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REVIEW

Hormonal control of cold stress responses in plants

Marina Eremina[1] · Wilfried Rozhon[1] · Brigitte Poppenberger[1]

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Abstract Cold stress responses in plants are highly sophisticated events that alter the biochemical composition of cells for protection from damage caused by low temperatures. In addition, cold stress has a profound impact on plant morphologies, causing growth repression and reduced yields. Complex signalling cascades are utilised to induce changes in cold-responsive gene expression that enable plants to withstand chilling or even freezing temperatures. These cascades are governed by the activity of plant hormones, and recent research has provided a better understanding of how cold stress responses are integrated with developmental pathways that modulate growth and initiate other events that increase cold tolerance. Information on the hormonal control of cold stress signalling is summarised to highlight the significant progress that has been made and indicate gaps that still exist in our understanding.

Keywords Abiotic stress · Freezing tolerance · Hormones · Plant

Abbreviations

ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AHK	Arabidopsis histidine kinase
AHP	Histidine phosphotransfer protein
AOPP	L-α-Aminooxy-β-phenylpropionic acid
ARR	Arabidopsis response regulator
AVG	2-Aminoethoxyvinyl glycine
BES1	BRI1-EMS-suppressor 1
BRI1	Brassinosteroid insensitive 1
BRs	Brassinosteroids
BZR1	Brassinazole-resistant 1
CAMTA	Calmodulin-binding transcription activator
CAS	Cold-acclimation-specific
CBF	C-repeat binding factor
CK	Cytokinins
COI1	Coronatine insensitive 1
COR	Cold regulated
CPD	Constitutive photomorphogenesis and dwarfism
CPKs	Calcium-dependent protein kinases
CPR1	Constitutive expression of PR genes
CRT/DRE	C-repeat/dehydration-responsive element
CTR1	Constitutive triple response 1
DREB	Drought-responsive element-binding protein
DWF4	Dwarf 4
EDS5	Enhanced disease susceptibility 5
EIL1	EIN3-like 1
EIN2	Ethylene-insensitive 2
EIN3	Ethylene-insensitive 3
ENO2	Enolase 2
ETR1	Ethylene-responsive 1
GAI	Gibberellic acid insensitive
GAs	Gibberellins
GID1	GA-insensitive dwarf 1
GNC	Gata, nitrate-inducible, carbon-metabolism involved

✉ Brigitte Poppenberger
brigitte.poppenberger@wzw.tum.de

[1] Biotechnology of Horticultural Crops, TUM School of Life Sciences Weihenstephan, Technische Universität München, 85354 Freising, Germany

GNL	GNC-like
HHP	Heptahelical protein
HR	Hypersensitive response
IAA	Indole acetic acid
IBA	Indole butyric acid
ICE1	Inducer of cbf expression 1
ICS	Isochorismate synthase
JA	Jasmonic acid
JAZ	Jasmonate zim domain
KIN	Cold induced
LOS2/AtMBP-1	Low expression of osmotically responsive genes 2/Arabidopsis thaliana c-MYC binding protein
MAPKs	Mitogen-activated protein kinases
OST1	Open stomata 1
PAL	Phenylalanine-ammonia-lyase
PIF	Phytochrome-interacting factor
PIN	PIN-formed
RGA	Repressor of gai
RGL	RGA-like
SA	Salicylic acid

Introduction

Plants have developed a remarkable ability to adapt to harsh environmental conditions, and thrive in habitats characterised by abiotic stresses such as temperature extremes [1]. Species-specific differences in temperature tolerance have evolved in plants that occupy different geographic zones. Annual crops from temperate climates, such as *Triticum aestivum* (wheat), *Avena sativa* (oats), *Hordeum vulgare* (barley) or *Pisum sativum* (pea), display a certain degree of basal (intrinsic) freezing tolerance, which they can further increase by utilising complex signalling events. In contrast, most species from subtropical or tropical climates, such as *Zea mays* (maize), *Oryza sativa* (rice) or *Solanum lycopersicum* (tomato), suffer damage at chilling temperature (often well above 0 °C) [1]. This represents a significant management challenge for agriculture and horticulture because these crops are regularly cultivated in geographical regions where temperature preferences of the plant are not fully met during the growing season. Moreover, extreme short-term weather events, such as late frost during spring, impact yields, particularly of fruit crops and spring cereals [2]. Consequently, chilling and freezing stress constitute some of the most severe abiotic factors that reduce crop productivity [3, 4]. Because reliable and high-quality crop yields are critical for food security, an understanding of the molecular modes that underpin cold stress resistance is essential to further optimise horticultural and agricultural crop breeding and production.

Cellular changes in response to cold

Temperate plants utilise a repertoire of mechanisms to avoid damage by freezing. These include the timing of developmental programs, such as germination and flowering, on the basis of the environmental information perceived [5]. In addition, they have the ability to further increase their basal freezing tolerance following prior exposure to chilling temperatures. This process, termed cold adaptation, requires marked transcriptional reprogramming to alter the expression of diverse classes of genes with a wide range of biological functions [6].

A key response to cold is growth repression (Fig. 1), which is thought to be utilised to re-allocate resources from growth to processes that increase cold stress resistance [7], although this hypothesis remains to be validated. In addition to pronounced effects on morphology drastic biochemical and physiological changes occur, which have been summarised in a number of excellent recent reviews [1, 4, 5]. In brief, these changes include an increase in cellular calcium contents, an accumulation of reactive oxygen species (ROS) and the formation of cryoprotective proteins and cryoprotective metabolites such as soluble sugars and amino acids. In addition, changes in the structure and composition of membranes and numerous other events occur [1], which are all dedicated to reducing the damage caused by cold temperatures or cellular freezing [1]. Interconnected molecular circuits that are governed by the activity of plant hormones control these events, and in the current review, we will summarise our understanding of the hormonal control of cold stress resistance in plants, with a particular focus on latest developments in the field.

