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Cold sensing in grapevine—Which signals are upstream of the microtubular "thermometer"

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Abstract

Plants can acquire freezing tolerance in response to cold but non-freezing temperatures. To efficiently activate this cold acclimation, low temperature has to be sensed and processed swiftly, a process that is linked with a transient elimination of microtubules. Here, we address cold-induced microtubules elimination in a grapevine cell line stably expressing a green fluorescent protein fusion of *Arabidopsis* TuB6, which allows to follow their response *in vivo* and to quantify this response by quantitative image analysis. We use time-course studies with several specific pharmacological inhibitors and activators to dissect the signalling events acting upstream of microtubules elimination. We find that microtubules disappear within 30 min after the onset of cold stress. We provide evidence for roles of calcium influx, membrane rigidification, and activation of NAD(P)H oxidase as factors in signal susception and amplification. We further conclude that a G-protein in concert with a phospholipase D convey the signal towards microtubules, whereas calmodulin seems to be not involved. Moreover, activation of jasmonate pathway in response to cold is required for an efficient microtubule response. We summarize our findings in a working model on a complex signalling hub at the membrane-cytoskeleton interphase that assembles the susception, perception and early transduction of cold signals.

KEYWORDS

calcium, cold stress, grapevine (*Vitis rupestris*), microtubules, signalling

1 | INTRODUCTION

Plants as sessile organisms cannot run away when facing abiotic or biotic stresses. In order to avoid that their growth and development is damaged, they must adjust themselves, which requires a rapid response of several intracellular and intercellular signalling pathways that are often interconnected at the level of perception, transduction, and response.

Cold stress is a major constraint for many crop plants, and the adverse influence on growth and development is a significant factor that confines the spatial distribution of plant species (Repo, Mononen, Alvila, Pakkanen, & Hanninen, 2008). When a plant is exposed to suboptimal, low temperatures, many cellular functions are disturbed, leading to various changes in hormone levels, membrane lipid composition, surface lesions, discoloration, tissue breakdown, accelerated senescence, and imbalance of oxidative and ionic homeostasis (Lyons & Raison, 1970; Sharma, Sharma, & Deswal, 2005). Cold acclimation has evolved as a crucial factor that allows plants to extend their habitat. Various physiological and biochemical changes, such as the synthesis of sugars or cryoprotective proteins allow circumventing freezing damage (Janska, Marsik, Zelenkova, & Ovesna, 2010; Zhao, Chen, Zhang, & Zhang, 2009). To efficiently activate cold acclimation, low temperature has to be sensed and processed swiftly.

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

In this respect, the microtubular cytoskeleton may be important, because microtubules (MTs) disassemble in the cold and play an important role for signalling by sequestration and release of molecules involved in transduction. MTs can not only support the assembly of such factors into complexes, but also can provide their directional transport by guiding the polar movement of molecular motors (Etienne-Manneville, 2010; Gundersen & Cook, 1999).

A role of MTs for cold sensing in plants is supported by different observations: In winter wheat, a transient elimination of cortical MTs has been shown to be necessary and sufficient to initiate cold adaptation, which is accompanied by subsequent formation of stable MTs bundles (Abdrakhamanova, Wang, Khokhlova, & Nick, 2003). Due to their close relationship with the plasma membrane as major platform for signal perception and transduction, MTs and microfilaments have been suggested as downstream targets of various signalling pathways including cold stress (Heidarvand & Maali Amiri, 2010). MTs are extremely cold-sensitive in chilling sensitive species, whereas they persist at low temperatures in chilling-tolerant species (Jian, Sun, & Lin, 1989). In cotton, a chilling-sensitive species, anti-microtubular drugs significantly accelerate and enhance chilling injury, whereas a pretreatment with abscisic acid prevents chilling injury and counteracts the sensitizing effect of antimicrotubular drugs. Chilling injury is accompanied by a destruction of the microtubular network, and abscisic acid prevents this microtubular destruction (Rikin, Atsmon, & Gitler, 1980, 1983). Because MTs and actin filaments can modulate the opening of cold-responsive Ca^{2+}channels (Örvar, Sangwan, Omann, & Dhindsa, 2000; Thion et al., 1996), they apparently participate in the generation or transduction of a cold signal.

Calcium has been known for a long time (Marme, 1985) as central a second messenger, because it rapidly responds to different stimuli (for instance, Gilroy, Read, & Trewavas, 1990) and can transduce the signal to various Ca^{2+}-binding proteins, such as calmodulin, thus inducing a panel of different intracellular responses (reviewed in Bush, 1995). In fact, a transient influx of Ca^{2+} into the cytoplasm is among the earliest events triggered by a cold shock (Monroy & Dhindsa, 1995; Örvar et al., 2000). One of these events is a transient disassembly of cortical MTs (Abdrakhamanova et al., 2003). This disassembly has been ascribed to the activation of calmodulin by cold-triggered calcium influx, but the quality of the microtubular response to calmodulin seems to be qualitatively different, depending on the concentration of calcium (Fisher, Gilroy, & Cyr, 1996), which indicates that several and partially antagonistic pathways coexist. In addition to direct interaction of calmodulin with MTs, modulation of MT-associated proteins (MAP) by calmodulin known to occur in neural cells (Pirollet, Margolis, & Job, 1992) might be a further mechanism. Irrespective of these complexities that are far from understood, not only the microtubular response, but also, the modulation of Ca^{2+} is necessary to initiate cold acclimation as concluded from experiments, where calcium chelators and channel blockers were found to suppress cold adaptation in alfalfa and *Arabidopsis* (Knight, Trewavas, & Knight, 1996; Tahtiharju, Sangwan, Monroy, Dhindsa, & Borg, 1997). Pharmacological elimination of MTs or actin filaments was shown to amplify cold-induced Ca^{2+} influx (Mazars et al., 1997), suggesting that MTs act not only downstream of calcium, but might also fulfill a second function very early in cold signalling.

This second function of MTs might be linked with changes in the fluidity of the plasma membrane. With decreasing temperature, the membrane becomes more rigid due to the high melting point of saturated fatty acids (Wang, Li, Li, & Welti, 2006). It has been proposed that low temperature could be perceived by actually measuring these changes in membrane rigidity (Vigh et al., 2007; reviewed in Nishida & Murata, 1996). Two implications of this membrane rigidity model have been confirmed experimentally: Rigidification of the membrane by DMSO can mimic cold responses that could induce the expression of a cold-activated promoter in the absence of cold stress, and, conversely, cold responses are inhibited, when the membrane is rendered more fluid by chemicals such as benzyl alcohol (BA) (Sangwan, Foulds, Singh, & Dhindsa, 2001). Likewise, mutants affected in desaturases show enhanced cold responses such as cold activation of diacylglycerol kinase (Vaultier et al., 2006).

