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Advances in physiological and molecular aspects of plant cold tolerance

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\textbf{ABSTRACT}

Abiotic stress is one of the main causes of crop reduction globally. Among the different abiotic stresses, cold is an essential factor that limits crop productivity worldwide. Low temperature affects the growth, development and distribution of agronomic species throughout the world. Cold stress is a serious threat to the sustainability of crop yields. Indeed, cold stress can cause major crop losses. A significant number of researches have been reviewed and discussed in this study in order to improve the understanding of the physiological and genetic nature and function of plant cold tolerance. Recent developments in determining the mechanism of genes with roles in freezing tolerance and the systems involved in low-temperature gene regulation and signal transduction are described. The roles of a family of \textit{Arabidopsis} transcription factors, the \textit{CBF/DREB1} proteins, have been described and its role in controlling the expression of a regulon of cold-induced genes (COR) that increase plant freezing tolerance has been explained. Moreover, this study has reviewed the recent application applied to improve the cold tolerance of plants such as molybdenum. The use of infrared camera to study the process of plant injuries caused by low temperature has also been reviewed.

\textbf{Plant abiotic stress}

Plant growth and productivity are limited by both biotic and abiotic factors (Seki et al. 2003). A series of morphological, physiological, biochemical and molecular changes can be triggered in plants under the effect of abiotic stresses which eventually adversely affect the growth and productivity of crops. Therefore, plants respond and adapt in order to survive at the molecular and cellular levels in addition to physiological and biochemical responses (Sanghera et al. 2011). It was reported that abiotic stresses can cause a decrease in yield of crops up to 50% resulting in very high economic losses (Vij & Tyagi 2007). The world population is increasing rapidly and at the same time food production is decreasing due to the effect of various abiotic stresses (Mahajan & Tuteja 2005). Therefore, the minimization of these losses is one of the main objectives for plant and crop specialists and since it is very difficult to control abiotic stress resulting from climate change and human activities, the development of stress-tolerant crop genotypes is necessary (Mahajan & Tuteja 2005).

Different scientists have defined stresses differently depending on their mode of studies. Lichtenthaler (2006) defined stress as any substance or condition which stops a plant’s metabolism, development or growth. Stress was also defined by Mahmood (2002) as any element that decreases plant reproduction and development below the genotype’s potential. However, plant response to stress is very complex, including morphological, physiological, biochemical and molecular changes. Some plants have morphological adaptations in order to avoid undesirable conditions and others can alter their physiology metabolism, gene expression and various developmental activities to tolerate the effect of specific stresses.

\textbf{Low-temperature stress (frost damage)}

Among the different abiotic stresses, cold is an essential factor that limits crop productivity worldwide. Low temperature affects the growth and development of agronomic species throughout the world (Pearce 2001). The survival of plants in freezing temperatures depends on their capacity for cold acclimation (McKhann et al. 2008). Low temperature limits the geographical distribution of plant species and significantly decreases the yield of several crops around the world (Pearce & Fuller 2001). It is very important to study the frost damage mechanism and to breed cold-tolerant varieties since the average minimum temperature is below 0°C in about 64% of the earth’s land area and it is below −10°C in about 48% (Deane 1994).

Frost technically means the formation of ice crystals and this includes a phase change from vapor or liquid to solid. Ice formation (crystallization) consists of two main steps: (a) nucleation; (b) crystal growth. Nucleation is the process when the molecules start to gather, forming clusters at the nanometer scale. However, these clusters are required to grow to a specific size in order to form stable nuclei and at this point the atoms arrange in a defined manner to form the crystal structure. Subsequently, the nuclei keep growing to form ice and cause damage effects on plant cells (Sakai & Larcher 1987).

