Metabolic and Hormonal Responses of Plants to Cold Stress

Joanna Lado and Matías Manzi

Abstract The adaptation and survival of plants in challenging environments involves changes at cellular and molecular levels. Plants inherently possess sophisticated mechanisms to timely perceive environmental stimuli and respond accordingly. Temperature and water availability are the major environmental factors that significantly influence geographical distribution of plants. In particular, cold acclimation includes structural and morphological modifications, changes in cell membrane composition and the accumulation of compatible solutes among others cryoprotective compounds. In order to survive in such non-optimal conditions, plants have developed complex mechanisms to perceive external signals and trigger crucial responses. These phenomena are mediated by complex phytohormone networks that are also involved in the adaptation to other abiotic and biotic stresses. Through hormonal signaling, cold stress modifies biomass and bioactive compounds accumulation in medicinal plants, both of special interest regarding their biological activity and therefore, their pharmacological potential. In this chapter we review the latest information regarding cold signaling in plants, phytohormones and how integrated crosstalk underpins cold tolerance together with a special mention of cold effect on bioactive compounds accumulation, with emphasis in medicinal and aromatical plants.

Keywords Abscisic acid · Bioactive compounds · Brassinosteroids · Chilling · Ethylene · Jasmonic acid · Salycilic acid

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Introduction

Plants have developed a striking set of different mechanisms to adapt and survive in challenging environments facing biotic and abiotic stresses. Cold is one of the most harmful abiotic stresses for plants due to its notable impact on different developmental processes and its huge influence on crop productivity and geographic distribution (Chinnusamy et al. 2007; Rahman 2013). Cold stress directly affects plant growth and development, inducing several physiological, metabolic and genetic mechanisms (Ruelland et al. 2009). Under cold conditions plants rapidly arrest plant growth and modify plant morphology which are believed to underpin the reallocation of resources to other processes aiming to increase cold tolerance (Eremina et al. 2016). Structural and celular changes associated with cold response involve the alteration of membrane conformation, leading to a decrease in membrane fluidity (Sevillano et al. 2009; Upchurch 2008) together with a rapid generation of reactive oxygen species (ROS) which could cause oxidative stress if plants are not able to detoxify them by activation of the antioxidant system (Ruelland et al. 2009; Sevillano et al. 2009). In addition, low temperatures induce a rise in the content of cryoprotective proteins and metabolites such as soluble solids and amino acids. In order to adapt and survive, plants have also developed diverse cellular enzymatic and non-enzymatic mechanisms to cope with cold stress, the vast majority of which are mediated by phytohormones (Ruelland et al. 2009; Eremina et al. 2016).

Plants ability to minimize and survive oxidative stress depends on the effectiveness of enzymatic and non-enzymatic antioxidants, a complex system that is triggered in response to low temperature (Shi et al. 2014; Chinnusamy et al. 2010). As mentioned, many of these processes are mediated by phytohormones, involving the participation of stress-related hormones such as abscisic acid (ABA), etylene (ET), salicylic acid (SA) and jasmonic acid (JA) and a tight interaction with growth-promoting cytokinins (CKs), auxins (AUXs), gibberellins (GAs) and brassinosteroids (BRs). Phytohormones act as secondary signals initiating a cascade of events that may follow almost a generic pathway with the involvement of different components (Shi et al. 2014; Ahammed et al. 2015; Lado et al. 2016; Peleg and Blumwald 2011). Signal perception is followed by the generation of second messengers such as calcium, ROS or nitric oxide (NO), a generic signal transduction pathway that eventually involves a protein phosphorylation cascade to initiate transcription of specific sets of stress-regulated genes as well as biochemical changes at tissue and celluar levels (Fig. 1). The proper spatial and temporal coordination of all signaling molecules is essential for successful cold acclimation or adaptation (Ahammed et al. 2015).

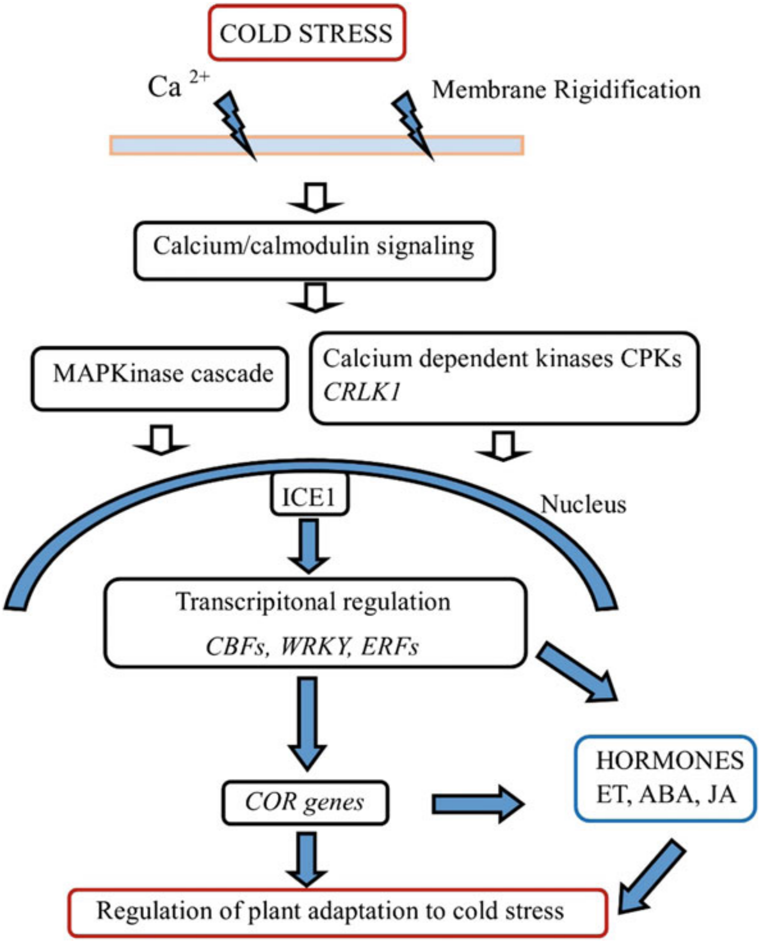

Fig. 1 Schematic representation of main pathways and interactions towards cold response and adaptation in plants