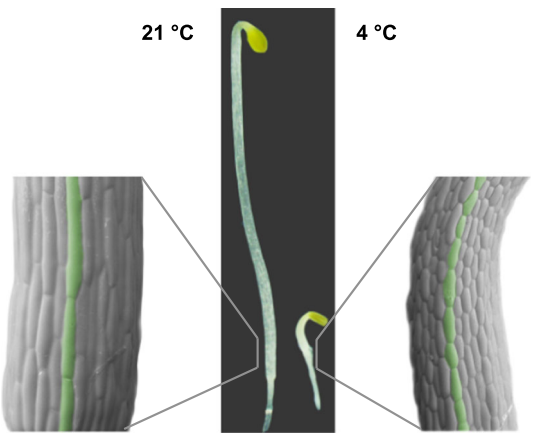

Fig. 1 Effect of cold treatment on cell elongation in arabidopsis hypocotyls. Seeds were germinated in the light and then moved to the dark at either 21 or 4 °C, where they were kept for 3 days. Electromicrographs of the indicated sections are shown. A representative cell row is marked in *green*

Cold-regulated gene expression

Cold-responsive transcriptomes differ between plant species [8–32]. In *Arabidopsis thaliana* (arabidopsis), a temperate plant that serves as a model species in plant molecular biology, approximately 10 % of all genes are cold regulated [33, 34]. A significant share of these cold-regulated (*COR*) genes, including *COLD-REGULATED 15A* (*COR15A*), *COLD-REGULATED 15B* (*COR15B*), *COLD-REGULATED 78* (*COR78*), and *COLD-INDUCED 1* and *2* (*KIN1* and *KIN2*), contain the C-repeat/dehydration-responsive element (CRT/DRE) in their promoters. CRT/DRE is a *cis* regulatory motif that is bound by the drought-responsive element-binding proteins (DREB), also known as C-REPEAT BINDING FACTOR (CBFs) [35], in response to cold and other types of abiotic stresses that cause cellular desiccation, for example, drought or high salt exposure. CBFs are APETALA 2 domain transcription factors (TFs) that increase resistance against freezing, drought and high salt exposure when overexpressed in different plant species such as arabidopsis, *Brassica napus* (canola), tomato and *Populus* spp. (poplar) [35].

The modes of CBF-controlled gene regulation have received a considerable amount of attention in arabidopsis, and the results of these studies are instructive. In arabidopsis, three different CBFs, namely CBF1, 2 and 3, exist, which are physically linked in a tandem array on the chromosome and act to control overlapping gene sets [34]. Interestingly, CBFs regulate their own activity in feedback regulatory loops: CBF2 represses *CBF1* and *CBF3* expression to adjust their mRNA abundance [36]. In addition, *CBF* expression is controlled by upstream TFs such as ZAT12, which acts as a repressor [37], and CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR (CAMTA), INDUCER OF CBF EXPRESSION 1 AND 2 (ICE1 and ICE2), which act as activators of *CBF* transcription [38–40]. Among those, the best characterised is ICE1, a MYC-like basic helix-loop-helix (bHLH) protein, which, in response to cold, is modified by SUMOylation involving the SUMO E3 ligase SIZ1. This promotes ICE1 binding to an E-box motif in the *CBF3* promoter and increases *CBF3* expression [41]. ICE1 protein abundance is controlled by phosphorylation [42, 43] and ubiquitination catalysed by the E3 ubiquitin ligase HOS1, which acts to maintain ICE1 equilibrium during cold acclimation [44].

Although it is established that the CBFs play an important role in cold acclimation they only control a share of the cold-responsive transcriptome [6, 17, 37]. Therefore, additional TFs take part, and one example is ZAT10, a negative regulator of *COR* genes whose activity is repressed by the bifunctional enolase LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 2/C-MYC BINDING PROTEIN (LOS2/AtMBP-1), in response to cold [45]. LOS2/MBP-1 is an interesting locus because in addition to the TF AtMBP-1, it encodes the enolase 2 (ENO2) that catalyses a key step in glycolysis. LOS2/AtMBP-1 utilises an elegant feedback regulatory mode to control its own activity: AtMBP-1 is alternatively translated from a second start codon [46] to represses LOS2/AtMBP-1 transcription and thereby regulates ENO2 abundance [47]. How LOS2/AtMBP-1 activity is controlled by cold is currently unknown, but clearly it would represent an interesting target for plants to utilise, when in response to low temperature, both glycolysis, as a central metabolic pathway and *COR* gene expression must be modulated. It will be important to dissect the roles of AtMBP-1 from those conferred by ENO2 to further define the role of the LOS2/AtMBP-1 locus in cold resistance.

Plant hormones control cold responses

Whereas the nuclear events that allow for TFs to directly control *COR* gene expression are fairly well defined, the upstream regulatory modes that govern these activities have remained largely elusive. The mechanisms allowing plants to perceive temperature remain unknown, although there is evidence that changes in membrane fluidity are contributory [1]. In addition, the modes transducing the cold stimulus to the nucleus are largely unspecified, although protein phosphorylation controlled by calcium-dependent protein kinases (CPKs) that are induced by increases in cellular calcium contents, and mitogen-activated protein kinases (MAPKs) have been implicated [48, 49]. That said, in recent years, accumulating data support a role of plant hormones, which additionally govern other signal events in cold stress responses and suggests that hormones function as governors of the pathways.

Hormones are small molecular weight compounds that signal information from a site of synthesis to a site of action. Biosynthesis, catabolism and transport adjust hormone homeostasis; the receiving tissues determine sensitivity by the presence and responsiveness of dedicated receptors that initiate signal transduction events to alter cellular processes [50]. Different classes of hormones exist that have specific as well as overlapping functions. Moreover, hormones display synergistic or antagonistic effects on the biosynthesis and signalling outputs of other hormones, creating a complex network of hormonal interactions [50]. This hormonal signalling network is utilised to integrate external information into endogenous developmental programs and/or activate stress response pathways leading to resistance. It is therefore perhaps not surprising that plants utilise hormones to signal cold stress

responses; however, our knowledge of the responsible molecular modes remains patchy. This may be attributed to the fact that hormone action in cold stress responses is influenced by cross-talk with signalling cascades conferring responses to other environmental stimuli such as light [51, 52] and is additionally impacted by endogenous developmental programs, in particular those that result in developmental phase transitions [53]. Moreover, hormone modes of activity vary between plant species complicating research in this field.