In addition to calcium influx, fluidity dependent modulation of NADPH oxidase might stimulate the production of reactive oxygen species (ROS) in the apoplast. Whereas ROS have been traditionally seen as by-products of disequilibrated electron-transfer reactions and symptoms or causes of cellular injury (Suzuki & Mittler, 2006), they have meanwhile emerged as central (and complex) signals, whose rapid accumulation and dynamic turnover orchestrates adaptive responses to stresses (Mittler et al., 2011). There is an evidence for the regulation of Ca^{2+} channels (reviewed in McAinsh & Pittman, 2009) and calcium activated MAPK cascades (reviewed in Colcombet & Hirt, 2008) by ROS. Similar to MTs, ROS could act both, upstream and downstream, because the activation of diacylglycerol kinase and/or phospholipase D (PLD) in response to decreased membrane fluidity will modulate the activity of NADPH oxidase. As further candidate component that might modulate the plant cold response, nitric oxide (NO) should be considered, because NO can reduce the abundance of activated oxygen species (Mittler et al., 2011).

The early players discussed above (membrane rigidification, calcium influx, apoplastic ROS, and MTs) must converge somehow to generate and adjust the signal(s) inducing the appropriate response. As central components of this signal processing, G-protein-dependent activation of PLD has been discussed (Munnik, 2001; Neer, 1995). In fact, phospholipase C and D are activated as early as 15 s after cold treatment (Ruelland, Cantrel, Gawer, Kader, & Zachowski, 2002) and generate, by hydrolysis of membrane phospholipids, phosphatidic acids (PtdOH) as membrane-located secondary messengers. PLD is associated with MTs and therefore might transduce alterations in the plasma membrane to the cytoskeleton (Gardiner et al., 2001).

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

to perception, and then further from perception to early signal transduction culminating in cold adaptation is central to identify genetic factors that promote cold hardiness. Using a transgenic grapevine cell line expressing a fluorescent tubulin marker (Guan, Buchholz, & Nick, 2015), we dissect the early events of cold signalling upstream of MTs. We show that calcium influx, membrane rigidification, and activation of NAD(P)H oxidase contribute to signalling and that a G protein in concert with PLD convey the signal towards MTs, whereas calmodulin seems to be not involved. However, we find that the cold-induced activation of the jasmonate pathway is required for an efficient MT response. These insights can be used in the future to design strategies targeted on improved cold tolerance, either by molecular-assisted breeding, or, alternatively to genetic changes, by chemical manipulation of early signalling events in order to improve cold tolerance of cultivars that are otherwise cold-sensitive in temperate climates.

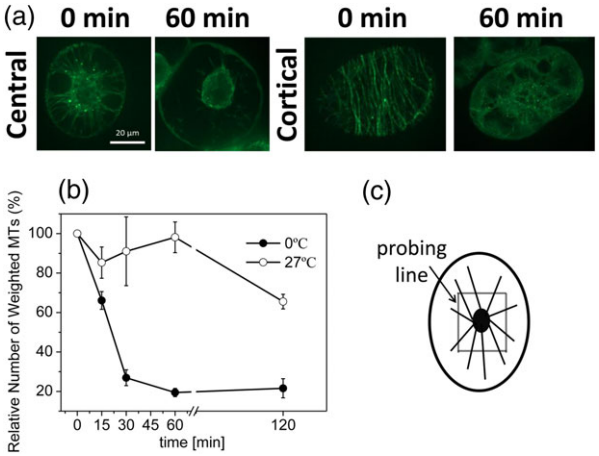

FIGURE 1 (a) Response of central and cortical microtubules (MTs) to cold stress (0 °C) shown by time-lapse series recorded in the MTs marker line *V. rupestris* GFP-TuB6. Zero minute shows the situation under normal temperature (27 °C); 60 min represents the situation after 60 min of incubation in ice water. Observations are representative of at least four independent experimental series with a population of 50 individual cells for each treatment. Bars, 20 µm. (b) Quantitative analysis of the cold response of central MTs. Relative number of weighted MTs relates to the value observed in the control at time 0 min. Each value of the relative number of weighted MTs represents the average of 50 individual cells. (c) Schematic set-up of the probing lines used to quantify microtubule integrity

2 | MATERIALS AND METHODS

2.1 | Plant material

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

2.2 | Cold treatment

For the experiments, the cells where either used in the exponential phase (3 days after inoculation), or in the stationary phase (5 days after inoculation). To administer cold stress (0 °C), either the entire Erlenmeyer flask with the suspension or aliquots of 2 ml in reaction tubes (Eppendorf, Hamburg) were placed in a bath of ice water and

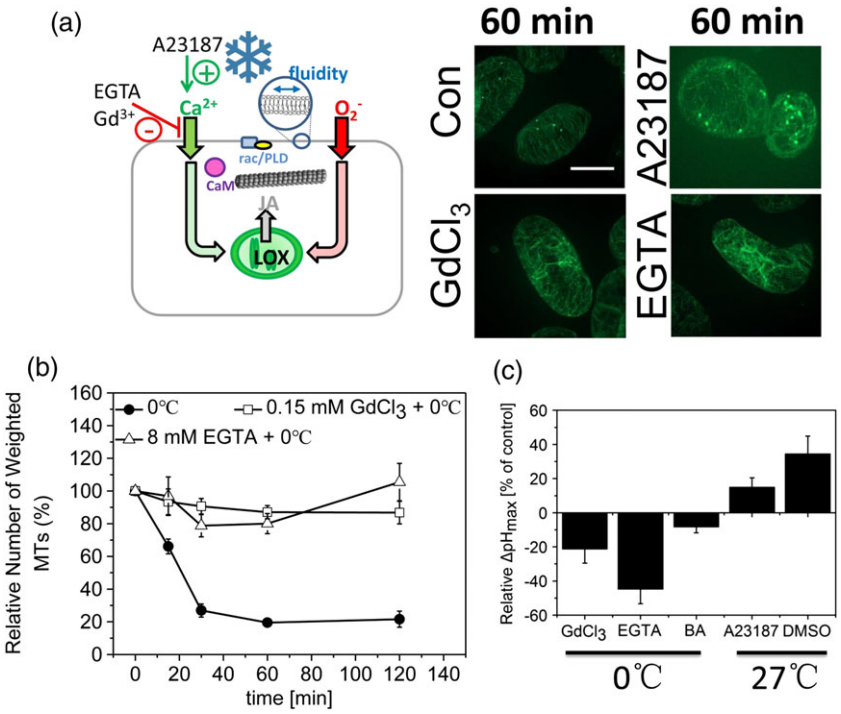

FIGURE 2 (a) Effect of $GdCl_3$, EGTA, and the calcium ionophore A23187 on the microtubular response to cold stress (0 °C) and normal temperature (27 °C) in the *V. rupestris* GFP-TuB6 cell line. Representative images for the response of cortical MTs after 60 min of treatment to 0.15-mM $GdCl_3$, 8-mM EGTA, and 50-µM A23187 at 27 °C are shown here. Observations are representative of at least three independent experimental series with a population of 30 individual cells for each treatment. Size bars, 20 µm. (b) Quantitative analysis of the central microtubules (MTs) response to cold stress (0 °C) in presence of $GdCl_3$ and EGTA. (c) Apoplastic alkalinization in response to cold stress (0 °C) in presence of $GdCl_3$, EGTA, and BA, as well as the response to A23187 and DMSO under normal temperature (27 °C). Data in (b) and (c) represent mean values and standard errors from three independent biological replicates

shaken in the dark on a horizontal shaker. The temperature cooling rate from 27 to 0 °C was 6.75 °C min^{-1} for cells treated at Day 3 after subcultivation and 4.70°C min^{-1} for cells treated at Day 5 after subcultivation. Samples of 3 days' cells for central MTs observation and 5 days' cells for cortical MTs observation or chemical treatment were collected at specified time points during the cold treatment.