Physiological, Biochemical and Molecular Responses to Cold Stress

Low temperature causes morphological and structural changes, alters membrane fluidity, triggering changes the activity of macromolecules and osmotic potential in the cell. Cold-induced damage in photosynthetic apparatus includes inhibition of CO_2 assimilation, photoinhibition of photosystem I (PSI) and PSII, and inhibition of diverse enzymatic activities. There is also an enhancement in the production of ROS which induces damage to membranes, proteins, lipids, DNA and RNA, among other cell constituents (Sevillano et al. 2009). Transgenic plants overexpressing

ROS scavengers or mutants with enhanced ROS scavenging capacity usually exhibit increased tolerance to cold stress (Ahammed et al. 2015).

It is still no clear how low temperatures are perceived in plants, however, the involvement of membranes appears to be crucial in this process (Shi et al. 2014). Lipid composition is a key factor in the stabilization of membranes and therefore plays a role in the enhancement of cold tolerance. Unsaturated fatty acids have been associated with cold tolerance since a greater amount was determined in the plastid membrane of cold-resistant compared to cold-sensitive plants (Upchurch 2008). The fluid mosaic physical state of the plasma membrane is vital for the structure and function of the cells, as well as to sense temperature variations. At low temperatures plant membranes undergo transition from a liquid crystalline to a gel-like phase with reduced fluidity while ion leakage and deactivation of membrane-integrated proteins occurs (Upchurch 2008), where free, esterified and glycosylated sterols play a crucial function (Valitova et al. 2016; Grunwald 1971). Lipid changes detected only in the cold-acclimating wild potato (*Solanum comersonii*) species included an increase in phosphatidylethanolamine, a decrease in sterol to phospholipid ratio, an increase in linoleic and a decrease in linolenic acid (Palta et al. 1993). Sterol acylglycosides are present in plant cells in minor amounts but it was demonstrated that relative changes in their concentration in the plasma membrane, along with other lipids, are associated with plants adaptation to low temperatures (reviewed in Valitova et al. 2016).

Calcium-mediated signaling is believed to play a pivotal role in plant responses to cold stress. Currently, a widely accepted hypothesis states that the reduction in membrane fluidity appears to be a primary event of cold perception, activating Ca^{2+} channel in higher plants. Ca^{2+} acts as secondary messenger in this process, triggering responses at the nuclear level (Shi et al. 2014). It was also described that receptor-like kinases (RLKs) are involved in this perception, amplification and transmission of environmental stimuli via signaling cascades that finally modulate gene expression, protein activation and cell adjustment (Ye et al. 2017; Wang et al. 2017). *CRLK1*, a calcium/calmodulin-regulated RLK is mainly localized in the plasma membrane and its protein levels increase notably after cold exposure, suggesting that it is involved in a cold-related oxidative stress signal transduction pathway (Fig. 1). Numerous reports have indicated that low and high temperature as well as hormones induce specific changes in the expression of *CDPK* genes in many plants (Ye et al. 2017; Wang et al. 2017; Xiao et al. 2017). Moreover, *CRLK1*-knockout mutant plants exhibited increased sensitivity to cold and had delayed induction of cold-responsive genes compared to wild type plants. These results indicated that *CRLK1* is a positive regulator of cold tolerance in plants and acts as a bridge in calcium signaling and cold signaling (Ye et al. 2017; Yang et al. 2010). Similarly, it was recently described that *MaCDPK7* gene is involved in regulating banana fruit ripening and chilling resistance (Wang et al. 2017) whereas its orthologue in rice (*OsCDPK7)* is induced in response to cold and its overexpression confers an enhanced cold tolerance (Saijo et al. 2000).

In the same line, the LRR receptor kinases are one of the largest and most renowned classes of RLKs, playing important functions towards cold stress tolerance. The perception of cold stimuli by receptors can activate large and complex intracellular signaling cascades, leading to the generation of secondary signaling molecules including $Ca^{2+,}$ ROS and inositol 1, 4, 5-trisphosphate (reviewed in Ye et al. 2017). Therefore it can be suggested that plant cells can perceive cold stress via membrane rigidification (Shi et al. 2014) and interestingly, histidine kinases (HKs) are also an important class of hormone receptors (Ha et al. 2012), being a possible point of convergence between cold sensing and phytohormone responses (Shi et al. 2014).

In addition to the plasma membrane, chloroplasts are among the most severely impacted organelle and could be contributing to ambient temperature sensing (Kratsch and Wise 2000). The maintenance of polyunsaturated fatty acid levels in chloroplast membranes has been shown to contribute to plant survival under low temperatures and to the normal formation of chloroplastic membrane in plants under cold stress (Upchurch 2008). Saturated phosphatidylglycerol (PG) content may be related to the phase transition temperature and thus related to the low-temperature adaptability of plants, being its proportion 40% or higher in cold-sensitive plants and lower in cold tolerant species (Upchurch 2008). Notable changes also occur at the photosynthetic apparatus; under low temperature, the ability to use the absorbed light energy decrease leading to an overreduction of PSII and consequently, to enhance the generation of ROS (Janda et al. 2014). Furthermore, cold-acclimated plants usually show less photoinhibition of PSII than cold-sensitive plants being therefore a good indicative of cold tolerance (Janda et al. 2014). Further, protein folding is influenced by temperature changes. Therefore, plant cell can sense cold stress and act through membrane rigidization, protein/nucleic acid conformation, and/or metabolite concentration, either a specific metabolite or the redox status (Chinnusamy et al. 2010).