Gibberellin (GA) activity is altered in response to cold stress

Growth-promoting hormones, in particular GAs, play central roles in conferring plants the ability to adapt growth to fluctuating external conditions, such as changes in the light environment [51]. GAs promote cell elongation and division and are synthesised from *trans*-geranylgeranyl diphosphate in a biosynthetic pathway in which small families of oxygenases, the KAO-oxidases, GA 20-oxidases and GA 3-oxidases produce bioactive GAs. GA cellular homeostasis is maintained by catabolic inactivation utilising GA 2-oxidases as well as by feedback suppression of GA biosynthesis via the GA signalling pathway. In GA signalling, DELLA activity is central [54]. DELLAs are GRAS proteins of which five members exist in arabidopsis, GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF GAI (RGA) and RGA-LIKE 1, 2 and 3 (RGL1, RGL2 and RGL3). They act as repressors of TFs such as PHYTOCHROME-INTERACTING FACTOR 3/4 (PIF3/PIF4) [55, 56], and brassinosteroid-insensitive 1 (BRI1)-EMS-SUPPRESSOR 1/BRASSINAZOLE-RESISTANT 1 (BES1/BZR1) [57], which control GA-responsive gene expression. DELLA repressive action is released upon perception of the hormone by the GA receptor GA-insensitive dwarf 1 (GID1), which initiates DELLA ubiquitination and degradation via the 26S proteasome [54].

Both GA metabolism [58] and signalling [59] are targeted by cold stress, and there is evidence that the CBFs take part. A first indication was that in arabidopsis, tobacco and tomato overexpression of *CBFs* resulted in a reduction of bioactive GA, which was correlated with suppressed growth and late flowering and could be restored by exogenous GA application [58, 60–62]. When studying the underlying molecular modes, it was found that cold-induced *GA2ox* expression, which resulted in increased hydroxylation and inactivation of bioactive GA (Fig. 2). In addition, CBF1, when overexpressed, affected transcript levels of the DELLA *RGL3* and enhanced DELLA accumulation, likely through posttranslational control [58].

Other studies provided evidence that GA signalling components can affect the low temperature stress responses of plants, because they showed that GA-insensitive or GA-deficient mutants have altered chilling and freezing tolerance both in arabidopsis [58, 59] and rice [64]. The arabidopsis *gai* mutant, which is a constitutive signalling mutant, was more freezing tolerant, and the DELLA knockout line *gai-t6 rga-24* was hypersensitive to freezing, both before and after cold acclimation [58]. Similarly, the

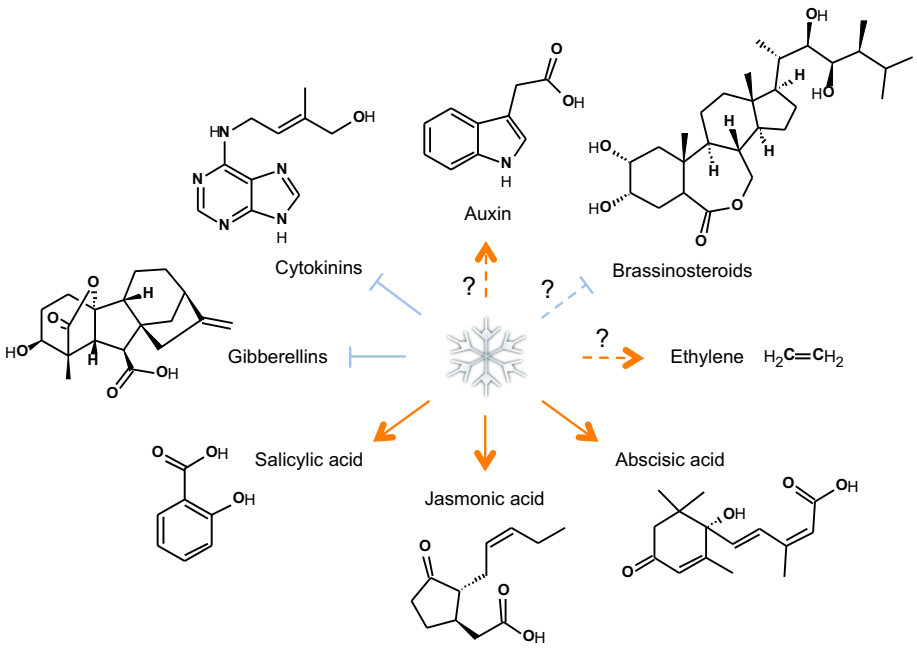

Fig. 2 Present understanding of the impact of cold stress on hormone levels in plants. *Orange arrows* and *blue inhibition lines* represent activation and suppression processes, respectively. *Dotted lines* are used in case no or controversial data on hormone levels were published. Please note that species-specific differences can occur; these are detailed in the text

ga1 mutant, which is impaired in GA production, had a greater degree of freezing tolerance than the wild-type. Interestingly, *ga1* additionally showed elevated levels of *CBF1*, *CBF2* and *COR15A,* and there is evidence that this is, at least partially, mediated by the action of the GA-regulated GATA TFs GNC and GNL [59, 64]. Overexpression of *GNC* or *GNL* enhanced *CBF2*, *COR15A* and *COR15B* expression and increased survival rates of transgenic seedlings before and after cold acclimation [59]. Because *GNL* and *GNC* expression are increased in GA signalling-deficient mutants, GNC and GNL are proposed to act in promoting freezing tolerance in the absence of GA [59] (Fig. 3).