2.3 | Pharmacological treatments

2.3.1 | Manipulation of Ca^{2+} influx at the plasma membrane

Ca^{2+} influx into tobacco protoplasts was enhanced by disruption of MTs and actin filaments (Mazars et al., 1997). Thus, the Ca^{2+} ionophore A23187 (Sigma-Aldrich, Germany), dissolved in DMSO, was used to promote Ca^{2+} in the absence of cold stress (at 27 °C), at different concentrations of 10 and 50-μM (Monroy & Dhindsa, 1995). In contrast, to prevent Ca^{2+} influx, either 8-mM EGTA (Roth, Germany) as calcium chelator, dissolved in H_2O, or 0.15-mM $GdCl_3$ (Sigma-Aldrich, Germany) as inhibitor of calcium-influx channels were used. The concentration of EGTA and $GdCl_3$ was based on the results of previous studies in grapevine cells (Liu, Ji, et al., 2013). For the A23187 treatment, the cells were observed by microscopy directly after addition of the agent to the cells at 27 °C. As negative control, cells were treated with the same concentration of the solvent DMSO. For the $GdCl_3$ experiment, the cells were first treated for half an hour with the respective compound at 27 °C before transfer of the cells to 0 °C.

2.3.2 | Manipulation of membrane fluidity

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

2.3.3 | Manipulation of ROS and NO

Diphenyleneiodonium (DPI) inhibits the production of ROS by specifically binding to flavin-binding enzymes, particularly the NAD(P)H oxidase (Li & Trush, 1998). Sodium nitroprusside (SNP) can be used as donor for the important stress-signalling molecule nitric oxide (Delledonne, Xia, Dixon, & Lamb, 1998). The suspension cells were treated with different concentrations of DPI (Sigma-Aldrich, Germany), diluted from a 100-μM stock solution in DMSO, for 2 min at 27 °C, before transfer to 0 °C. As negative control, cells were treated with the same concentration of the solvent DMSO. Aliquots collected from the cold-treated population at specific time points were directly analyzed by microscopy. For the SPN treatment, the suspension cells were treated with 1-mM SNP (Sigma-Aldrich, Germany), dissolved in water at 27 °C for 1 hr and then observed by microscopy. The concentrations of these treatments were derived from preparatory experiments with respect to the absence of toxic symptoms.

2.3.4 | Manipulation of calmodulin

Calmodulin is a Ca^{2+} binding protein that has been implicated in cold-triggered MT disassembly (Fisher & Cyr, 1993). Trifluoperazine (TFP) is a widely used antagonist of calmodulin (Feldkamp, O'Donnell, Yu, & Shea, 2010). Thus, the suspension cells were treated with 100 μM TFP (Sigma-Aldrich, Germany), dissolved in water, for 2 hr at 27 °C

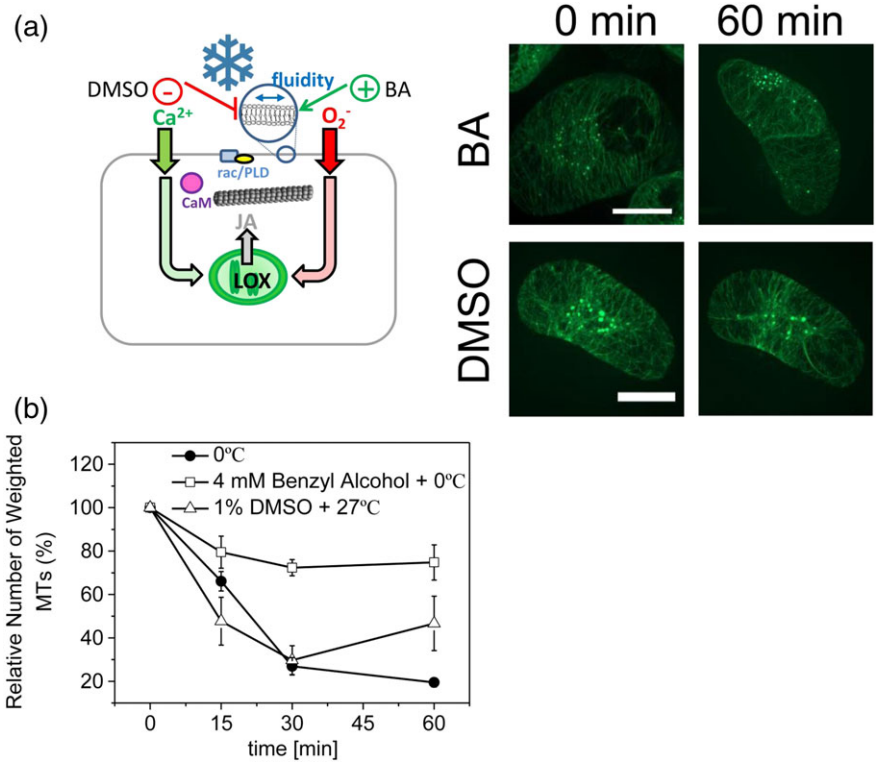

FIGURE 3 (a) Effect of benzyl alcohol (BA) and DMSO on the microtubular response to cold stress (0 °C) and normal temperature (27 °C). The response of cortical microtubules on 60 min to 4-mM BA and to 1% DMSO are shown. Observations are representative of at least three independent experimental series with a population of 30 individual cells for each treatment. Size bars, 20 μm. (b) Quantitative analysis of the response of central microtubules to BA and DMSO under cold stress (0 °C) and normal temperature (27 °C)

and then transferred to 0 °C and observed by microscopy directly after sampling at specific time points.

2.3.5 | Manipulation of the jasmonate pathway

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

2.3.6 | Manipulation of PLD

PLD activity can be diverted by replacing water as acceptor of the transphophatidylation by small primary alcohols such as *n*-butanol. As a consequence, instead of the active signalling compound phosphatidic acid (PA) inactive phosphatidyl alcohols are generated (Munnik, Arisz, deVrije, & Musgrave, 1995). Secondary butanol can be used as a negative control. Cells were therefore treated with 1% (*v*/v) *n*-butanol and *sec*-butanol (Roth, Germany), respectively, either under cold stress (0 °C), or at room temperature (27 °C). Furthermore, cells were also treated with 0.1% and 1% (v/v) *tert*-butanol (Sigma-Aldrich, Germany) for half an hour, then treated under cold stress (0 °C) for 1 hr.

2.3.7 | Manipulation of G proteins

PLD is activated by a trimeric G protein. Therefore, pertussis toxin (Sigma-Aldrich, Germany), a blocker for trimeric G proteins (Warpeha, Hamm, Rasenick, & Kaufman, 1991), was used. Cells were pretreated for 30 min with 10 ng ml^{-1} of pertussis toxin at 27 °C, then transferred to 0 °C and observed by microscopy directly after sampling at specific time intervals. The G-protein activator AlF_4^- (Bigay, Deterre, Pfister, & Chabre, 1985) was freshly prepared as described in Campanoni and Nick (2005), and then added directly to the suspension cells at 27 °C, directly following the cellular response by microscopy at 27 °C.