Regarding molecular changes in response to cold stress, *Lotus japonica* exposed during 24 h to low temperatures upregulated genes related to lipid, cell wall, phenylpropanoid, sugar, and proline metabolism, while downregulated genes involved in photosynthesis and chloroplast development (Calzadilla et al. 2016). Moreover, the same authors described a total of 41 cold-inducible transcription factors, including members of the AP2/ERF, NAC, MYB, and WRKY families. In this sense, the best understood cold signaling pathway is mediated by *ICE1/CBF/COR* transcriptional cascade. The *C-repeat* (CRT)-*binding factors* (CBFs)/*dehydration-responsive elements* (DREBs) are induced by cold, binding to the promoter of *cold responsive* (*COR*) genes, activating their transcription. There are evidence showing that this pathway is tightly regulated at transcriptional, postranscriptional and postranslational levels (Shi et al. 2014; Kurepin et al. 2013). *CBF14* gene expression is considered as a marker of cold-treatment in wheat and was induced in response to cold treatment in wheat plants, in proportion with their freezing-sensitivity/tolerance being its basal levels higher in cold-tolerant genotypes (Ba et al. 2016). It is noteworthy that *CBF* genes are rapidly and transiently induced by cold while induction of *COR* genes by cold is much slower. A peak of *CBF1* and *CBF3* genes expression after 1 h of cold expossure was measured but expression levels drop off further on while no relevant changes were observed in *CBF2*

(Calzadilla et al. 2016). A higher expression of these TFs in different herbaceous and woody plants has been associated with an increased tolerance to low temperatures (Chinnusamy et al. 2007; Ba et al. 2016; Zhou et al. 2011; Champ et al. 2007; Zhou et al. 2014). Therefore CBFs appear to play a pivotal role in cold stress signaling and response in different plants showing a fine specializaton for the different isogenes. Key components of the ET, GA and JA signaling pathways have been shown to also modulate CBFs in different extents (Achard et al. 2008). Moreover, certain *CBF* genes are induced by exogenous application of ABA, contributing to sustain cold tolerance (Knight et al. 2004).

In Arabidopsis, the CBF/DREB1 pathway is controlled by an MYC-type factor known as ICE1 (Fig. 1). Overexpression of *ICE1* confers increased freezing tolerance whereas *ice1* mutant exhibited high chilling and freezing sensitivity (Chinnusamy et al. 2003). Similarly, in chilling-tolerant tomato (*Solanum chilense*) a higher constitutive expresson of *ICE1* was described (Nosenko et al. 2016). Around 40% of *COR* genes and nearly 46% of cold-regulated transcription factors genes are regulated by ICE1 which suggests that ICE1 functions as a master regulator controlling *CBF3/DREB1A* and many other *COR* genes (Lee et al. 2005). Interestingly, it was recently demostrated that CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 3 (CAMTA3) and CAMTA5, which bind to the CGCG-box in ICEr2 in order to activate the expression of *DREB1B* and *DREB1C*, only respond to a rapid but not to a gradual decrease in temperature. Moreover, plants appear to differentially regulate daily temperature changes, since CAMTA acts during both day and night whereas usual *DREB1*-regulating circadian components such as CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL 35 (LHY) only function during the day. These findings demostrate that the adquisition of tolerance to low temperatures could follow different pathways depending on the speed of temperature decrease (Kidokoro et al. 2017). In addition to the above mentioned CBF-dependent pathway, some CBF-independent components have a function in cold signaling. In fact, transcriptome analysis, indicated that only ~12% of the *COR* genes are controlled by CBFs (Fowler and Thomashow 2002). The Arabidopsis *esk1* mutant shows constitutive freezing tolerance that is independent of the CBF regulon (Xin and Browse 1998). Loss of *HOS9*, a homeobox transcription factor, causes reduced freezing tolerance without affecting the expression of CBFs and their target genes (Zhu et al. 2004). Also, *GIGANTEA* (*GI*), which encodes a nuclear-localized protein involved in flowering and the circadian clock, is induced by low temperature. The *gi-3* mutant shows both decreased constitutive cold tolerance and impaired cold acclimation ability without affecting *CBF* expression (Cao et al. 2005). It was also suggested that ABA, GA and CK could also regulate cold responses via CBF-independent pathways (Shi et al. 2014; Kosová et al. 2012). Thus, mechanisms dependent and independent of CBF signaling cascade appear to mediate in plant responses to cold and both could be behind the interaction with different phytohormones.

Key Phytohormones Involved in Plant Cold Tolerance

Biological activity of hormones depends on its availability which is controlled by its synthesis, transport and conjugation and catabolism as well as by the efficiency of signal perception and transduction. This processes are also integrated with tight cross-talks among different phytohormones and the interaction of other biological process and signaling molecules (reviewed in Deb et al. 2016). Then, the final plant response to low temperature is not determined by the specific activity of a single hormone but rather by a complex interactive network of diverse signaling molecules. Nearly half of the genes differentially expressed in response to a one-day cold shock in wheat were related to hormone metabolism (Ba et al. 2016), illustrating their key relevance in plant's cold stress response.

Growth-Promoting Phytohormones

Despite what could be first guessed based on their promoting role towards plant development, the phytohormones GAs, AUXs and CKs also display relevant functions in plants cold stress response. In general, growth rate decline at the beginning of cold stress meanwhile plant architecture is modified generally in response to a decrease in GA, AUX and CKs metabolism (Rahman 2013; Vanková et al. 2014).