A second level of CBF control by GAs may be mediated by PIF4. PIF4, which is controlled by phytochromes and DELLAs [55, 56], was found to directly bind to the promoters of all three CBFs and repress their expression in long-day growth conditions in arabidopsis [65]. The aforementioned study complemented earlier work that had elucidated a function of phytochrome signalling in *CBF* expression [66]. Because PIF4 additionally controls auxin biosynthesis at high temperatures [67] and is controlled in its activity by brassinosteroids (BRs) [68], a second class of growth-promoting hormones, PIF4, and redundant factors may act as central nodes for the integration of multiple environmental stimuli into growth processes (Fig. 3).

Although the role of CBFs in GA-mediated growth suppression is established, our understanding of the molecular control of these events remains ill defined. The mechanism used by CBFs to control GA2ox and DELLA transcription or posttranslational processing remains unknown. In addition, there is evidence that cold may inhibit growth by additional means, because ZAT10, ZAT12 and further TFs that are induced by cold in primary

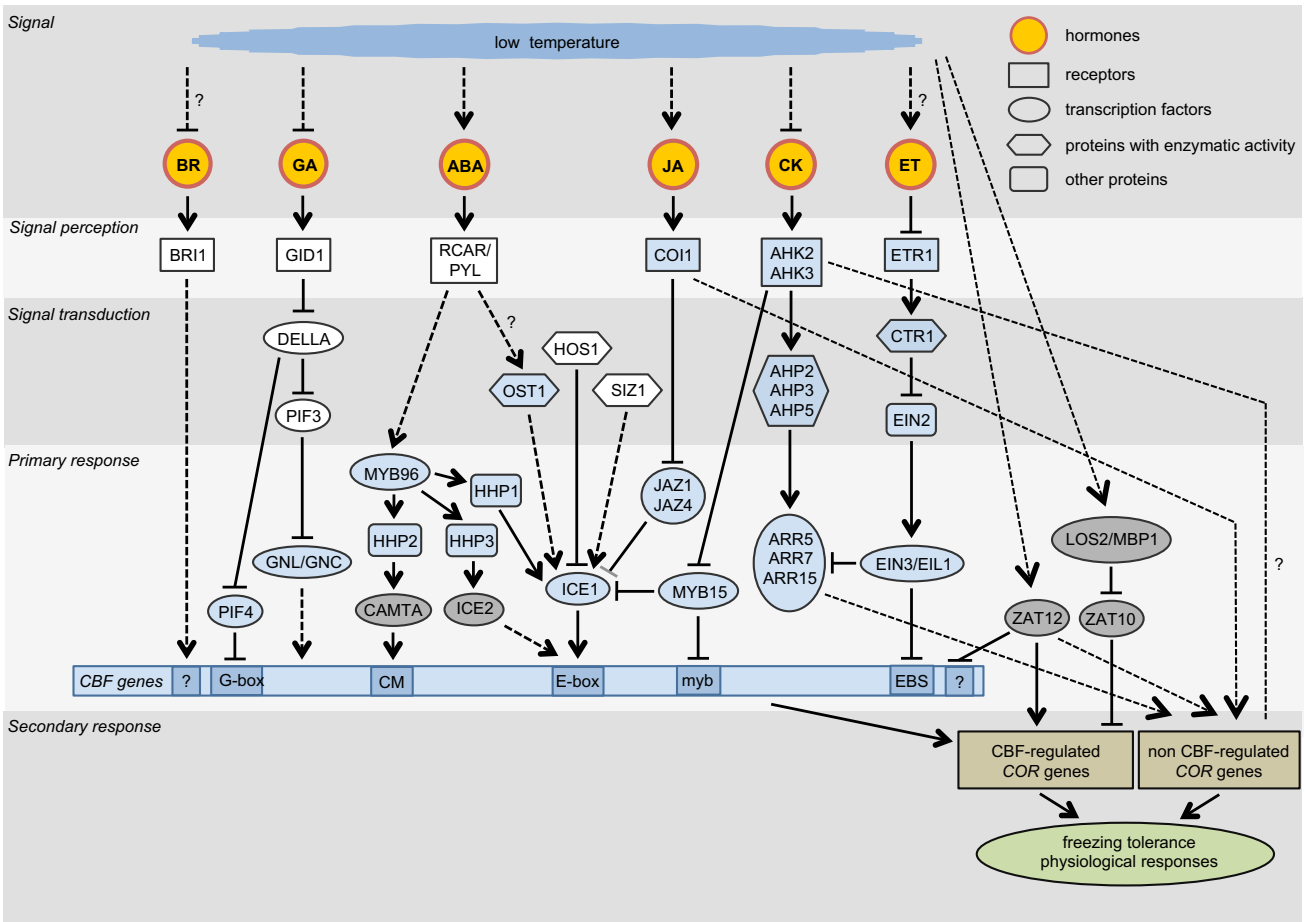

Fig. 3 Model summarising the current understanding of signalling events that confer hormone activity in cold adaptation. In *blue* are transcription factors (TFs) and components of hormone signalling pathways, which are regulated by both cold and hormone. In *grey* are TFs regulated by cold but not by hormone. In *white* are components of hormone signalling pathways, which are not known to be cold-regulated. *Arrows* and *inhibition lines* represent activation and suppression processes, respectively. *Solid lines* indicate a direct interaction; *dotted lines* represent indirect interactions

responses and control non-CBF regulons, also suppress growth when overexpressed. This growth repression was not correlated with reduced *GA2ox* or *RGL3* expression [34], and it will therefore be interesting to determine which other growth regulatory pathways participate.

BRs promote cold resistance

A class of hormones that closely interacts with GAs are the BRs. BRs are polyhydroxylated steroids with a well-established role in growth promotion [69]. In arabidopsis this function is conferred by an intimate interplay with GAs where both hormones impact on the other's biosynthesis [70, 71] and contribute signalling components that interact to control multiple overlapping developmental programs [68, 72, 73]. In cold responses, there is as yet no evidence for a synergistic activity of BRs and GAs. Quite on the contrary, as opposed to GAs, BRs are thought to positively control cold stress responses, because it has been shown that BR application increases cold tolerance of many plant species, including chilling-sensitive crops such as maize and *Cucumis sativus* (cucumber) [74–76].

