve cold tolerance by genetic approaches (for a review, see Guy, 1990). Transcription factor cascades in particular have been studied intensively in this context (reviewed in Thomashow, 1999, 2010; see also the most recent comprehensive review by Ding *et al.*, 2019*b*), because they regulate the expression of cold-responsive (COR) genes that encode enzymes for detoxification of reactive oxygen species (ROS), 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 made up of the Inducer of CBF expression 1 (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 the control of several signalling pathways. As will be described 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, has 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 recognized 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 to cold tolerance (Rikin *et al.*, 1980; Jian *et al.*, 1989). 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 has even 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 contradictory results. In some studies, cold hardiness was linked with cold stability of microtubules; in others, stabilization 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 can have 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 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 a result of the signalling. In each of these two set-ups, two variations have to be considered, depending on whether the trigger is of a chemical or 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).

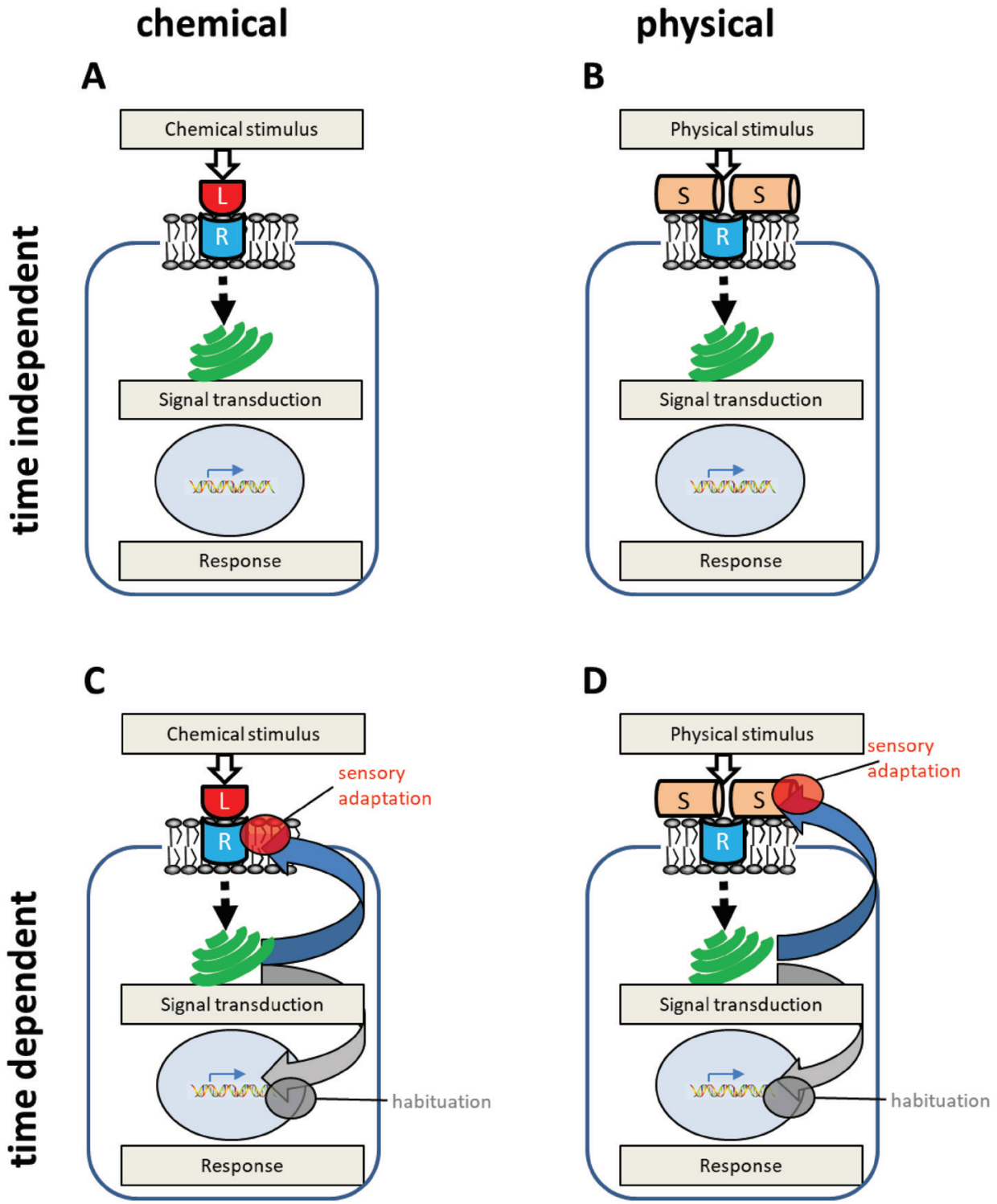

Fig. 1. Conceptual framework to discuss the role of microtubules in cold signalling. The logical matrix is ordered according to the dependence of perception on time, and on the nature of the stimulus. In chemical signalling (A, C), a ligand (L) is binding to a receptor (R), which will deploy signalling, 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 co-exist 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).