A decrease in GA content in mostly all organs of wheat plants has been described in response to cold stress (Vanková et al. 2014) mainly due to a rise in the expression of GA-catabolizing *GA2ox* gene, and a repression of GA-biosynthetizing GA20ox enzyme (Kurepin et al. 2013). Besides, *CBF1/DREB1B*-overexpressing plants showed growth retardation, explained in part by the accumulation of DELLA proteins (negative regulators of GA signaling), a phenotype that could be easily rescued by the exogenous application of GAs (Achard et al. 2008). Therefore, it was suggested that *CBF1* enhances the accumulation of DELLA proteins by reducing GA content through the stimulation of *GA2ox* genes expression (Achard et al. 2008). These data indicate that *CBF/DREB1*-dependent signaling pathway partially regulates plant growth through GAs modulation by the regulation of DELLA proteins. It was also shown in tobacco that *CBF* overexpression enhances cold tolerance and causes growth inhibition through the interaction with GAs and cell cycle pathways. Moreover, the observed dwarfism as well as the cold-induced *CBF* expression and its increased cold tolerance were arrested by exogenous GAs application (Zhou et al. 2014), showing that a reduction in the level of this phytohormone is necessary to develop cold tolerance in plants. Additionally, a GIBERELLIC ACID INSENSITIVE (*gai*) mutant showed an impaired GA production and a higher cold tolerance (correlated with elevated levels of CBF1, CBF2 and COR15A proteins), a response mediated by the action of the GATA TFs (Eremina et al. 2016).

The knowledge about the exact role of AUXs in cold stress signaling is still limited. An increase in endogenous IAA level was reported in rice seedlings in response to cold stress (Du et al. 2013a) however cold tolerance was higher in mutants with a lower IAA level (Du et al. 2013b). It is generally considered that intracellular AUX transport and hence, AUX gradient, plays a major role in controlling hormonal crosstalk with other phytohormones and therefore, in the regulation of plant growth and development under cold stress conditions (Rahman 2013). It was described that cold stress inhibit certain processes governed by this phytohormone, such as root gravity response (Shibasaki et al. 2009) which suggests that cold adaptation involves a decrease in internal levels of indol-3-acetic acid (IAA). However, since AUX signaling mutants (*axr1* and *tir1*) showed a reduced gravity response but a normal cold tolerance, it was suggested that cold stress affects more AUX transport rather than AUX signaling (Shibasaki et al. 2009). It was also demonstrated by direct transport assays that cold stress inhibits AUX transport, suggesting that its effect is linked to the inhibition of intracellular transport (Rahman 2013; Shibasaki et al. 2009). Besides that, a crosstalk among IAA, JA and ABA has been reported since low temperature induce JA and IAA accumulation in rice seedlings (Du et al. 2013a) whereas lines overexpressing an enzyme (OsGH3-2), that catalyzes IAA conjugation into amino acids (with the concomitant reduction in free IAA content), finally derived in a rise in ABA levels and in a higher cold tolerance (Du et al. 2012).

As described for GAs and AUX, there is also a drop in CKs content as well as in its direct precursors in response to cold stress (Ba et al. 2016). There is also a differential regulation of genes from CK metabolism under cold stress in wheat plants (Ba et al. 2016) while CK receptors function as negative regulators in plant responses to low temperatures through an still unknown mechanism (Shi et al. 2014; Jeon et al. 2010). CK signaling is based in a multistep two-component system involving the membrane sensor Arabidopsis histidine kinase CKs receptors (AHK2-AHK4), Arabidopsis histidine phosphotransfer proteins (AHP1-AHP5) and Arabidopsis response regulators (ARRs), being the last localized in the nucleus (Jeon et al. 2010). Among ARRs (DNA binding TFs) two types (A and B) have been descibed, being the former negative and the second positive regulators of CKs signaling. It was demonstrated that *AHK2* and *AHK3* and the cold-inducible ARRs (A-type) play a negative regulatory role in cold stress signaling via inhibition of ABA response, occurring independently of the cold acclimation pathway (Jeon et al. 2010).

Cytokinin response factors (CRFs) are transcripiton factors localized in the nucleus, that belong to the APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) superfamily, being transcriptionally regulated by CKs and it is proposed that they dislpay an integrative role between environment and hormonal cues (Kim 2016). ARR1, ARR10 and ARR12 were described to upregulate a *CRF2* expression by binding direclty to its promoter in response to cold stress. However, *CRF3* expression remains unaltered in the two-component system mutants, suggesting that cold response of *CRF3* occurs independently of this two-component system (Kim 2016). Both CKs response factors (*CRF2* and *CRF3*) integrate the environmental cold signal into lateral root development, contributing root adaptation to cold stress (Jeon et al. 2016). Similarly, *CRF6* was described to mediate a connection between CKs and oxidative stress response in part by repressing the expression of CK-related genes (Zwack et al. 2016). Apart from CKs singnaling, *CRF*s have been described to be involved in the regulation of other phytohormones such as ABA, SA, JA, AUXs and BRs. Moreover, *CRF*s mediate a subset of CKs responses, acting not only through CK signaling but also independently of CKs (reviewed in Kim 2016). Certain CRFs like *CRF2* (but not *CRF3*) are also capable of transcriptionally regulating AUX efflux carriers (*PIN FORMED-PIN* genes), participating also in the fine-tunning of auxin transport (Šimášková et al. 2015). It was therefore proposed by these authors that *CRF*s represent a missing cross-talk component that regulate auxin transport capacity downstream of CKs signaling to control plant development, exerting a special function under cold stress conditions.