Research about the mechanism of ice initiation in plants has greatly decreased recently because of its essential role in specific aspects of cold hardness (Gusta et al. 2009; Aryal & Neuner 2010; Hacker et al. 2011; Walters Jr et al. 2011). Ice formation may take place either in the intracellular or extracellular spaces depending on the cooling conditions (Guy 1990). Intercellular freezing can happen when cooling...
rates are high or after a significant supercooling. Supercooling is defined as when liquid water cools below the melting temperature (0°C) without any ice formation. The supercooling point is the minimum lower temperature reached before ice formation. Extracellular freezing takes place in the spaces between cells in water transporting elements (xylem and phloem) or on the external surfaces of the plant. Ice will grow and spread from the initial formation point (nucleation site) through the extracellular spaces in contiguous water. Moreover, as long as the plasma membrane is unharmed and the cooling rate is low, the ice will stay in the extracellular spaces and it will draw the water from the cells since water has a lower osmotic potential in the ice (solid) phase than that in the liquid phase. This process will continue until equilibrium of water potential is achieved resulting in a dehydration effects on the plant cells (Wisniewski & Fuller 1999). Several forms of membrane damage can result as a consequence of dehydration caused by freezing effects. This could include expression-induced lysis, lamellar-to-hexagonal-phase transitions and additional jump lesions (Steponkus et al. 1993). McKersie and Bowley (1997) reported that freezing contributes to membrane damage through the induction of reactive oxygen species (ROS) production. It has been demonstrated that protein denaturation caused by the effect of low temperature could cause cellular damage (Guy et al. 1998). Takahashi et al. (2013) reported that the alternation of plasma membrane compositions and functions during cold acclimation is one of the most important adaptation mechanisms for plant cold tolerance.

Generally, plants can be classified into three different classes according to their low-temperature tolerance (Stushnoff et al. 1984). The first group includes frost-tender plants that are sensitive to chilling injury and can be killed by short periods of exposure to temperature just below freezing point. Beans (Phaseolus lunatus), corn (Zea maizze), rice (Oryza sativa) and tomatoes (Solanum lycopersicum) are examples of plants in this category. Low-temperature acclimation of plants in the second group allows them to tolerate the presence of extracellular ice in their tissues. Their frost resistance ranges from the broad-leaved summer annuals, which are killed at temperatures slightly below freezing point, to perennial grasses that can survive exposure to −40°C. As temperatures decrease the outward migration of intracellular water to the growing extracellular ice crystal causes dehydration stress that will eventually result in irreversible damage to the plasma membrane. The final group is made up of very cold hardy plants that are predominantly temperate woody species. Like the plants in the previous group, their lower limits of cold tolerance are dependent on the stage of acclimation, the rate and the degree of the temperature decline and the genetic capability of tissues to accommodate extracellular freezing and the accompanying dehydration stress. Deep supercooling allows certain tissues from plants in this group to survive low temperatures without formation of extracellular ice. However, plants have adapted two mechanisms to protect themselves from damage due to below freezing temperatures. First, supercooling is a low-temperature tolerance mechanism that is usually associated with acclimated xylem parenchyma cells of moderately hardy woody plants. The second and most common low-temperature response mechanism is acclimation. Acclimation is a gradual process during which there are changes in just about every measurable morphological, physiological and biochemical characteristic of the plant. These changes are determined by genotype and environmental interactions that are quite complex (George et al. 1982).

**Cold acclimation and frost stress tolerance**

Cold acclimation is defined as the exposure of plants to low non-freezing temperatures leading to significant positive effect on the cold tolerance of the plants (Thomashow 2010; Pearce et al. 2013). Extensive researches have been conducted to improve the understanding of the biochemical and molecular basis of the cold acclimation response and the changes that take place throughout this process (Lissarre et al. 2010). However, the increase in cold tolerance obtained by acclimation is not static. It depends on the season and it is lost quickly (deacclimation) when plants are exposed to warm temperatures. Wanner and Junttila (1999) also reported that cold acclimation is a photosynthesis-activity-demanding process. The authors reported that light plays a role in cold acclimation and moderate to high light intensity is required for a sufficient cold acclimation process. Ivanov et al. (2012) reported that the strength of light and the ambient temperature could be integrated into electron sink alternation in chloroplasts inducing a signal transduction pathway. They reported that the energy balance in chloroplasts could play a role in sensing ambient temperature. However, a plastid signal contributes to the rhythm of CBF expression and downstream of cold-responsive genes expression during the day/night cycle (Norén et al. 2016). Moreover, cold-tolerant species such as winter wheat, spinach, etc. have the capacity to maintain a high level of CO₂ assimilation rate whereas sensitive cold species do not have this capacity (Yamori et al. 2010). Gusta and Wisniewski (2013) reported that energy is an essential factor to drive acclimation and that sugars have important effects on plant freezing tolerance, resiliency of photosynthesis under stress condition. Dahal et al. (2012) reported a significant correlation between the accumulation of sugars and the development of cold tolerance in plants. The timing of cold acclimation and deacclimation, the onset and release from dormancy, as well as the timing of bud break play an integral role in the life cycle of woody plants and their adaptation to the external environment (Ríos et al. 2014; Wisniewski et al. 2015).