BRs are synthesised from campesterol in a biosynthetic pathway that relies mainly on the activities of cytochrome P450s including DWARF 4 (DWF4) and CPD (CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM). *DFW4*, when overexpressed, enhanced cold stress resistance of arabidopsis and additionally increased *COR15A* mRNA levels [77]. Moreover, BR application enhanced expression of *CBF1* and the CBF target *COR47* in arabidopsis following low temperature treatment, which indicated that BRs promote *CBF* expression and cold tolerance [78] (Fig. 3).

In agreement, a BR receptor BRI1 mutant, *bri1-116*, showed enhanced ion leakage following freezing stress, thereby providing evidence that BR signalling promotes acclimation to cold [79]. However, contradictory results were obtained in a different study. In this earlier work, plants overexpressing BRI1 showed elevated basal *CBF1-3* expression levels but had reduced cold tolerance, whereas BRI1 loss-of-function in *bri1-9* showed increased resistance [80]. Therefore, although a role of BRs in increasing cold responses appears likely, further verification of these results is required.

Auxins in cold stress? As yet, not much is known

Auxin has been heavily studied for its role in growth adaptation, in particular, in response to light and gravitropic stimuli. However, somewhat surprisingly, little is known regarding roles of auxins in cold stress responses of plants, although it has been shown that a significant number of auxin-regulated genes are additionally affected by cold in arabidopsis and rice [81, 82] and that application of auxin analogues on canola (*B. napus*) stimulated accumulation of freeze-protective metabolites and soluble sugars during cold hardening [83].

Biologically active auxins are indole acetic acid (IAA) and indole butyric acid, which are synthesised from tryptophan. Cold stress appears to affect IAA levels with differences depending on plant species, developmental context and physiological settings. In wheat, following 21 days of exposure to 4 °C, a significant increase in IAA concentrations was found in crown tissues but not in leaves [84]. In addition, whereas IAA levels were not affected by cold stress in spring wheat, significant increases occurred in winter wheat after 12 days of cold exposure [85]. Contradictory results were published for rice. Whereas one study reported decreased IAA contents following low temperature treatment [86], another study found that IAA contents were elevated after 1 day of cold treatment and remained elevated for 5 days [87]. This correlated with an increased expression of auxin biosynthetic genes of the YUCCA family and a reduction of the OsGH3 family, which catalyses auxin inactivation in rice [87]. Therefore, in rice, auxin levels may be increased by activation of auxin biosynthesis and suppression of auxin inactivation (Fig. 2).

Studies of auxin may be complicated by the sophisticated transport events, catalysed by auxin influx and efflux carriers that determine spatial gradients of auxin concentrations and are required for directional growth and many other processes [88]. In this respect, it is known that low temperature affects gravitropic growth of plants, because in stems and roots of arabidopsis and rice, gravitropic responses were inhibited by cold [89–91]. For arabidopsis, it was shown that short-term cold treatment slowed down gravitropic bending and elongation of roots, whereas a 24-h long-term treatment blocked gravitropic responses of roots completely [91]. This was correlated with changed auxin distribution patterns in arabidopsis roots and repressed basipetal auxin transport caused by impaired intracellular cycling of the auxin efflux carriers PIN2 and PIN3 [91]. Interestingly, application of the membrane rigidifier DMSO, which was previously shown to reduce membrane fluidity, suppressed elongation growth of arabidopsis roots at 23 °C but did not affect root gravitropism or intracellular PIN trafficking [91], indicating that root gravitropic responses in low temperatures are not caused by membrane rigidification.

Cytokinins control both CBF-dependent and CBF-independent regulons

A class of hormones that strongly cross-talks with auxins in plant developmental processes is the cytokinins (CKs). CKs are adenine derivatives with isoprenoid or aromatic side chains that, in addition to many other aspects, control directional growth processes such as gravitropism. The most abundant CKs in higher plants are isopentenyladenine and zeatin [92], and external application of these hormones is known to enhance freezing tolerance of arabidopsis [93, 94]. Because *CBF1* expression is not increased by CK treatment and is neither elevated in CK-overaccumulating plants nor decreased in the CK-deficient mutant *35S:AtCKX2-2*, which overexpresses CK oxidase, there is evidence that CKs increase freezing tolerance in a CBF1-independent manner [93]. Somewhat puzzling is the finding that although increased CK contents enhance freezing tolerance, CK levels decrease in response to chilling temperature in different plant species including wheat, rice and arabidopsis [84, 86, 95]. In rice, this reduction was correlated with a significant down-regulation of CK biosynthetic gene expression [86], providing evidence for a model in which a decrease in CK levels would be required for cold responses (Fig. 2).

Also for CK signalling results were published that are contradictory. In arabidopsis, CKs initiate signalling utilising three histidine protein kinases, namely arabidopsis histidine kinase (AHK)2, AHK3 and AHK4, which act as CK receptors. Upon CK binding, the receptors autophosphorylate and transfer the CK signal via arabidopsis histidine phosphotransfer proteins (AHPs) to nuclear localised arabidopsis response regulators (ARRs), TFs that regulate expression of CK target genes. In non-acclimated and acclimated *ahk* knockout plants, freezing tolerance was promoted, although transcript levels of *CBFs* in response to cold were found to be unchanged [93]. However, a later study reported that microarray analysis of a cold-treated *ahk2 ahk3* double-mutant line showed that these mutations promoted expression of *MYB15* and repressed expression of its target *CBF3*, which additionally correlated with suppressed expression of a set of CBF-controlled genes [96]. Moreover, non-CBF-regulated sets of cold-responsive genes were differentially regulated in *ahk2 ahk3*. Therefore, although AHK2 and AHK3 appear to be important for the modulation of both CBF-dependent and CBF-independent cold-responsive pathways, the question of whether AHKs have positive or negative roles in the freezing tolerance of plants remains to be conclusively answered.