2.4 | Superoxide assay

The suspension cells were treated with 1 μM DPI (Sigma-Aldrich, Germany), diluted from a stock solution in DMSO (100 μM), for 30 min at 27 °C, before transferring them to 0 °C. As negative control, cells were treated with the same concentration of the solvent DMSO (1% v/v). The cells were collected at different time points to do the measurement of O_2^-.

To detect apoplastic O^{2-}, the nitroblue tetrazolium method was employed (Vacca et al., 2004) harvesting 1 ml of cell suspension. Cells were collected by centrifugation (10,000 g, 5 min, 25°C), and O_2^- concentration was measured in the supernatant by monitoring the reduction of nitroblue tetrazolium (100 μM) at 530 nm. The amount of O_2^- was then calculated using an absorption coefficient of 12.8 mM^{-1} cm^{-1} (Murphy, Vu, & Nguyen, 1998). The increment of superoxide at 10 and 30 min were plotted relative to the value in the 0 min (after 30 min DPI treatment).

2.5 | MDA assay

Malone dialdehyde (MDA), a product of lipid peroxidation, can be used as readout for oxidative damage using a colorimetric assay based on conversion of 2-thiobarbituric acid (TBA) according to Hodgson and Raison (1991) with minor changes in sample preparation as follows: Cells were collected in aliquots of 1 ml and spun down for 10 min at 10.000 *g* to remove the supernatant and record fresh weight of the cells. Immediately after recording fresh weight, cells were shock frozen in liquid nitrogen, and homogenized with steel beads (Tissue Lyser, Qiagen/Retsch, Germany) in a standardized manner (twice 30 s at 25 Hz). Each sample was complemented with 1 ml of 10-mM sodium phosphate buffer (pH 7.4), homogenized, and briefly spun down. 200 μl of the supernatant were added to a reaction mixture containing 100 μl of 8.1% (*w*/*v*) sodium dodecyl sulphate, 750 μl of 20% (*w*/*v*) acetic acid (pH 3.5), 750 μl of 0.8% (*w*/*v*) aqueous TBA, and 200 μl of Milli-Q water. An identical reaction mixture, where the supernatant from the sample was replaced by an equal volume of buffer, was used as blank. Both reaction mixtures were then incubated at 98 °C for 1 hr. After cooling to room temperature, the mixtures were centrifuged for 5 min, and absorbance measured at 535 nm (specific signal) and 600 nm (background). Lipid peroxidation could then be calculated as μM MDA from A535 to A600 using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.6 | Measurement of extracellular alkalinization

Extracellular alkalinization was measured by combining a pH meter (pH 12, Schott Handylab) with a pH electrode (LoT 403-M8-S7/120, Mettler Toledo). The suspension cells were pre-equilibrated on an horizontal shaker for around 60 min and then treated with either 0.15 $GdCl_3$, 8 EGTA, or 4-mM BA, respectively, while incubating the sample in ice water (0 °C). In contrast, the pH responses to 10 μM A23187 or 1% (*v*/*v*) DMSO were measured at 27 °C. All samples were kept in the dark on a horizontal shaker. Alkalinization was followed over 1 hr, and peak values (corrected for the basal value established during pre-equilibration) reached at around 35 min after the onset of treatment were plotted relative to the value in the control (treatment with 0 °C without addition of any compounds). In addition to this ice water control without any chemical treatment, a second control kept at room temperature (27 °C) was included—in this second control, no fluctuation of apoplastic pH was observed.

2.7 | Microscopy and image processing

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

were captured and processed using the ZEN software (Zeiss, Oberkochen) to generate orthogonal projections from the recorded stacks and to export them in TIFF format.

2.8 | Quantification of microtubule integrity

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

$$f(x_i) = x_i^{'}/\mathrm{ABS}\left(x_i^{'}\right)$$

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

3 | RESULTS

3.1 | Central and cortical microtubules disassemble under cold stress

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

For radial MTs, already after 15 min of cold treatment (0 °C), the number of MTs decreased significantly, although most cells still retained a small number of MTs. After 30 min, most MTs had disassembled, and only small remnants of MTs were seen. When the cold treatment exceeded 60 min, most of the MTs disappeared (Figure 1a). One hour was sufficient for a disassembly of transvacuolar MTs. We therefore used a cold treatment over 2 hr to ensure that central MTs were completely eliminated. The response of cortical MTs was qualitatively similar-largely depolymerized within 1 hr under cold stress (0 °C). However, in contrast to radial MTs, the cortical MTs became disordered during partial depolymerization over the 30 min (Figure S1).

In order to statistically verify the microtubular response to cold stress (0 °C), density and integrity of MTs were quantified by a parameter termed "relative number of weighted MTs" (for details, see Section 2), which allowed to measure the responses of radial MTs over time (Figure 1b). Without cold treatment, this parameter remained stable over the first 60 min of observation and did not exhibit any significant changes. Only during the second hour, it would decrease slightly by around 30%. This indicated that potential effects of photo bleaching on the green fluorescent protein (GFP) labelled MTs were negligible. Moreover, from this time course, it could be concluded that observation of a given cell over 1 hr should yield reliable and stable results. In contrast to the stable behavior for the relative number of weighted MT under control conditions, this parameter dropped rapidly under cold stress to approximately 20% of the initial value within 30 min and then remained stable at a very low level over the remaining 90 min of the observation period. In preparatory experiments, the graphs generated from plotting "number of weighted MTs" were found to match closely the situation seen in the confocal images (Figure 1a).

3.2 | Calcium influx into the cytoplasm is necessary and sufficient to trigger cold-induced depolymerization of microtubules

Calcium as an important secondary messenger for the signalling of different stresses has also been proposed to be responsible for MT disassembly. In order to verify this hypothesis, we either blocked calcium influx by 150-μM $GdCl_3$ or removed calcium ions from the medium by chelation with 8-mM EGTA for 30 min before following the response of MTs to cold stress (Figure 2a). We observed that both treatments mostly suppressed the elimination of MTs under cold stress (Figure 2a). This means that calcium influx from the exterior was necessary for cold-induced MT disassembly. To test, whether calcium influx would also be sufficient for MTs disassembly, the cells were treated with 10 and 50-μM of the calcium ionophore A23187 at 27 °C. In response to 10 μM most of the central and cortical MTs persisted, however, when the concentration was raised to 50 μM, the cortical MTs partially depolymerized in 1 hr treatment (Figures 2b and S2) suggesting that calcium influx is sufficient to activate MTs disassembly. Furthermore, to probe for a potential role of calmodulin, we used 100 μM of TFP, but this treatment did not inhibit the disassembly of central and cortical MTs under cold stress (Figure S3).