Extensive physiological and biological changes occur during cold acclimation (Figure 1) starting with a reduction in the growth rate and water content of various plant tissues (Levitt 1980). Through the cold acclimation process, programming of gene expression and various modifications in the metabolism take place (Chinnusamy et al. 2010). Acclimation also causes an increase in the production of antioxidants, abscisic acid (ABA) and compatible osmolytes such as soluble sugars and proline (Lynch & Steponkus 1987; Koster & Lynch 1992; Chen et al. 1993; Kishitani et al. 1994; Uemura & Steponkus 1994; Murelli et al. 1995; Nomura et al. 1995; Dörrfling et al. 1997; Tao et al. 1998). Denesik (2007) reported that cold treatment affects membrane fluidity resulting in an increase in the membrane rigidity. This more fluid state of cell membranes helps to protect cells under the effect of low-temperature stress through maintaining the cell shape and preventing cellular component from water loss. Moreover, the rigidity of membrane could reduce cells collapsing during extracellular freezing by creating a negative pressure in the cells (Rajashekar & Lafta 1996; Heidarvand...
Takahashi et al. (2013) reported that the alternation of plasma membrane composition and function is one of the most important mechanisms of plant cold tolerance. A proteome profiling to characterize alternations of the *Arabidopsis* microdomain during cold acclimation has been conducted by Minami et al. (2009) who reported the increase of clathrins and dynamin-related proteins in the microdomain during cold acclimation. Many proteins such as P-type ATPases, aquaporins and tubulins have been reported to accumulate under the effect of low-temperature treatment (Minami et al. 2009; Takahashi et al. 2013) (Figure 2). For more details, please read Takahashi et al. (2013).

Cold acclimation leads to large-scale transcriptome changes that program the production of an array of proteins to help plants surviving freezing stress (Kocsy et al. 2010; Winfield et al. 2010). Recent studies describing full genome transcripts and mutational and transgenic plant analysis have provided a great deal of information about the complex transcriptional system that functions under cold acclimation (Jan et al. 2009). Various regulatory pathways were found to be involved in the cold response when transcriptome profiling of around 8000 genes of *Arabidopsis* was carried out. It was observed that 300 genes were affected by cold, of which 218 showed an increase of transcripts expression and 88 showed a decrease of transcripts expression within 7 days of cold treatment (Fowler & Thomashow 2002). The use of microarray systems has enabled researchers to demonstrate that a large number of genes are induced under abiotic stresses (Seki et al. 2001; Kreps et al. 2002; Bray 2004; Maruyama et al. 2004; Vogel et al. 2005; Jan et al. 2009). The genes affected by low temperature have been classified into two main groups (Seki et al. 2002): the first group involves the proteins that function as a response to cold stress such as LEA proteins (Cushman & Bohnert 2000); the second group contains proteins which have roles in the further regulation of gene expression in cold stress conditions. Some of these pathways are considered to have multiple functions since they have also been reported to be involved in stress responses to drought and high salinity (Seki et al. 2003).

The majority of studies to improve the cold tolerance of plants have been conducted with the *Arabidopsis* plant (Gery et al. 2011). However, Li et al. (2012) reported that *Brachypodium distachyon* could be an important model to investigate the molecular mechanism of low-temperature tolerance in core Pooidae species since *B. distachyon* have cold-responsive IRIP. Moreover, a large C repeating binding factor genes CBF3 subfamily was identified in *B. distachyon*. The authors also confirmed that FST-motifs and cold treatment that increased fructan accumulation were intensely different in *B. distachyon* compared to core Pooidae species.