Brassinosteroids are a class of plant steroid hormones whose general effects are the promotion of cell elongation, cell division, differentiation, disease resistance, stress tolerance, exerting also a role in plant senescence. Thus, BRs possess significant growth-controlling activity as well as a function in reproductive and vascular development, membrane polarization and proton pumping. They also influence various other developmental processes like seed germination, rhizogenesis, and flowering (Bartwal et al. 2012). A key point is that BRs function strictly depends on tissue concentration, since opposite effects can be observed at high or low levels (i.e. stomatal aperture or photosynthesis regulation (Ahammed et al. 2015)).

A positive role of BRs in chilling stress tolerance was described in certain crops such as maize, cucumber, tomato and rice (Divi and Krishna 2009). Exogenous application of BR induces the expression of stress-related genes, leading to the maintenance of photosynthetic activity, activation of antioxidant enzymes, accumulation of osmoprotectants and induction of other hormones responses. The former suggest a role for BRs in promoting cold tolerance in Arabidopsis seedlings (Divi and Krishna 2010). Furthermore, mung bean epicotyls whose growth was initially suppressed by chilling partly recovered their ability to elongate after treatment with 24-epibrassinolde (a bioactive BRs). In this experiment, 17 proteins involved in methionine assimilation, ATP synthesis, cell wall construction and the stress response were down-regulated by chilling but re-up-regulated in response to exogenous application of BRs (Huang et al. 2006). The same bioactive BR was applied to young grapevine seedlings causing an enhancement in antioxidant enzymes activities (CAT, SOD and APX) and consequently, a reduction in ROS and lipid peroxidation. In addition, the exogenous treatment greatly increased the contents of free proline, soluble proteins and soluble sugars. These results indicated that exogenous BRs treatment could enhance the antioxidant defense system and reduce oxidative damage caused by ROS and lipid oxidation in plants (Xi et al. 2013). It was also suggested that BRs participate in the enhancement of cold acclimation in winter ryegrass by regulation of stress-related signaling compounds such as JA and ET but not SA (Pociecha et al. 2017).

BRs have been also suggested to underpin cold tolerance in the medicinal plant *Withania somnifera,* an effect that could be linked to the stimulation of ABA or ET biosynthesis (Bartwal et al. 2012). There was also reported that the increased cold tolerance of BRs-treated plants may be attributed to the induced changes in membrane stability, a key process affected by chilling (Bartwal et al. 2012). Recent studies described the existence of several BRASSINAZOLE-RESISTANT (BZR) transcription factors (positive regulators of BRs signaling) which are upregulated in response to exogenous ABA treatment and showed differential expression in response to low temperature stress in *Brassica rapa* plants. These TFs were proposed to activate CBF-mediated cold responses in this species, displaying a key regulatory function in cold tolerance (Saha et al. 2015). In addition to various stimulatory effects on growth, development and stress tolerance in field plants, BRs were described to improve post-harvest quality of fruits and vegetables through extension of shelf life under chilling stress (Ahammed et al. 2015). However other studies support the idea that downregulation of these compounds could be also part of certain plant responses to low temperatures, therefore BRs role in cold tolerance is still elusive (Calzadilla et al. 2016).

Stress-Responsive Phytohormones

ABA regulates many physiological and developmental processes in plants and it is also considered as the most important hormone in the response to abiotic stress (Gómez-Cadenas et al. 2015). In fact, nearly 10% of the protein encoding-genes are regulated by ABA (Nemhauser et al. 2006). ABA accumulation in plant tissues constitutes a common response in order to cope with abiotic stresses, including low temperature (Eremina et al. 2016). However, there are many genes which are induced in response to cold stress but remain irresponsive to ABA (Shinozaki et al. 2003), indicating the existence of both ABA-independent and ABA-dependent signal transduction pathways (Yamaguchi-Shinozaki and Shinozaki 2006). Exogenous application of ABA before the onset of stress generally favors cold tolerance in different plants (Li et al. 2014; Fu et al. 2017). Most of these responses are related to an enhancement of antioxidant defense system, reducing the harmful effect of ROS and other molecules such as NO (Fu et al. 2017; Liu et al. 2011), a key system that is considered the major factor involved in the tolerance to cold stress (Du et al. 2013b). In this sense, both, enzymatic and non-enzymatic antioxidants are stimulated by ABA application during chilling exposure (Wang et al. 2013).

There is still some controversy regarding the precise involvement of ABA in cold stress. Plant stress responses have been divided into ABA-independent, such as the above mentioned CBF and ICE1-regulated; and ABA-dependent responses, including bZIP transcription factors known as ABA Responsive Element Binding Protein/Factors (AREB/ABF) (Nakashima et al. 2014). Moreover, a parallel effect of dehydration/osmotic stress occurs during cold stress, being water deficit also common consequence of low temperatures, inducing therefore overlapping responses between dehydration and low temperatures (Fu et al. 2017). The implication of ABA on the tolerance to water deficit is well-known (Gómez-Cadenas et al. 2015; Gómez-Cadenas et al. 2014) and involves ABA-dependent and ABA-independent mechanisms (Nakashima et al. 2014), whereas cold stress mostly triggers and ABA-independent pathway (Yamaguchi-Shinozaki and Shinozaki 2006; Fu et al. 2017; Vishwakarma et al. 2017). However, ABA has been recently proposed to control *COR* genes expression through the regulation of CBFs (Eremina et al. 2016). Thus, there appears to be an intricate interaction among genes which is regulated by dehydration and low temperatures, evidenced by a reduced expression of cold and drought stress related genes in an Arabidopsis *los5* mutant (negatively affected in ABA biosynthesis) which also showed an extreme sensitivity to low temperatures (Xiong et al. 2001). ABA content increases during plant exposure to cold being the more tolerant genotypes which showed a faster and higher ABA accumulation compared to the sensitive ones (Kosová et al. 2012; Janowiak et al. 2002). Moreover, key enzymes (β-carotene-3-hydroxylase-βCHX, zeaxanthin epoxidase-ZEP and 9-*cis*-epoxycarotenoid dioxygenase-NCED1) involved in ABA biosynthesis were induced in wheat plants in response to low temperature, being this rise more intense in cold-tolerant varieties (Ba et al. 2016). ABA catabolism was also down-regulated in those cold tolerant plants through the repression of a cytochrome P450 8-hydroxylase gene (*CYP707A2*), which catalyzes the first step of ABA catabolism toward phaseic and dihydrophaseic acids (Ba et al. 2016). Furthermore, a negative interaction between ABA and CKs signaling was described in rice seedlings under cold stress, showing that the cold-induced ABA increase is linked to CKs signaling (Maruyama et al. 2014).