Model 2: perception by susceptor–receptor interaction

The ligand–receptor model can be used as a first approximation to describe the response to chemical signals. However, some of the most relevant signals for a plant are of a physical nature. This includes gravity and mechanical stimulation, but also heat and cold. Since signal transduction is usually of a chemical nature (examples would be calcium, phosporylation 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 a 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 with ligand-induced sensing, the perceptive structure is bipartite (susceptor and the actual sensor). Nevertheless, this perception will otherwise fulfil 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 a 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 for the ligand, or their ability to deploy a signal, are down-modulated in response to ligand binding (Fig. 1C). This phenomenon is termed sensory adaptation and prevents the continuous presence of a potential stimulus leading to a continuous cellular response (which would be not only meaningless but, in the case of stress signalling, even deleterious). The response amplitude is usually not dependent on the absolute magnitude of the stimulus, but on its relative change, a rule that is known as the 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 to the way in which 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, for both 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 (Li *et al.*, 2017; Liu *et al.*, 2017; Zhang *et al.*, 2017; Zhao *et al.*, 2017; Ding *et al.*, 2018; Liu *et al.*, 2018, 2019). The most recent and comprehensive review of these findings is given in Ding *et al.* (2019*b*). 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 fall rather into the

realm of habituation. Interestingly, microtubules, while mostly being acknowledged as upstream factors 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.* (2019*b*) uses 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 susception 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 in membrane fluidity should culminate in a mechanic force that can be used as perceptive event is rarely asked, but is far from trivial to understand. The fluid-mosaic model of 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 the 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 susceptive structure, together with heterogeneous rigidification of the membrane (Fig. 2A, ①).

The third element of the plant cold susceptor seem to be plasma membrane-located calcium channels (Fig. 2A, ①).