Other components of CK signalling that have been implicated in cold stress responses are the type A ARRs. Transcript levels of *ARR5*, *ARR7* and *ARR15* were induced in response to cold in a AHK-dependent manner [93, 94] and *arr5*, *arr6* and *arr7* single knockout lines showed slightly increased tolerance to freezing, both constitutively and following cold acclimation [93, 94]. Moreover, overexpression of ARR7 slightly decreased freezing tolerance after cold acclimation. Because the induction of CBF-regulated genes was not impaired in this line, ARR7 was proposed to act as a negative regulator of cold signalling by CBF-independent means [93]. Again, seemingly in contradiction, a different study found that seedlings overexpressing *ARR7* as well as *ARR5* or *ARR15* were more tolerant to freezing stress before and after cold acclimation; however, *CBF* and CBF-regulated gene expression were unchanged in these lines, lending support to the notion that ARRs do not impact on CBFs in cold responses [94] (Fig. 3).

Abscisic acid (ABA), a central regulator of cold stress signalling with emerging roles in the CBF-dependent pathway

Abscisic acid is an isoprenoid hormone that plays a central role in seed dormancy, abscission and abiotic stress signalling [97], and its function in cold stress responses is well established. An increase in ABA levels correlates with increased ABA biosynthesis in arabidopsis and rice [86, 98, 99] and occurs in response to cold in many plant species (Fig. 2). Exogenous application of ABA promotes freezing tolerance, and ABA mutants exhibited altered cold resistance. However, because it was previously thought that ABA does not act on *CBF* expression, the postulation was that ABA-controlled cold responses are regulated by CBF-independent means [97, 100].

Recently, evidence has accumulated that suggests otherwise. In this context, two publications are of particular importance. In the first work it was shown that MYB96, an ABA-induced TF, which is a central regulator of ABA-responsive gene expression, is cold induced and controls *CBF* induction in cold adaptation because it was compromised in a *myb96* knockout line and was enhanced in a MYB96 overexpressor [101]. Furthermore, was provided evidence that MYB96 interacts with the HEPTAHELICAL PROTEIN (HHP) proteins HHP1, HHP2 and HHP3, which in turn interact with ICE1, CAMTA and ICE2, respectively [101, 102]. By these means, the authors propose that the MYB96–HHP complexes control expression of all three CBFs, and it will be of interest to observe whether this can further be verified by genetic approaches in the future.

The second work found that Open Stomata 1 (OST1), a Ser/Thr protein kinase activated by ABA [103], additionally regulates *CBF* expression. OST1 activity was induced

by cold, *ost1* knockout lines were hypersensitive to freezing and OST1 overexpressing plants were more resistant. Importantly, the molecular modes of the OST1 function were addressed, and it was revealed that OST1 interacts with and phosphorylates ICE1 to stabilise the protein and promote its transcriptional activity under cold stress. Evidence was provided that this is enabled by OST1 phosphorylation of ICE1 S278, because mimicking phosphorylation at this site as well as the presence of OST1 inhibited ICE1-HOS1 interaction and stabilised the protein [43]. Intriguingly the activation of OST1 by cold appears to be independent of ABA biosynthesis [43] and it will therefore be important to identify upstream regulatory components. In summary, in addition to its CBF-independent modes, ABA may impact on *COR* gene expression by controlling CBF transcription (Fig. 3) and appears to play a more central role in the cold stress tolerance of plants than previously assumed.

Ethylene: a gaseous hormone with versatile roles in cold responses

Ethylene is a gaseous hormone that is synthesised from the amino acid methionine utilising a biosynthetic pathway, in which the rate-limiting step, the conversion of *S*-adenosyl-L-methionine to 1-aminocyclopropane-1-carboxylic acid (ACC), is catalysed by isoforms of ACC synthase (ACS). Whereas the role of ethylene in abiotic stress resistance and additionally in cold responses is clear [104], the question of whether it has positive or negative regulatory roles remains. Some studies reported increases in ethylene contents in response to cold in different plant species including tomato [105], *Secale cereale* (rye) [106], *Phaseolus* spp. (bean) [107], wheat [84] and *Medicago sativa* (alfalfa) [108]. In arabidopsis these increases were correlated with increased expression of biosynthetic enzymes [109] (Fig. 2). However, other studies presented evidence for a reduction of ethylene contents in response to cold. For example, in *Medicago truncatula*, ethylene levels decreased rapidly by 94 % and the production of the ethylene precursor ACC was reduced by 45 % following cold treatment [110]. Although changes in transcript levels of the biosynthetic enzymes ACC oxidase (ACO) and ACS were not observed following low temperature treatment, the activity of ACO, an enzyme catalysing the last step of ethylene biosynthesis by converting ACC to ethylene, was reduced [110].

A reduction of ethylene in response to cold would fit well to the proposed role as a negative regulator of freezing tolerance in plants. Freezing tolerance was inhibited following treatment with ethylene or ACC in *M. truncatula* [110] and arabidopsis [94] and was promoted by treatment with ethylene biosynthesis inhibitors, including 2-aminoethoxyvinyl glycine (AVG), in both plant species [94, 110]. Moreover, *M. truncatula* plants were hypersensitive to freezing when treated with the ethylene releaser ethephon, which was correlated with a compromised induction of expression of *MtCBF1* and *MtCBF3* as well as *MtCAS15* that belongs to the *COLD ACCLIMATION-SPECIFIC* (*CAS*) gene family, counterparts of *COR* genes in *M. truncatula*. Under non-acclimated conditions, treatment with AVG caused a significant induction of *MtCBF* genes, thereby complementing these results [110].