**Gene expression induction in response to low temperature**

Cold acclimation affects the expression of a huge number of genes by either up- or down-regulations (Hannah et al. 2005). Furthermore, there is crosstalk between the response of different abiotic stresses and some of the genes up-regulated by drought or salt stress (Thomashow 1999; Seki et al. 2003; Zhao & Zhu 2016). Fowler and Thomashow (2002) reported that cold acclimation activates different gene expression pathways which in turn means that cold-induced genes could be members of more than one cold regulon. However, it should be mentioned that plant response to abiotic stresses is mediated by a common group of reactions, cooperatively known as signal transduction (Heidarvand & Maali Amiri 2010). Therefore, plant response to low temperature goes through specific process starting by cell recognition of low temperature (sensing) followed by the signaling process in order to induce the cold-responsive genes resulting in an increase in plant cold tolerance (Denesik 2007). Free Ca$^{2+}$ has been observed to increase rapidly in the cytoplasms of plant cells during the acclimation process and, therefore, it is most likely to be involved in this process (Eckardt 2009). Protein phosphorylation seems to be involved since the treatment of alfalfa plants with the protein phosphatase inhibitor okadaic acid increased the transcript levels of the alfalfa cold-responsive cas15 gene at normal temperature and the transcript level of this gene did not increase to normal levels when plants were exposed to low-temperature treatment after they were treated with the protein kinase inhibitor staurosporine. Furthermore, cold effects significantly decreased the protein phosphatase 2A activity which has been reported to be dependent on calcium influx. Thus, calcium is believed to prevent phosphatase 2A activity leading to the phosphorylation of one or more proteins.
proteins that could have parts in the induction of genes involved in cold tolerance and acclimation (Monroy et al. 1998 cited from Thomashow 2001).

Knight and Knight (2000) indicated that the free cytoplasm Ca\(^{2+}\) observed under low-temperature effects comes from extracellular and intracellular calcium stores. Significant evidence have confirmed the essential role of Ca\(^{2+}\) signaling on cold induction of CBF pathways (Knight & Knight 2000). Ca\(^{2+}\) is considered to be the main signal transducer in signaling cascades motivated in response to plant abiotic stress types. Ca\(^{2+}\) also seems to be the crosstalk between abiotic stress pathways since the level of this cation increases under the effects of different types of abiotic stresses. Ca\(^{2+}\) binding proteins (Ca\(^{2+}\) sensor) mediate the signal activated by Ca\(^{2+}\) influx under the effect of abiotic stresses. Jenks and Wood (2010) reported three main Ca\(^{2+}\) proteins in plants: Ca\(^{2+}\)-dependent proteins kinases, calmodulin (CaM) and calcineurin B-like proteins. Rudd and Franklin-Tong (2001) reported that CaM, which a highly conserved protein, is the first sensor to the Ca\(^{2+}\) cytosolic level in all eukaryotic cells. Therefore, calmodulin genes, which control calcium fluxes, are considered to be the main sensors of environmental triggers such as extreme temperatures. Calmodulin is a calcium-binding protein which binds to a regulatory element in the CBF gene promoter which plays a role in controlling the CBF regulon and freezing tolerance (Doherty et al. 2009). Eckardt (2009) reported that CAMTA play a significant part in transduction of low-temperature–induced cytosolic Ca\(^{2+}\) signals into downstream regulation of gene expression. However, Marc et al. (2010) reported clear evidence that physical changes in membrane fluidity initiated by the stoichiometry of lipids could be the main trigger for calcium influx into the cell.