The activity of calcium influx channels in the membrane is linked with the cotransport of protons. The resulting extracellular

alkalinization can therefore be used to conveniently monitor the activity of calcium influx, a strategy that has been widely used to follow plant defence (Felix, Regenass, & Boller, 1993). The correlation of extracellular alkalinization with influx of calcium has also been shown for the salinity response of the *V. rupestris* cell strain measuring calcium by atomic ion spectroscopy (Ismail et al., 2014). We compared this pH shift for cold treatment alone with that produced after pre-treatment with 150-μM $GdCl_3$ or 8-mM EGTA, respectively. The alkalinization proceeded after a few minutes and reached a peak at 35 min after start of the cold treatment. These peak values were used as readout for the amplitude of the pH response. Both chelation of calcium by EGTA and inhibition of channel activity by $GdCl_3$ effectively inhibited alkalinization, whereas A23187 promoted alkalinization slightly (Figure 2c). The early activation of alkalinization, along with the fact that alkalinization was inhibited by EGTA and $GdCl_3$ in the same way as the cold response of central and cortical MTs was inhibited, lends further support to a role of calcium influx as upstream signal in cold-induced MTs disassembly.

3.3 | Membrane rigidification is necessary and sufficient to trigger microtubule disassembly

Membrane fluidity is reduced in low temperature, and this change has been discussed as a primary event of cold perception. Therefore, BA was used to inhibit membrane rigidification under cold stress, and DMSO to increase membrane rigidification at 27 °C, to investigate the response of MTs. In presence of 4-mM BA, central and cortical MTs appeared normal, and cell proliferation was not affected. However, cold-induced disassembly was suppressed mostly (Figure 3b). In contrast, treatment with 1% DMSO at 27 °C eliminated central MTs within 30 min (Figures 3b and S4). However, cortical MTs although getting disordered, disappeared only partially (Figure S4). This difference is also evident, when the geometrical projection of the z-stack is considered (Figure S4)—although initially, the entire cell is brightly labelled, after 30 min, only the cortical MTs have persisted. These data suggest that membrane rigidification was necessary for cold-induced disassembly of MTs. Further, rigidification induced by DMSO was able to mimic cold stress with respect to MT disassembly.

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

3.4 | NADPH oxidase activity is required for cold induced microtubule disassembly

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

In presence of DPI, cells incubated at 0 °C kept MTs intact over the entire observation time of 1 hr (for longer treatment, DPI caused progressive mortality) no matter whether central or cortical MTs were observed (Figures 4a and S5B). This was in marked contrast to cold treatment in the absence of the inhibitor, where the weighted MTs number decreased rapidly (Figure 4b), demonstrating that inhibition of NADPH oxidase activity effectively prevented cold-induced disassembly of MTs.

As mentioned above, the presumed inhibition of the activity of NADPH oxidase by DPI could block the cold-induced disassembly of MTs. To verify whether this cold treatment could, in fact, induce the

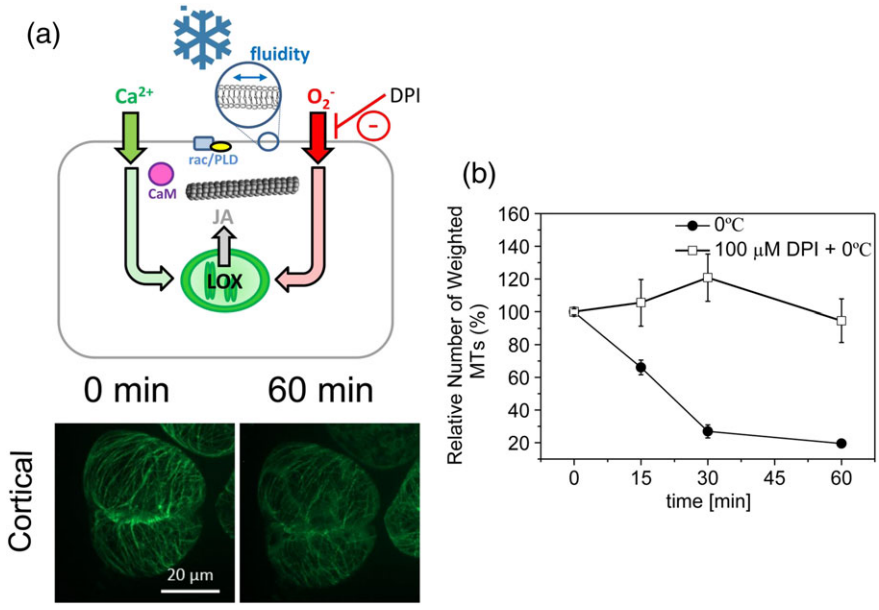

FIGURE 4 (a) Response of microtubules (MTs) to cold stress (0 °C) to in presence of 100-μM diphenyliodonium (DPI) at different time points after the onset of cold stress. Observations are representative of at least three independent experimental series with a population of 30 individual cells for each treatment. Size bars, 20 μm. (b) Quantitative analysis of the response of central MTs in presence of 100 μM DPI under cold stress (0 °C)

synthesis of superoxide (O_2^-) and that the effect of DPI pretreatment was linked with an inhibition of this superoxide induction, we measured superoxide using nitroblue tetrazolium. As shown in Figure S5C, without DPI treatment, the concentration increased nearly by 0.4 μM over the resting level seen at time 0, within the first 10 min of cold treatment (0 °C) and then declined to the initial level within 30 min after the onset of the treatment. This transient induction of superoxide was almost completely abolished after pretreatment with DPI consistent with a model of cold-induced NADPH oxidase activity.

Membrane lipids are primary targets of ROS, generating oxidized reaction products of fatty acids including MDA. The level of MDA can therefore be used as marker to monitor lipid peroxidation. However, over 2 hr of cold treatment, we could not observe any significant changes of MDA concentration (Figure S5A). Because DPI has been discussed to inhibit also the activity of nitric oxide synthase, we were also interested to see, whether nitric oxide might be able to induce MT disassembly. We therefore used the NO donor sodium nitroprusside (1 mM), but did not observe any response of neither radial, nor cortical MTs, even for prolonged observation over 2 hr (Figure 5). This indicates that exogenous NO cannot induce the disassembly of MTs and, if it plays a role in the cold response at all, must act downstream of MTs, or in a pathway not related to MTs.

3.5 | Jasmonic acid participates in cold-induced microtubule disassembly

JA is involved in the signal pathways of different biotic and abiotic stresses and therefore was investigated with respect to the microtubular response to cold. When the cells were treated with 50-μM JA in the absence of cold stress, central MTs disassembled rapidly (Figure 6b) within 30 min, although cortical MTs were still persisting to a residual extent (Figure S6). This demonstrated that JA could mimic cold stress

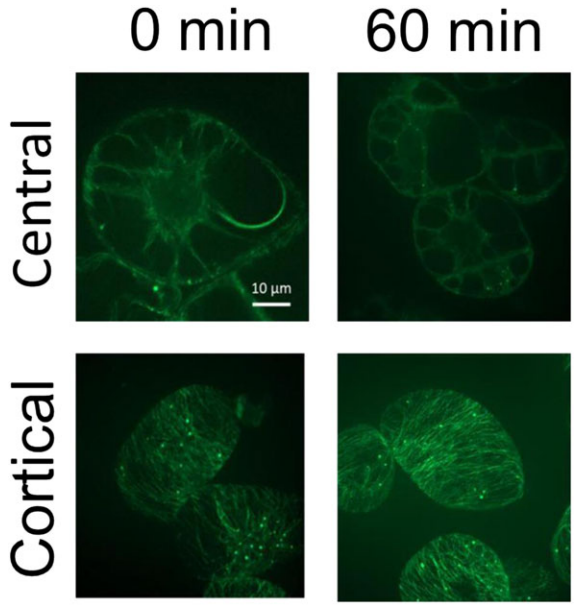

FIGURE 5 Effect of 1-mM sodium nitroprusside on the cold response of central and cortical microtubules in *V. rupestris* GFP-TuB6 cell line. Observations are representative of at least three independent experimental series. Size bars, 10 μm

in triggering the response of MTs. In the next step, we asked, whether jasmonates are also necessary for cold-induced MTs disassembly. We therefore used 4 mM of the lipoxygenase inhibitor phenidone to inhibit the production of JA under cold stress and tested the changes of MTs. Central and cortical MTs were slightly affected under cold stress in presence of phenidone (Figure 6a), resulting in a reduction of the weighted number of MTs by 40% over 1 hr (Figure 6b). However, this decrease was significantly mitigated as compared to the control without phenidone. This means that exogenous JA is sufficient to induce the disassembly of MTs in the absence of cold stress and that JA is necessary to convey efficient disassembly of MTs under cold stress but that it is not the exclusive signal.