Interestingly, exogenous application of certain molecules increases the ABA levels leading to an alleviation of cold stress. In this sense, melatonin, which was recently discovered in plants and it is present in high amounts in several medicinal plants such as feverfew (*Tanacetum parthenium*), St John's wort (*Hypericum perforatum*), and Huang-qin (*Scutellaria baicalensis*) (Bajwa et al. 2014), was shown to induce the antioxidant defense via ABA-dependent and ABA-independent pathways in the grass *Elymus nutans* (Fu et al. 2017). Melatonin increases ABA levels but there were no evidence of a reciprocal effect of ABA on melatonin endogenous content, suggesting that this compound should be acting upstream of ABA (Fu et al. 2017). Similarly, celastrol, a triterpenoid isolated from *Tripterygium wilfordii* which is used to treat some systemic illnesses, was demonstrated to enhance cold tolerance in cucumber by inducing the activities of antioxidant enzymes, reducing the oxidative stress and increasing the ABA levels by upregulating its biosynthesis through the expression of *NCED2* gene (Zhu et al. 2017).

ET is involved in abiotic stress resistance and specifically in cold stress response however, the question of whether it exerts a positive or negative role remains still elusive. Some works report changes in ET production in response to low temperatures (Kazan 2015). In Arabidopsis, an increase in ET production was related to a higher activity of biosynthetic enzymes ACC synthase and ACC oxidase in response to cold while the application of the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) enhanced cold tolerance (Catala et al. 2014). Similarly, the application of the ET biosynthesis inhibitor 1-methylcyclo- propene (1-MCP) reduces cold tolerance in tomato, suggesting a positive ET effect on cold tolerance (Zhao et al. 2009).

Ethylene perception and response in plants is carried out by a two-component receptor kinases system. ET binds to its receptors ETHYLENE-RESPONSIVE1 (ETR1) which are a superfamily of negative regulators of this signal and activates the signaling cascade through CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), a Ser/Thr kinase that dimerizes when active and suppresses the ethylene response. CTR1 controls ethylene-insensitive 2 (EIN2) cleavage, a membrane integral protein that induces downstream nuclear TFs such as ETHYLENE INSENSITIVE 3 (EIN3) and ein3-like 1 (EIL1) which stimulates ethylene response genes (Merchante et al. 2013). In the absence of ET, active receptors negatively regulate EIN2 through phosphorylation via the protein kinase CTR1. Upon ethylene perception, the receptors become inactivated, which in turn facilitates EIN2 activation to mediate ethylene signaling through the nucleus, inducing the activity of EIN3/EIL1 and ERFs (Merchante et al. 2013).

ERFs are TFs located downstream in the signaling pathways of many hormones such as ET, JA and ABA, and are possibly a cross-talk point among these signaling pathways. ERFs family is huge and each member responds to different biotic and abiotic stresses, regulating many biosynthetic pathways (Mizoi et al. 2012). The overexpression of soybean *GmERF9* in tobacco enhanced the accumulation of proline and soluble carbohydrates as well as cold tolerance compared to wild type, indicating a positive role of this TF cold tolerance (Zhai et al. 2017). Tomato *ERF2* showed a role in regulating ethylene biosynthesis and a higher expression was determined under cold stress, implying that this gene may be involved in cold response through ethylene modulation. Moreover, overexpression of this gene in tobacco showed a higher expression of *COR* genes, an enhanced cold tolerance and a reduced membrane damage (Zhang and Huang 2010). ET can also induce the expression of calcium dependent kinases (*CDPK* genes), which positive role in cold tolerance has been previously described (Ye et al. 2017) in many different plants including banana (Wang et al. 2017), tomato (Chang et al. 2009), grape (Zhang et al. 2015) and rubber tree (Xiao et al. 2017). However this family is integrated by at least 30 different members which exhibit different expression patters, suggesting that their roles are distinct during plant development and stress response (Xiao et al. 2017). *LeCFB1* expression and cold tolerance in tomato plants and fruits was induced by ET as well as by ethephon application, underpinning a positive role of this hormone on cold tolerance (Zhao et al. 2009).

Contrastingly, other studies determined at least a 90% reduction in ET content in *Medicago truncatula* plants after cold exposure together with a lower ACC oxidase activity (Zhao et al. 2014). This reduction is consistent with a negative role of ET towards cold tolerance in accordance with the fact that its exogenous application (or the application of its direct precursor ACC) causes a significant drop in *M. truncatula* freezing tolerance. Moreover, the application of ET inhibitors promoted this tolerance through *CBF3* expression regulation (Zhao et al. 2014). Supporting also this negative role of ET, cold tolerance was reduced in Arabidopsis *ethylene overproducer1* as well as after the application of ethylene precursor ACC whereas it was increased with the application of an inhibitor of ET biosynthesis or perception (Shi et al. 2012). Moreover, ET insensitive mutants (*etr1-1, ein4-1, ein2-5, ein3-1,* and *ein3 eil1*) showed an enhanced freezing tolerance and they proposed that ET negatively regulates cold signaling at least partially through the direct transcriptional control of *CBF*s and *type-A ARR* genes by EIN3 (Shi et al. 2012). Therefore, it appears that the role of ET on cold tolerance could be species-dependent and that certain differences would exists between cold and freezing tolerance for this phytohormone.