Ethylene binds to the ethylene receptor ETHYLENE-RESPONSIVE 1 (ETR1) to activate the signalling cascade. In response, CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), a negative regulator of the cascade, controls phosphorylation-dependent cleavage of ethylene-insensitive 2 (EIN2), a NRAMP-like integral membrane protein that then induces downstream TFs such as ETHYLENE-INSENSITIVE 3 (EIN3) and ein3-like 1 (EIL1) [111]. Ethylene signalling mutants of arabidopsis have increased freezing tolerance both before and after cold acclimation [94]. Moreover, overexpression of EIN3 resulted in hypersensitivity to freezing, which additionally correlated with suppressed expression of *CBF*s, whereas in an *EIN3 EIN1* double mutant, *CBFs* and CBF-regulated genes were constitutively induced. When addressing the underlying molecular modes, it was found that EIN3 functions as a repressor of *ARR5*, *ARR7* and *ARR15* transcription. ChIP experiments demonstrated that EIN3 directly binds to the promoters of these ARRs and to all CBFs to regulate their expression (Fig. 3). Interestingly, although EIN3 was shown to act as a negative regulator of cold responses, cold increased EIN3 protein stability in an ethylene signalling-dependent manner [94].

In *M. truncatula*, a mutant of SICKLE (MtSKL1), an orthologue of arabidopsis EIN2, was slightly more tolerant to freezing than the wild-type without cold acclimation but less tolerant to freezing after cold acclimation. Surprisingly, in *skl1* plants *MtCBF1*, *MtCBF3* and *MtCAS15,* transcripts were suppressed both with and without cold treatment, suggesting that ethylene has a positive regulatory role in cold responses [110]. In addition, results for arabidopsis were published that dispute the negative regulatory role of ethylene in cold stress signalling. An arabidopsis ethylene biosynthesis ACS *octuple* mutant that contained extremely low levels of ethylene was hypersensitive to freezing before and after cold acclimation. In agreement, under cold treatment, induction of *CBF1*, *CBF2* and *COR* genes was significantly lower in the ACS *octuple* mutant plants than in the wild-type. In support, the authors demonstrated that ethylene levels increased transiently after cold treatment in soil-grown arabidopsis plants, which was correlated with a transient increase in transcripts of different ethylene biosynthetic genes. Moreover,

microarray data were presented, which showed that a significant overlap exists between genes induced by ACC treatment and those induced by cold in arabidopsis and that approximately 48 % of genes suppressed in ACS *octuple* mutant plants are activated by low temperature [109].

A positive role of ethylene in cold tolerance and the regulation of *COR* genes was additionally proposed for chilling-sensitive plant species. Overexpression of the tomato ethylene response factor TERF2/LeERF2, whose expression is induced both by ethylene and cold, stimulated ethylene biosynthesis and enhanced freezing tolerance in transgenic tobacco, tomato and rice plants, which was associated with an induction of *COR* genes and reduced amounts of ROS in the transgenic plants [112, 113]. Moreover, application of the ethylene precursor ACC promoted freezing tolerance in tobacco and tomato [114]. Moreover, an ethylene-insensitive tomato mutant was hypersensitive to severe chilling, thereby supporting a positive regulatory role of ethylene in cold stress responses of this plant species [105]. Because ethylene functions as a repressor of plant growth and development [115], it is possible that ethylene is an additional hormone that may serve to restrict growth in response to cold.

Salicylic acid (SA) contributes to cold stress-induced growth repression

Salicylic acid is another hormone that appears to contribute to the low temperature-induced growth retardation of plants. SA is a phenolic compound that is in particular known for triggering defence reactions against biotrophic pathogens such as the hypersensitive response (HR). Mutants with elevated SA levels are severely dwarfed and exhibit necrotic lesions caused by excessive HR induction [116].

Plants synthesise SA from chorismate, and in the SA-deficient *NahG* and *enhanced disease susceptibility 5* (*eds5*) mutants growth is promoted at 5 °C, resulting in higher biomass and larger rosette leaf area as compared to wild-type plants [7, 117]. Moreover, SA-overaccumulating *constitutive expression of PR genes* (*cpr1*) mutant plants show reduced growth rates in low temperatures [7]. Interestingly, at low temperature, SA deficiency in *NahG* plants was correlated with enhanced transcription of the CK-regulated D-type cyclin CYCD3;1 that promotes the G1/S phase transition in arabidopsis [117]. This finding supported earlier results that had suggested that, in response to environmental cues, SA could affect cell cycle progression in plants [118] and this may contribute to SA-induced growth inhibition in the cold.

Cold-induced increases in SA levels were reported for both chilling-sensitive and freezing-tolerant plant species [7, 84, 119–121]. In cucumber seedlings exposed to 8 °C, levels of free and conjugated SA increased three- to five-fold [119], which was correlated with an induction of the SA biosynthetic enzyme phenylalanine-ammonia-lyase (PAL). Interestingly, in cucumber, organ-specific changes in SA levels were observed, whereas in roots, SA accumulated rapidly during the first 24 h following the cold treatment; SA accumulation was much slower in leaves where a maximum was reached after 72 h only [119].

In addition, SA contents increased following cold application in arabidopsis; however, a significant accumulation was detected only during the second week of low temperature treatments [7, 120], whereas growth inhibition occurred immediately [7]. Therefore, SA may act to mediate growth repression in later stages of cold exposure, when the role of GA becomes less pronounced [58]. In contrary to the chilling-sensitive species cucumber, SA biosynthesis in arabidopsis was induced through the isochorismate synthase (ICS) pathway, because *ICS1* transcript levels were induced after 1 week of exposure to low temperature, and a loss-of-function of *ICS1* abolished low temperature-induced SA accumulation [120] (Fig. 2).

The mechanism used to control SA biosynthesis in response to cold is currently unknown, although in *LOS2* knockouts and overexpressing plant SA levels were increased. These effects were attributed to a loss of *ENO2* function, which impaired lignin biosynthesis and resulted in collapsed walls; the damage caused to cellular structures was thought to increase SA levels as a secondary response [47]. Because LOS2 is cold stress induced [45], it appears possible that in response to cold, ENO2 function in glycolysis is altered, and this may impact on SA production.