3.6 | PLD is involved in cold stress signalling and modulates the state of microtubules

PLD has been known for more than a decade to control the association of cortical MTs with the membrane and to modulate MT disassembly. This enzyme also represents an important signalling hub, where signalling through the enzymatic product, the PA, diverges from a different signal (possibly linked with membrane association of MTs) that is independent of PAs. PLD can be activated by different alcohols, for instance butanols, and this activation can be used to steer the two diverging pathways differentially: On the one hand, *n*-butanol can activate PLD and act at the same time as acceptor for the resulting PA, such that the PA-dependent signalling will be interrupted. In contrast, *sec*-butanol can also activate PLD, but does not bind to PA, such that the PA-independent branch of signalling can proceed. We observe that 1% of *n*-butanol can, in the absence of cold stress, induce a partial disassembly of central and cortical MTs by about 40% within 30 min (Figure 7). In contrast, 1% of *sec*-butanol has no effect on MTs under normal temperature (Figure S7A) but can suppress the majority of cold-induced MT disassembly, even for a treatment extending over 1 hr (Figure 7). Because *sec*-butanol has been reported to exert effects on MTs independent of PA(Peters, Logan, Miller, & Kropf, 2007), we tested a second butanol, *tert*-butanol. Both 0.1% and 1% *tert*-butanol treatments were not able to block cold-induced MTs disassembly (Figure S7B). As in cells not treated with any butanol, MTs were completely eliminated in response to cold, suggesting that the effects of *n*-butanol and *sec*-butanol were specific.

Because PLD is activated through a trimeric G protein, we tested the effect of pertussis toxin (PTX), a specific G-protein inhibitor. After pretreatment with 10 ng/ml PTX for 30 min at 27 °C, the cells were transferred to ice water. We observed that PTX efficiently suppressed cold-induced disassembly (Figure 8a,b). In the next step, we used aluminum tetrafluoride, a G-protein activator. A treatment with 100-μM of this compound caused MTs disassembly in the absence of cold stress within 30 min (Figure 8a,b), reaching almost the same degree of disassembly as incubation at 0 °C and thus partially mimicking the effect of cold stress. These data suggest that cold-induced activation of a trimeric G protein is required for cold-induced MTs disassembly. At the same time, pharmacologically induced activation of this G protein is sufficient to activate MTs disassembly in the absence of cold.

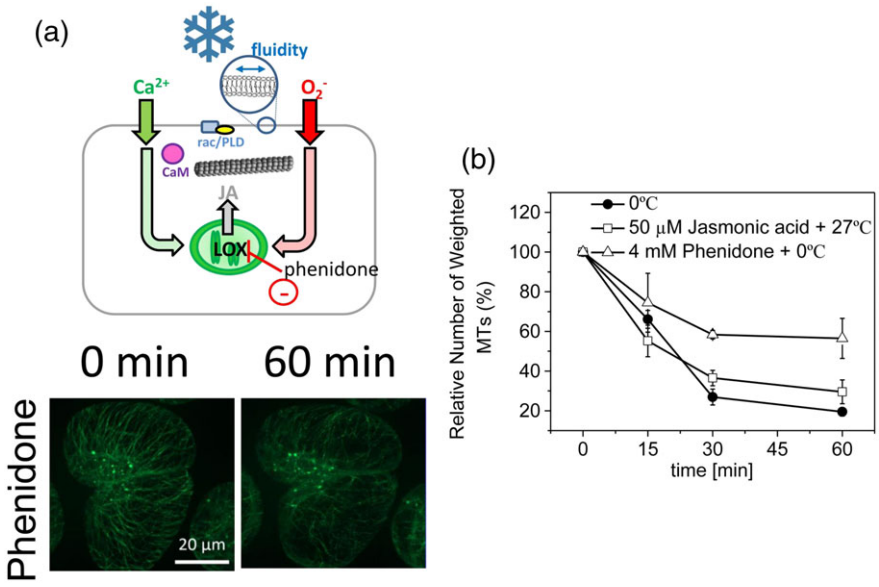

FIGURE 6 (a) Effect of phenidone on the microtubular response to cold stress (0 °C) followed over time in a representative cell file. Observations are representative of at least three independent experimental series with a population of 30 individual cells for each treatment. Size bars, 20 µm. (b) Quantitative analysis of the cold response of central microtubules (MTs) in presence of phenidone and jasmonic acid along with the response under normal temperature (27 °C)

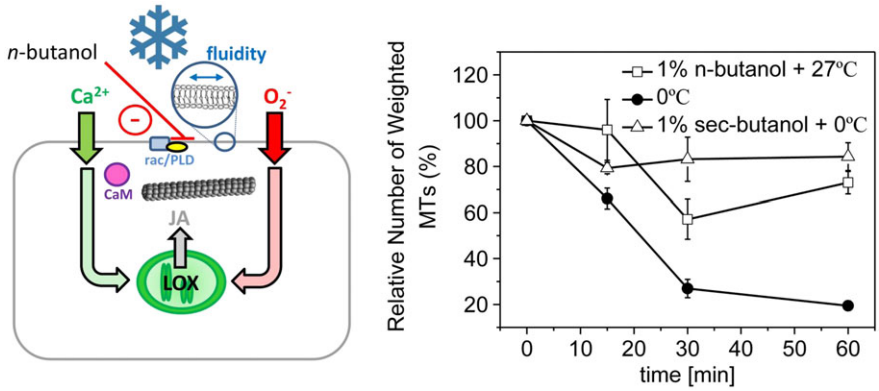

FIGURE 7 Quantitative analysis of the cold response of central microtubules (MTs) to *sec*-butanol and *n*-butanol compared to their response under normal temperature (27 °C). Each value represents the average of 10 individual cells