JA positive role in cold and freezing tolerance was recently unveiled by different works, suggesting that cold exposure elevates endogenous JA levels by the induction of biosynthetic genes such as *LOX1, AOS1, AOC1* and *JAR1* in Arabidopsis (Hu et al. 2013) and *OsAOS, OsOPR1* and *OsLOX2* in rice (Du et al. 2013a). Furthermore, JA positively regulates Arabidopsis response to freezing since its exogenous application significantly enhanced freeze tolerance whereas the blockage of its endogenous biosynthesis and signaling rendered cold-hypersensitive plants (Hu et al. 2013). JA is described as a positive regulator of the ICE1 protein transcriptional activity (Zhao et al. 2013), which exerts a key role in cold signaling cascade (Fig. 1) as well as an inducer of CBFs expression in Arabidopsis (Hu et al. 2013), modulating cold stress responses. Therefore, jasmonate function is a critical upstream signal in ICE1-CBFs pathway to positively regulate low temperature tolerance in Arabidopsis (Hu et al. 2013).

In the same line, cold-tolerant tomato species showed a higher expression of the *FAD7* gene (Nosenko et al. 2016), which plays a role in JA biosynthesis, encoding a chloroplast ω-3 fatty acid desaturase involved in temperature-dependent remodeling of the thylakoid membrane fluidity (Upchurch 2008). Similarly, in the leaves of *Camellia sinensis* a higher expression of *FAD7* and *FAD8* genes was associated with a reduced damage in thylakoid membranes after cold exposure (Ding et al. 2016). An induction of JA and its precursor, α-linolenic acid production were described in *Camellia japonica* during cold stress (Li et al. 2016) and the rise in expression of JA biosynthetic genes indicate that JA and its signaling pathway may play critical roles in the early stages of cold acclimation in *C. japonica*. In this plant, genes from GAs, AUXs, ET, ABA, JA, CKs and BRs pathways were also identified to be differentially expressed during cold acclimation (Li et al. 2016). Thus, GA, ET, ABA and JA signaling were predominantly induced while the AUX, BR and CK pathways were mostly inhibited (Li et al. 2016).

A recent and detailed review about cross-talk among different phytohormones in rice suggest a tight interaction towards abiotic stress survival in plants (Deb et al. 2016). CKs conjugation is regulated by ABA and BRs whereas BRs biosynthesis is affected by both, ABA and CKs. JA and ET biosynthesis appears to be modulated by ABA through DREB factors and JA and SA pathways are also co-regulated but unlikely to influence each other production. Thus, a complex regulatory network is spun by multiple hormones which may modulate hormone biosynthesis pathways where biosynthesis of one hormone is affected by several others.

Apart from phytohormones, other compounds were described to exert as messengers among different plant tissues and to trigger mechanisms associated with cold tolerance in plants. Such is the case of melatonin, which local exogenous application in *Citrullus lanatus* confers cold tolerance not only at the application site but also in distant tissues. Such induction is associated with an enhanced antioxidant capacity and optimized defense gene expression such as heat shock proteins, peroxidases and lipoxygenases (Li et al. 2017). Moreover, many of the observed upregulated genes were related to phytohormone signaling; melatonin pretreatment alleviated cold-induced down-regulation *ERFs*, key genes in ET signaling network, and up-regulated other *ERF* transcription factors. Other intermediaries in AUX, CK and GA metabolisms were also regulated by melatonin treatment and ABA receptor (*PYL8*) was notably suppressed by melatonin plus cold application but not by cold alone (Li et al. 2017), suggesting an important role of all these phytohormones in the observed cold tolerance induction by melatonin pre-application.

Medicinal Plants and Cold Stress Response

Many phryganic plants (*Thymus sibthorpii, Satureja thymbra, Cistus incanus, Phlomis fruticosa, Teucrium polium*) are aromatic or medicinal and display the phenomenon of seasonal dimorphism (Lianopoulou and Bosabalidis 2014), developing mechanical and chemical defensive barriers to cope with cold stress. These plants induce morphological, anatomical, cytological, physiological, and biochemical defense mechanisms and most of these changes are mediated by phytohormones. In order to cope with cold conditions, *Origanum dictamnus* was described to induce structural and functional changes affecting leaves shape, size and distribution in favor of small and apical ones. Mesophyll cells develop large intracellular spaces with the aim of accumulating air at higher temperature (Lianopoulou and Bosabalidis 2014). This plant also stimulates the development of a thick and dense layer of non-glandular trychomes as a protective shield together with a thick cuticular/waxy layer on the leaf epidermis in response to cold. Essential oils are more abundantly secreted by glandular hairs under cold, being the major constituent p-Cymene (60%) during the winter and Carvacrol (42%) during the summer (Lianopoulou and Bosabalidis 2014).

Similarly, *Salvia sclarea*, commonly known as clary sage and an important medicinal herb with high market demand, showed a reduction in individual leaf area but an increased length and number of spikes and a longer inflorescence with higher content of essential oils under cold conditions (Kaur et al. 2015). The mountain germander (*Teucrium polium*) and *Thymus sibthorpii* are also structurally affected by chilling stress since winter leaves are smaller and thicker, have more stomata and glandular hairs while epidermal and mesophyll cells contain in their vacuoles dark phenolic and calcium oxalate crystals (Lianopoulou et al. 2014a, b). Thus, in general, winter plants exhibit small, thick, and curled leaves, high densities of glandular and non-glandular hairs, compact mesophyll with developed palisade parenchyma, numerous stomata and sclerenchymatic fibers, and abundant phenolics in the epidermal and mesophyll cells (Fahn and Cutler 1992).