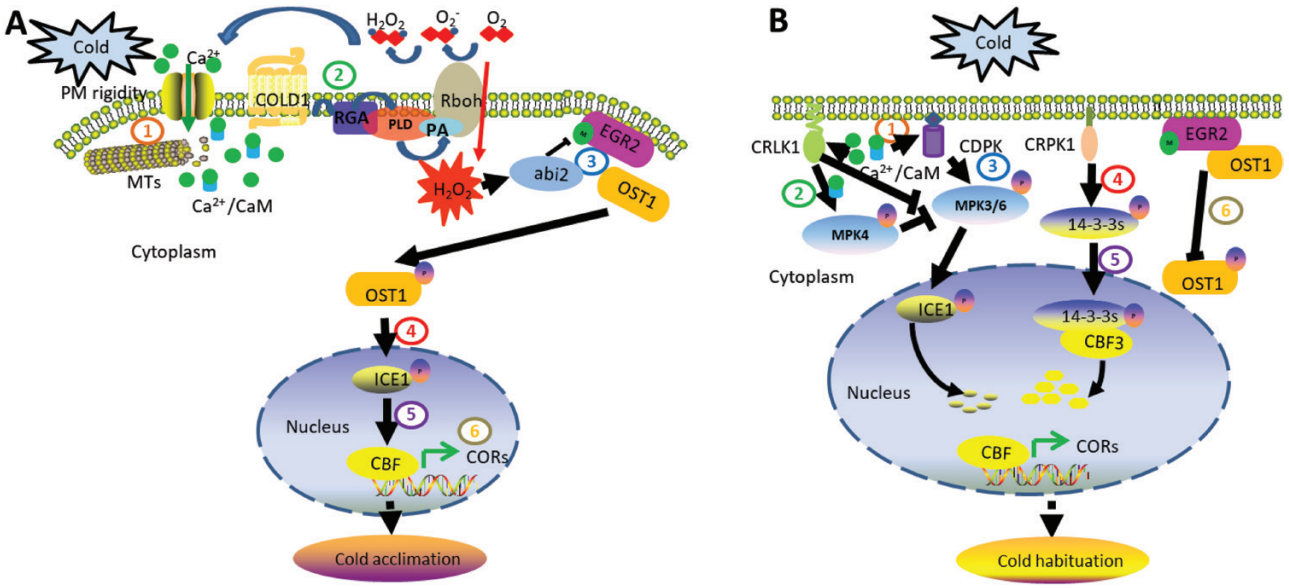

Fig. 2. Simplified model of cold signalling (A) and cold habituation (B) sorted by timing. (A) The cold susceptor system includes PM rigidity, MTs, and calcium channels (①), which in concert with the facilitator COLD1 (①) would deploy activation of a Gα protein (RGA), such that PLD would be triggered to produce phosphatidic acid, 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 of OST1 from the 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 Ca^{2+}/CaM (①), inducing the activity of MPK3/6 (③), such that ICE1 is phosphorylated and degraded. In parallel, CRLK1 is activated by Ca^{2+}/CaM as well (②), followed by activation of MPK4 and negative regulation of the MPK3/6 pathway. As a third mechanism of habituation, CRPK1 can phosphorylate the 14-3-3 protein (④), 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 myristoylation, such that the membrane association of OST1 with the plasma membrane is down-modulated (⑥). Abbreviations: PM: plasma membrane, MTs, microtubules; Ca^{2+}/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, O_2^-, superoxide anion; H_2O_2. hydrogen peroxide; OST1, Open Stomata 1; ICE1, Inducer of CBF expression 1; CBF, Cold Box Factor; COR, cold response genes; CDPK, calcium-dependent protein kinase; CRLK1, calcium/calmodulin-regulated receptor like kinase 1; CRPK1, cold response protein kinase 1; EGR2, clade-E growth-regulating 2.

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 present given the huge number of candidates: among the five known families of Ca^{2+}-permeable channels in Arabidopsis (reviewed in Kudla *et al.*, 2018), only one family belongs 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.*, 2014) 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 in its own right is still debated. The authors have tested this by expressing COLD1 in *Xenopus* oocytes to follow the resulting currents after cold treatment. They saw 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 (PLC; 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 in which COLD1 acts as a facilitator of calcium channels, but does not need to be a channel itself. Activation of Gα triggers phospholipase D (PLD) activity (Munnik *et al.*, 1995), and, in fact, the accumulation of phosphatidic acids (partially from PLD, partially from the concerted activity of diacylglycerol kinase and PLC, which is also activated by G-proteins) is part of 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 millimolar 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 PLD, 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 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 can be concluded from scavenging by exogenous catalase (Chang *et al.*, 2011); and (iii) increased levels of phosphatidic acid. As detailed 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 ICE1 have to increase to activate a transcriptional cascade. This master switch is constitutively synthesized, but also continuously degraded in the proteasome, such that, in the 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 expected, more than one signal travels this path from the membrane to the nucleus, whereby the discussion has focused on two mitogen-activated protein kinase (MAPK) cascades: the one cascade culminating in activation of MPK6 from ~5 min and MPK3 from ~15 min after the onset of cold stress (Zhao *et al.*, 2017) will lead to phosphorylation of ICE1 at three sites (Ser94, Thr366, and Ser403), recruiting this master switch to the proteasome. The other cascade, culminating in activation of MPK4 from ~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 therefore cannot be this first step. The only candidate for the primary positive signal which we could locate from screening the literature of the last decade is the kinase Open Stomata 1 (OST1). This kinase can phosphorylate ICE1 at Ser278 (i.e. at a site different from those targeted by MPK3 and MPK6), and this modification prevents ICE1 being 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 an 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 a 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 CBFs and activate their transcription (Chinnusamy *et al*., 2003), a process that can be detected from ~15 min after the onset of cold stress (Thomashow, 2010; Fig. 2A, ⑤). The CBFs, in turn, will activate downstream COR genes, a process initiating in the range of 2 h and leading to a significant modulation in the steady-state transcript levels of ~1000 genes that are up-regulated, while another 1000 genes are down-regulated (Fig. 2A, ⑥).