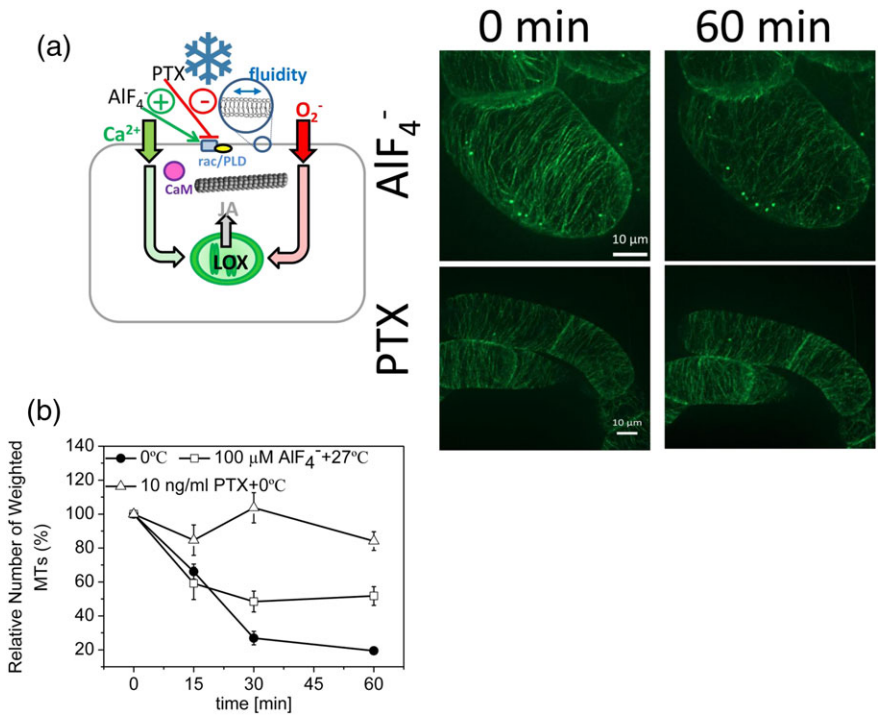

FIGURE 8 (a) Effect of 10 ng mL^{-1} PTX (pertussis toxin) and 100-µM AlF_4^- (aluminum tetrafluoride) on the microtubular response to cold stress (0 °C) and normal temperature (27 °C). Two representative cell files are shown for 60 min of treatment compared to the situation prior to addition of the compound. Observations are representative of at least three independent experimental series with a population of 30 individual cells. Size bars, 10 µm. (b) Quantitative analysis of the response of central microtubules (MTs) response to cold in presence of PTX and AlF_4^- under normal temperature (27 °C)

4 | DISCUSSION

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

MTs are endowed with innate dynamics, whereby α/β tubulin heterodimers polymerize and depolymerize continuously. This dynamic instability shows a distinct polarity, with assembly dominating at the plus end, and disassembly at the minus end. Modulated by associated proteins, this dynamic equilibrium can be shifted to rapid decay (so called MT catastrophe) in a nonlinear fashion. Using life-cell imaging based on GFP-tagged tubulin, cortical MTs of *Arabidopsis* have been shown to treadmill through the cell cortex with both plus- and minus-ends contributing to dynamics (Shaw, Kamyar, & Ehrhardt, 2003). Modulation of dynamic instability in response to environmental stress factors is a common phenomenon in animal cells (for review, see Walczak, 2000). For instance, the cold tolerance of Antarctic fishes correlates with a reduced cold sensitivity of MTs (Detrich, Parker, Williams, Nogales, & Downing, 2000; Walczak, 2000). In plants, cold stability of MTs can be regulated by stress-induced hormones, such as abscisic acid (Rikin et al., 1983; Sakiyama & Shibaoka, 1990), but also by cold-induced factors that differ from abscisic acid (Wang & Nick, 2001). Because cold sensitive MTs can be rendered cold stable by cleavage of the β-tubulin C-terminus (Bokros, Hugdahl, Blumenthal, & Morejohn, 1996), binding or dissociation of associated proteins at this domain is a prime candidate for cold-induced elimination of MTs. Molecular candidates for such proteins are MAP65 that can increase cold stability of animal MTs in vitro (Mao, Jin, Li, Liu, & Yuan, 2005) or calmodulin that, dependent on the concentration of calcium, has been reported to decrease MT stability in lysed protoplasts of carrot (Fisher et al., 1996).

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

On the basis of the published records, we interpret the fact that already within 30 min of cold treatment most MTs had disassembled, as sensory event. We think that this rapid disassembly of MTs acts to sense and amplify the signals generated by the cold-dependent reduction of membrane fluidity. In the current paper, we have now tried to dissect the events acting upstream of this sensory event:

Under cold stress, changes of membrane fluidity modulating the level of cytosolic Ca^{2+} are widely accepted as the primary signal for cold perception. However, their relationship to the cold response of MTs has remained unclear. A role of MTs for cold signalling has been demonstrated by a study, where the activation of the *Brassica napus* BN115 promoter had been mapped by pharmacological manipulation (Sangwan et al., 2001). Here, oryzalin, a compound that eliminates MTs by sequestering tubulin heterodimers, could mimic the effect of low temperature, whereas taxol could suppress the activation of this promoter by cold. Likewise, gadolinium ions and BA could prevent the induction of this reporter by low temperature, and, conversely, the calcium ionophore A23187, or DMSO could activate the reporter in the absence of cold treatment (Sangwan et al., 2001). Our findings that the calcium ionophore A23187 (50 μM) as well as the membrane rigidifier DMSO cause depolymerization of MTs under normal temperature, whereas the calcium channel blocker $GdCl_3$, the calcium chelator EGTA, or the membrane fluidizer BA could block the disassembly of MTs under cold stress are consistent with a model, where MTs transduce the condition of calcium and membrane as upstream events that are modulated under cold stress. This pattern is exactly congruent with the sequence found for the cold activation of the *Brassica napus* BN115 promoter (Sangwan et al., 2001) and supports a model, where cold-induced changes of membrane rigidity are transduced by modulations of calcium influx upon MT disassembly. Rapid increases of cytosolic calcium in response to cold stress have been shown repeatedly, for instance, by using transgenic plants expressing aequorin as a reporter that allowed to follow calcium signatures through monitoring changes of bioluminescence (Knight, Campbell, Smith, & Trewavas, 1991). Because calcium influx runs by coimport of protons, extracellular alkalinization is a convenient way to monitor calcium influx (Felix et al., 1993)-especially, when compared with imaging by fluorescent dyes, which requires a lot of skill and expertise. The fact that in our cell system, alkalinization correlates with intracellular calcium content as measured by atomic ion spectroscopy (Ismail et al., 2014) supports the feasibility of our assay. The most straightforward mechanism would be the activation of calmodulin. Cortical MTs have been shown to be decorated with calmodulin depending on the concentration of calcium (Fisher & Cyr, 1993), and calmodulin can induce their disassembly (Fisher et al., 1996). However, when we tested TFP, a widely used inhibitor of calmodulin (Feldkamp et al., 2010), we did not observe any inhibition of the microtubular cold response. This not only indicates a different second messenger, such as calcium-dependent protein kinases found to confer cold tolerance in rice (Saijo, Hata, Kyozuka, Shimamoto, & Izui, 2000), it also indicates that the upstream signalling cannot be reduced to influx of calcium activating calmodulin but must involve additional events. In fact, we are able to identify some of these additional upstream factors.