A link between cold-induced SA accumulation and biotic stress signalling was revealed when the chilling-sensitive (*chs*) mutants *chs2* [121] and *chs3* [122] were characterised. These mutants accumulate high levels of SA specifically when grown in the cold. The mutations were dominant and mapped to genes encoding resistance (R) proteins of the TIR-NB-LRR class [121, 122]. Therefore, certain R proteins appear to impact on the induction of SA biosynthesis under low temperature regimes and this warrants future investigation.

The downstream regulatory cascades that enable SA to participate in cold responses are not entirely clear. Whereas two studies reported that in SA-deficient mutants, *CBF* expression was not altered and no significant differences in freezing tolerance following cold acclimation occurred [117, 120], others concluded that SA suppresses *CBF* expression and freezing tolerance. This was based on the result that in *NahG* plants, increased freezing tolerance correlated with increased expression of *CBF3, COR47* and *KIN1* following cold acclimation. Moreover, the SA-overaccumulating lines *siz1-2* and *acd6* were

hypersensitive to freezing with and without cold acclimation, which correlated with suppressed expression of *CBF3, COR47* and *KIN1* [123]. Hypersensitivity to freezing and reduced *CBF3* expression of *siz1-2* and *acd6* was abolished in the *NahG* background [123], which is somewhat surprising for *siz1-2*, because in this background, ICE1 SUMOylation and activity in CBF3 transcription were compromised [41].

In the chilling-sensitive plant cucumber, SA was proposed to activate *CBF* expression. Application of the SA biosynthesis inhibitors paclobutrazol (which additionally acts on GA biosynthesis) or L-α-aminooxy-β-phenylpropionic acid (AOPP) suppressed expression of *CBFs* and *COR47* in low temperature-treated seedlings [119]. In addition, the chilling tolerance of such plants decreased compared with untreated controls. Low SA levels in treated plants were correlated with reduced photosynthetic efficiency and greater membrane damage, as well as reduced expression of genes encoding enzymes for carbon assimilation and synthesis of cryoprotectants such as sucrose and proline [119]. Because in arabidopsis, SA had no or little effect on carbon assimilation rates and photochemical efficiency [7], it appears that species-specific differences occur in SA function in photosynthesis under cold stress.

Jasmonic acid (JA) may participate in prioritising stress responses over growth

Similar to SA, JA is also involved in biotic and abiotic stress responses and is thought to repress growth in response to cold. JA is an oxylipin whose levels increase under cold stress in different plant species including wheat [84], rice [86, 87] and arabidopsis [124]. This increase is correlated with increased expression of JA biosynthetic genes in arabidopsis and rice [87, 124] and repression of genes encoding enzymes involved in JA catabolism in rice [87] (Fig. 2).

JA is synthesised from linolenic acid and is activated by conjugation with isoleucine, which enables binding to CORONATINE INSENSITIVE 1 (COI1), an F-box protein that acts as a JA receptor. COI1 initiates JA signalling by ubiquitinating and thereby stimulating proteasome-dependent degradation of JASMONATE ZIM DOMAIN (JAZ) proteins, which repress expression of JA-responsive genes. Exogenous application of JA enhanced induction of *CBFs* and CBF-regulated genes following cold treatment and promoted freezing tolerance in arabidopsis. Moreover, plants compromised in JA biosynthesis or signalling were hypersensitive to freezing [124]. It was proposed that this altered freezing tolerance is mediated by JAZ1 and JAZ4, which physically interact with ICE1 and ICE2 to repress their transcriptional activity. Overexpression of *JAZ1* and *JAZ4* inhibited expression of *CBFs* and their downstream targets and repressed freezing tolerance before and after cold acclimation (Fig. 3). Interestingly, microarray analysis of the JA signalling-deficient *coi1-1* allele following low temperature treatment demonstrated that some of the *COR* genes of non-CBF regulons were differently regulated, which indicated that JA may additionally modulate cold acclimation through CBF-independent pathways [124].

In regard to the role of JA as a growth repressor in the cold, it was shown that JA-mediated growth inhibition occurred through stabilisation of DELLA proteins, which was speculated to allow JA to prioritise defence mechanisms over growth [125]. Therefore, it appears that chilling-induced JA accumulation contributes to suppression of plant growth, at least partially through cross-talk with GA signalling.

Conclusion

In summary, it is clear that hormones act as central regulators of cold stress responses in plants. However, our knowledge of their regulatory activities remains limited and seemingly contradicting results have occasionally been published.

Several reasons may account for this fact. First, changes in hormone levels at the cellular levels are usually not determined, although it is known that local hormone concentrations control adaptive growth and development. Rather, whole plants are usually being used for measurements, which is due to the fact that most phytohormones are present in extremely low concentrations and quantification at the cellular level is very challenging. Thus, results may be more enlightening if hormone measurements are complemented with reporter studies that allow for high spatial resolution.

Second, the experimental setups are decisive for determining cold stress tolerance of plants. However, no standardised procedures exist as yet. Rather, systems as different as responses of seedlings grown on agar plates and adult plants grown in soil are being used. Because both intrinsic developmental programs and environmental factors impact cold stress responses, experiments should be conceived to account for these facts more frequently. This is challenging because even seemingly little details such as the daytime of the treatment have to be taken into account, as many implicated genes, for example CBFs [65], are under circadian control.

Third, hormone mutants often have strong morphological defects, which likely impact on the outcome of chilling or freezing tolerance assays due to secondary effects that are not related to cold signalling but rather to decreased

biomass, altered membrane morphologies and compositions or defective developmental phase transitions (such as flowering time control). Moreover, dwarfism as in GA, BR, auxin and SA mutants may reduce cold damage simply because there is less vegetative tissue exposed. In such cases, it will be necessary to additionally include weak alleles that are less impaired in growth and complement results of qualitative assessments such as survival rates with those of quantitative evaluations such as electrolyte leakage following freezing treatments [126].

Developmental biologists have already established techniques required for the study of plant hormone action under those highly challenging circumstances. In the future, it will be important that these systems are more readily adopted and optimised for the study of cold stress responses, which would further facilitate the rapid progress been made in the field in recent years.

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