Habituation of cold signalling

The constitutive activation of cold stress signalling would involve 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 an early step of primary cold signalling can be read by a plethora of binding proteins that allow for ample crosstalk between signal chains and certainly also for further amplification (for recent reviews, see Guo *et al*., 2018; Kudla *et al*., 2018). Examples are calmodulin (CaM), CaM-like proteins, calcium-dependent protein kinases (CDPKs), calcineurin B-like proteins (CBLs), and 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/CaM (Fig. 2B, ①). This would then lead to MAPK signalling activating the activity of MPK3 and/or MPK6 (Fig. 2B, ③), which phosphorylate ICE1 in the destabilizing sites (Ser94, Thr366, and 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/CaM: the calcium/calmodulin-regulated receptor-like kinase 1 (CRLK1), which is located at the inner side of the plasma membrane (tethered by an N-terminal transmembrane domain), and endowed with two calmodulin-binding domains (Yang *et al*., 2010*a*, *b*), interacts with MEKK1 (Fig. 2B, ②), which results in stimulation of MPK4 (an antagonist of MPK3/6 signalling), leading to the stabilization of ICE1. CRLK1 also down-regulates 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 with 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 cold response protein kinase 1 (CRPK1) is localized at the inner face of the plasma membrane and can phosphorylate 14-3-3 proteins (Liu *et al*., 2017; Fig. 2B, ④). These interact physically with CBF3 (Fig. 2B, ⑤) 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 takes place in the range of 1–3 h, 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*., 2019*a*; Fig. 2B, ⑥). The expression of EGR2 becomes detectable from 3 h and will therefore repress OST1 signalling in the long term. This repression of OST1 activity is independent of ABA (i.e. also independent of abi2). With prolonged cold treatment, the induction of EGR2 is considerably increased, which will lead to increasing levels of unmyristoylated product, loosening the tethering of OST1. Thus, the initial down-modulation 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 (e.g. 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*., 2019*a*).

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 green fluorescent protein (GFP) fusions of plant tubulin opens up 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 fluidization through benzyl alcohol was able to suppress the microtubule response to cold. We could further 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 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 stabilizing 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 various 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 a 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. In congruence 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 which proposed that the cytoskeleton in concert with 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 (Kerr and Carter, 1990; Bartolo and Carter, 1991; Abdrakhamanova *et al.*, 2003), 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 at 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 for 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 pre-treated 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 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 a marker for cold acclimation, the expression of 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 coignetiae* 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 with cold shock at 0°C. Using this molecular readout, the role of calcium influx and microtubules was assessed by their 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 and 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: (i) the expression of *CBF4* was tightly linked with calcium influx; (ii) the expression of *CBF4* was well correlated with cold hardiness; (iii) the expression of *CBF4* was independent of microtubules; (iv) transient elimination of microtubules induced cold hardening; (v) stabilization of microtubules by taxol induced cold hardening; and (vi) treatments that induced cold hardening did not induce stability of microtubules

The task of the following will be to integrate these apparently discrepant and partially even paradoxical findings into the conceptual framework (Fig. 1). As already demonstrated for the simplified model of cold signalling and habituation (Fig. 2), it is worth considering 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 were true, disassembly of microtubules by factors other than 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 stabilizer taxol and a transient elimination of microtubules by pronamide 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. (i) The signalling in response to deleterious cold stress (in the case of grapevine cells, a cold shock at 0 °C) should be less efficient compared with the signalling in response to mild cold stress (in the 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 >10-fold within 3 h after mild cold stress (8 °C), twice the treatment time was needed to induce a comparable induction under deleterious cold stress (0 °C). (ii) Stabilization of microtubules (taxol treatment) or mild and transient elimination of very dynamic microtubules (pronamide treatment) can increase the sensitivity of cold perception, such that a suboptimal signal (0 °C) can also 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 could improve cold hardiness with much shorter time scales (>1 h). Thus, microtubules are probably modulated as a 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 above, 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 quarter 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's modulus is comparable with that of glass (Gittes *et al.*, 1993)—are good candidates for such a cold susceptor (Fig. 3, ①). Stabilization of these microtubules as a long-term response to chilling should therefore improve the focusing of the minute forces originating from local drops of membrane fluidity, and thus render cold sensing more efficient (Fig. 3, 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 stabilization of microtubules by taxol.