ROS are central signals in the response to various stress factors. Originally just considered as toxic products produced in consequence of stress-induced damage, they are meanwhile seen as important signals mediating the transduction of stress adaptation (Mittler et al., 2011). Although numerous reports describe, how ROS are generated in response to cold stress, a role of ROS as signals has only been rarely addressed. Using inhibitors, hydrogen peroxide as well as nitric oxide has been shown to be required for the cold response of myo-inositol phosphate in alfalfa (Tan, Wang, Xiang, Han, & Guo, 2013). Similar to this report, our results suggest a role of ROS for cold-induced signalling. Inhibition of the NAD(P)H oxidase by DPI could effectively inhibit the depolymerization of central and cortical MTs. Moreover, the transient accumulation of O_2^- in response to cold treatment was suppressed. Because the superoxide anion will trigger lipid peroxidation leading to accumulation of the end product MDA, we probed for the accumulation of MDA using a colorimetric assay based on conversion of TBA (Hodgson & Raison, 1991) but did not see significant changes after 2 hr of cold treatment, indicating that the temperature sensitivity of the lipid peroxidation limits the accumulation of MDA, such that this widely used assay for ROS detection is not suited for this purpose. In contrast to the situation in alfalfa (Tan et al., 2013), we were not able to detect a role of NO, because generation of exogenous NO by nitroprusside could not induce the disassembly of MTs. This does not preclude that NO maybe one of the products generated under cold stress possibly targeted to other responses such as myo-inositol phosphate synthase, but it precludes that it is acting upstream of MTs. Calcium influx can activate the NADPH oxidase via a calcium-dependent protein kinase pathway (Dubiella et al., 2013), and apoplastic oxidative burst, in turn, can stimulate calcium influx (Rentel & Knight, 2004), constituting a self-amplifying signalling loop, which might be responsible for cold-induced MTs disassembly. The fact that the extracellular alkalinization peaked at 35 min after onset of cold stress, that is, at a time, when superoxide levels (peaking at 10 min) already had declined again would support a model, where oxidative burst acts upstream of calcium influx.

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

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

We obtained, however, some evidences for divergent signalling downstream of PLD, when we investigated the role of JA. JA and its derivatives respond to various biotic and abiotic stresses, such as wounding, drought, salt stress, and is thought to act as an important downstream compound activating different stress responses (Balbi & Devoto, 2008). Modulations of jasmonate and jasmonate-isoleucine levels have also been reported as responses to cold stress, for instance in winter wheat (Kosova et al., 2012). However, to the best of our knowledge, a role of JA as upstream signal of cold stress has not been reported so far. We find that phenidone, a growth regulator that can down regulate jasmonate levels by inhibition of lipoxygenases (Bruinsma et al., 2010) can partially suppress the depolymerization of

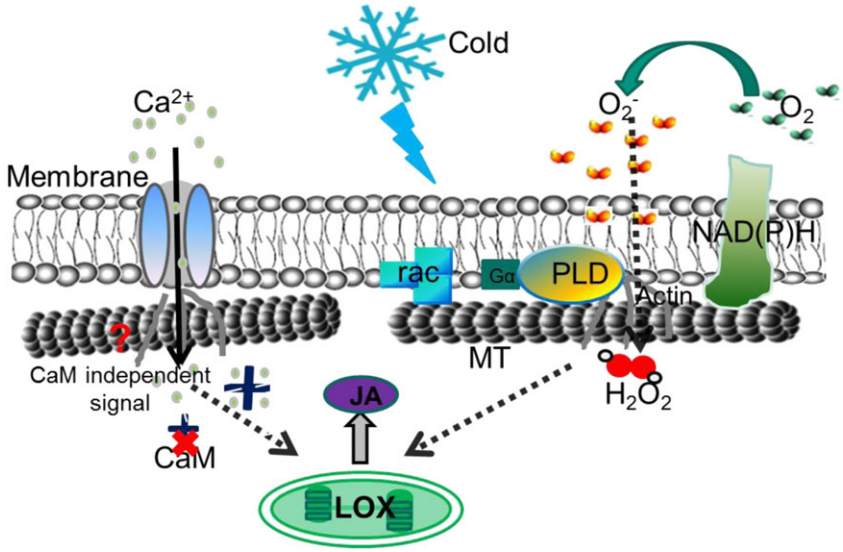

FIGURE 9 Working model of the early signal transduction under cold stress. Calcium influx, membrane rigidification, and activation of an NADPH oxidase and G protein in concert with a phospholipase D convey the signal towards microtubules (MTs) as factors in signal susception and amplification, whereas calmodulin seems to be not involved. Moreover, activation of the jasmonate pathway in response to cold is required for an efficient microtubule response. Ca^{2+} = calcium; rac = GTPase; PLD = phospholipase D; NAD(P)H, NAD(P)H oxidase; O_2 = oxygen; O_2^- = superoxide anion; H_2O_2 = hydrogen peroxide; CaM = calmodulin; LOX = lipoxygenase; JA = jasmonic acid

MTs (to around half of the level induced by cold stress), whereas, conversely, exogenous JA could mimic cold stress with respect to causing MTs disassembly. This shows that, although JA is sufficient to induce the MTs response, cold-induced JA only accounts for around half of the response, placing JA in a parallel pathway enhancing the signal conveyed by calcium influx, NADPH oxidase, and a trimeric G protein. The fact that a similar partial inhibition of cold-induced MT disassembly is observed, when PLD is blocked by *n*-butanol, points to a functional link between the two events. In fact, PLD has been shown to activate jasmonate synthesis in the wound response (Wang et al., 2000) leading to a working model, where jasmonate is placed downstream of cold-dependent activation of PLD.

We arrive at a model (Figure 9), where cold-induced membrane rigidification stimulates calcium influx and NADPH-oxidase mediated oxidative burst. These two early events seem to be connected by mutual amplification resulting in activation of a trimeric G protein that, in turn, activates PLD. Here, the pathway branches into a PA-dependent signal (possibly targeted to actin filaments), and a PA-independent signal (targeted to MTs). In parallel, activation of PLD activates the jasmonate pathway, which serves to sustain and stabilize the MT response. The link between PLD activation and activation of JA signaling is well established in the literature (Wang et al., 2000). MTs disassembly would thus function as final step of a thermometer triggering cold adaptation (reviewed in Nick, 2008). The downstream effects of this thermometer might be cold-induced voltage-dependent calcium channels resulting in an increased influx of calcium (Mazars et al., 1997) leading to further self-amplification. However, in consequence of cold adaptation, MTs acquire cold stability (Abdrakhamanova et al., 2003; Pihakaskimaunsbach & Puhakainen, 1995), which in turn, should constrain the activity of cold-activated calcium channels and thus prevent deregulated signalling. This mechanism would down regulate sensitivity upon prolonged stimulation, which represents a key element of biological sensing. This so called cold hardening has already been confirmed in our experimental model and will be dissected in a forthcoming publication.

The original model, where cold-induced MTs disassembly was seen as a part of cold-induced cellular damage, has over the years shifted into a model, where MTs disassembly is interpreted as a sensory process. At some point, this sensory event has to translate into changes of gene expression, requiring activation of transcription factors such as the dehydration-responsive element-binding protein 1 s (DREB1s)/ C-repeat-binding factors (Ito et al., 2006). Whether MTs are sequestering components required for this activation, or whether they modulate chromatin structure by mechanic tensegrity, will be addressed in forthcoming work.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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