All these morphological and structural changes are regulated by phytohormones, mostly ABA, which is also involved in stomatal closure, resulting in a decline in photosynthesis as occurred in winter leaves of *O. dictamnus* (Lianopoulou and Bosabalidis 2014). However, a higher photosynthetic rate and stomatal conductance was measured in *Teucrium polium* and *Thymus sibthorpii* winter leaves (Lianopoulou et al. 2014a, b), showing that physiological changes also depend on the plant species considered. Chloroplasts showed relevant modifications under cold weather, with the presence a few grana and the appearance of lipid grains in plastoglobuli (Lianopoulou and Bosabalidis 2014). Chloroplastic membranes are strongly affected by cold stress, showing signs of damage and lipid accumulation during low temperature exposure. Thylakoids swell and distort, starch granules disappear, and a peripheral reticulum (vesicles arising from inner membrane of chloroplast envelope) appears, driving finally to chloroplast disintegration after prolonged chilling (Kratsch and Wise 2000).

Apart from structural changes observed in many medicinal and aromatic plants, it was reported that certain environmental stresses are linked to a higher accumulation of antioxidant compounds in different medicinal plants (Lianopoulou and Bosabalidis 2014; Mir et al. 2015; Saema et al. 2016), contributing in most cases to increase their medical or nutritional value (Mir et al. 2015; Nourimand et al. 2012). Therefore, the knowledge of how cold stress is affecting medicinal plants and the possible impact on bioactive compounds accumulation in relevant species is valuable (Mir et al. 2015). Moreover, understanding how plant hormones are mediating in these responses could be a key tool for future manipulation of plants in the search of bioactive molecules.

Cold severely impacts on biomass production and slightly chlorophyll and β-carotene content in fennel (*Foeniculum vulgare*) seedlings growing at 2 °C for 2, 3 and 4 h whereas total antioxidant activity increased in response to cold (Nourimand et al. 2012). Relevant differences in membrane damage among 11 fennel varieties have been described (Xiao et al. 2011), showing that tolerance to cold and therefore, adaptation to different environments, is also dependent on the variety.

The Indian ginseng or ashwagandha (*Withania somnifera*) showed an increase in withanolide (steroidal lactones, key bioactive compound from this species) accumulation in the leaves in response to cold stress (Mir et al. 2015; Kumar et al. 2012) which also was linked to a better recovery after cold exposure in *W. somnifera* transgenic plants (Saema et al. 2016). Cold also caused a concomitant rise in antioxidant enzymatic activity of CAT, SOD, APX and GR in Indian ginseng (Mir et al. 2015) as well as in *Thymus sibthorpii* leaves (Lianopoulou et al. 2014b). This is part of the antioxidant system in charge of protecting aerial tissues from cold damage. Contrastingly, the root tissue showed a slight reduction in withanolides concentration during cold stress (Mir et al. 2015), suggesting that different plant organs have developed specific mechanisms towards cold survival and that an internal balance in the distribution of these compounds could be crucial upon survival. The medicinal properties of *W. somnifera* have been attributed to these withanolide compounds, synthesized in leaves and roots, which biosynthesis and accumulation are directly influenced by environmental factors. In this plant, seasonal low temperature appears to exert a key role in increasing the accumulation of bioactive withanolides (Kumar et al. 2012).

Certain enzymes have been studied in order to understand possible mechanisms behind cold tolerance in *W. somnifera.* Sterol glycosyltransferases (SGT) are enzymes that glycosylate sterols, which are crucial in plant adaptation to low temperatures. The *WsSGTL1* gene is specific for 3β-hydroxy position and has a catalytic specificity to glycosylate withanolides and sterols. Glycosylation not only stabilizes the products but also alters their physiological activities and governs intracellular distribution (Ullmann et al. 1993). The clonation of *WsSGTL1* gene in *Arabidopsis thaliana* showed an increase in cold tolerance in transgenic plants linked to a faster formation of sterol glycosides as well as a higher enzymatic activity (Mishra et al. 2013). Furthermore, the overexpression of this gene enhanced tolerance to cold together with a better photosynthetic performance and quenching regulation of PSI and PSII as well as a higher stomatal conductance after cold exposure (Saema et al. 2016). These enzymes are able to glycosylate also steroidal hormones, such as BR which function as growth promoters and development regulators (Bartwal et al. 2012). In this sense, the activation of SGTs in response to cold could be slowing down plant growth in order to prioritize cold survival through the regulation of BR metabolism (Mishra et al. 2013). SGTs in plants are also involved in sensitivity to stress-related hormones and in differences found in the relative tolerance to biotic and abiotic stresses (Chaturvedi et al. 2012).

Concluding Remarks

In this chapter, the latest literature about cold responses and phytohormones, with special emphasis in medicinal and aromatic plants was reviewed. Most mechanisms triggered by plants in order to cope with cold stress are conserved among different species involving changes in architecture and morphology as well as in primary and secondary metabolism. Therefore, examples about model plants and different crops were included to illustrate the complexity of these phenomena. Phytohormones act together as an orchestrated network in order to finely tune main changes in response to low temperatures, being their regulation crucial for plant acclimation and survival. Further studies should be focused on the modification of cold-signaling and response pathways with the aim of stimulating desired responses (i.e. accumulation of health-related phytochemicals that are induced by cold in medicinal plants) and plant adaptation to challenging environments.

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