However, it should be kept in mind that the susceptor in cold sensing not only comprises microtubules, but also the membrane as a 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, ⑥) to the membrane is down-modulated upon prolonged cold treatment, because myristoylation of the tether EGR2 phosphatase decreases, such that OST1 is more readily

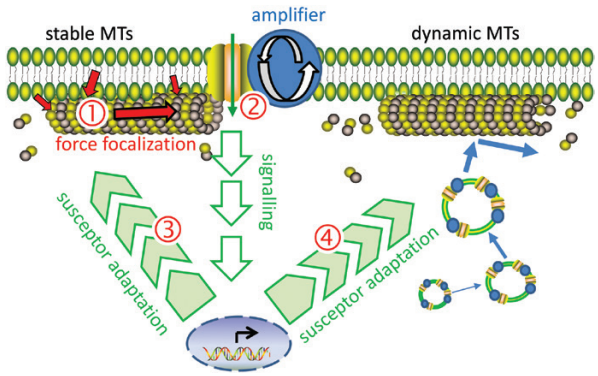

Fig. 3. Working model of the role of microtubules for cold acclimation. Microtubules as cold susceptors focus and transmit compression forces originating from membrane rigidification (①). This will deploy cold signalling through mechanosensitive calcium channels associated with the signal amplification loop detailed in Fig. 2A (②). As a consequence of cold signalling, the cold susceptor function of stable microtubules will be increased, leading to more efficient susception (③). In parallel, molecular components of cold signalling and signal amplification will be synthesized 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.

deployed in response to additional cold stress (integration of EGR2 phosphatase). Thus, the amplitude of cold signalling would increase after prolonged cold stress. However, this would not be sensory adaptation, but would represent a case of cold habituation (*sensu* Galland, 1991), since it is acting on the level of signal transduction, not on the level of perception.

Nevertheless, there also exists a true sensory adaptation as a 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 RbohH (Fig. 2A, ②)—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), namely a subpopulation differing from the stable microtubules that convey membrane tensions upon the calcium channel (Fig. 3, ④). 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 in respect to cold acclimation—a phenomenon that otherwise would remain a paradox.

It should be stated clearly and explicitly that this new model overturns 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, 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 of where signalling begins and where 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 is dealing 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 a perceptive step. Calcium influx, amplified by the self-amplifying 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 and membrane composition), perception (calcium influx channels), and signal amplification (COLD1, PLD, and RbohH) occur in parallel to habituation (i.e. as part of the signal response). It should be terminologically separated from mere cold adaptation (for instance as accumulation of sugars) as cold acclimation *sensu stricto*, with the argument that it will modulate future signalling. Cold acclimation would thus be a kind of 'memory' resulting from having successfully coped with a stress experience.

Towards a molecular and functional model of the microtubule cold susceptor

The microtubule susceptor model implies that microtubular dynamics and/or organization are modulated depending on the history of cold signalling. Cold-inducible microtubule-associated proteins would be prime candidates for such a feedback from signalling upon cold sensitivity of the microtubule susceptor. The microtubule cross-linker MAP65 might be interesting in this respect, because the isotype MAP65-2 was found to stabilize 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 would impact directly on force transmission in response to membrane rigidification and might be the microtubular correlate to the adaptive re-fluidization of the membrane by increasing the abundance of unsaturated fatty acids.

It should be kept in mind, however, that the feedback of cold signalling 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 a mechanism for ROS-triggered microtubule disassembly (Livanos *et al.*, 2014*a*, *b*), which would create a functional link between cold-induced activation of RbohH as an early event in cold signalling (Fig. 2A, ①) and modulation of the susceptive structure, microtubules. A second candidate would be PLD, which not only had originally been identified as a 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 signalling 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 destabilize 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 signalling-dependent modulations of microtubule stability or organization? They would be 2-fold, 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 as a consequence of preceding cold sensing.

Open questions for future work

While cold hardening or cold acclimation have been known for several decades, 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 stabilization of microtubules observed as a response to prolonged chilling in some (but not in all) systems (Pihakaski-Maunsbach and Puhakainen, 1995; Abdrakhamanova *et al.*, 2003) 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 shown by the example of *CBF4* in grapevine cells (Wang *et al.*, 2019). While naïve cells required 6 h of stringent cold stress for accumulation of *CBF4* transcripts, cold-acclimated cells were able to do so already after half that time, indicative of an ~2-fold increased sensitivity of the perceptive process.

The phenomenon whereby 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 often been used inappropriately, due to confusion with partial adaptation caused by the pre-treatment. To describe the cold-induced changes of microtubule-dependent susception, the term cold priming is, however, appropriate. The working model developed in this review stimulates a couple of new questions. Will it become possible to visualize and/or discriminate between the two functional subpopulations of microtubules (force-transmitting microtubules and 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 conceptualized in a manner that allows testing of 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 us 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 rather to develop such questions.

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