Nuclear Ca\(^{2+}\) is increased after cold treatment and the maximum increase is delayed at 5–10 s compared to the peak of cytosolic calcium (Miura & Furumoto 2013). Mauger (2012) reported that the increase in nuclear Ca\(^{2+}\) is caused by a nuclear envelope that is considered one of the major Ca\(^{2+}\) stores. Moreover, they indicated that the increase in nuclear Ca\(^{2+}\) can be amplified by cytosolic Ca\(^{2+}\) transients through the nuclear pore complex. Because of the similarity in pore complex system in both animal and plant nuclear membrane (Xu & Meier 2008), it was suggested that the nuclear Ca\(^{2+}\) signal could be started from the nuclear envelope and propagated by cytosolic Ca\(^{2+}\) transients in plants as well. However, nuclear Ca\(^{2+}\) has been reported to have an important role in controlling gene transcription in both plants and animals (Mazars et al. 2011; Mauger 2012).

ROS also play an important role as second messengers responding to various abiotic stresses (Tyystjärvi 2013). Rao et al. (2006) reported that abiotic stresses cause an oxidative burst and that a low level of ROS induces an increase in Ca\(^{2+}\) influx into the cytoplasm. The high level of Ca\(^{2+}\) activates NADPH oxidase in order to produce ROS through yielding O\(_2^–\) which is then converted to H\(_2\)O\(_2\) (ROS) under the effect of super oxidase dismutase (SOD). Therefore, the production of ROS is Ca\(^{2+}\) dependent and the concentration of Ca\(^{2+}\) is also regulated by the concentration of ROS by the activation of Ca\(^{2+}\) channels in the plasma membrane (Kwak et al. 2003). Therefore, a crosstalk between Ca\(^{2+}\) and ROS modulates the activity of specific proteins that control the expression-specific definitive defense genes in the nucleus. However, several other genes have been demonstrated to have roles in the abiotic stress responses such as kinases and transcription factors which in turn have roles in the crosstalk between signaling cascades involved in responses against two or more kind of stresses (Rao et al. 2006).

Kocsy et al. (2008) reported a crucial role of glutathione in the ascorbate-glutathione cycle. They reported that glutathione has an important role in the regulation of the concentrations of hydrogen peroxide (H\(_2\)O\(_2\)) in plants. When plants are exposed to low temperature (0-15°C), chilling and cold acclimation, an increase in H\(_2\)O\(_2\) level is observed. The accumulation of H\(_2\)O\(_2\) is a result of the reduction of oxygen by the excess electrons coming from the photosynthetic and respiratory electron transport chains under low-temperature conditions. This leads to the accumulation of superoxide radicals (O\(^{2–}\)) which are subsequently converted to form H\(_2\)O\(_2\).
via SOD. H$_2$O$_2$ has a damaging effect on the cell membrane and protein structures of the cells. Therefore, plants developed an effective mechanism for the removal of excess H$_2$O$_2$ through the conversion of reduced glutathione (GSH) to its oxidized form (GSSG). However, GSSH is reproduced under the effect of NADPH-dependent glutathione reductase (GR). The high level of GSH and GR observed under the effect of low temperature has been considered as an important indicator of a possible contribution of these materials to low-temperature tolerance in plants (Kocsy et al. 2008). It has been reported that the GSH/GSSG ratio and changes in H$_2$O$_2$ level can have an important role in the cold acclimation resulting in an activation of redox state of the cells and this in turn could activate special defense machines via a redox signaling chain under the effect of acclimation or low temperature. This hypothesis has been supported by the discovery of several defense genes that have antioxidant-responsive elements or GSG-binding sites in their regulatory regions (Kocsy et al. 2008).

Ghildiyal and Zamore (2009) indicated that micro-RNAs (miRNAs) and small interfering RNAs could be repressors of gene induction in both plants and animals. Many cold-regulated miRNAs have been recognized using bioinformatics, cloning and sequencing tools in different plant species (Zhou et al. 2008; Zhang et al. 2009; Lv et al. 2010; Chen et al. 2012). However, only a little is known about the mi target genes that are unregulated under the effect of low temperature (Miura & Furumoto 2013).

Although a large number of studies have investigated the cold-tolerance mechanism in plants, there is still a lack in the progress of improving the cold frost tolerance of crop plants and this could be caused by difficulty of synthetically creating the complex environmental conditions existing when plants acclimate in nature (Gusta et al. 2009). Plants acclimated in growth chambers might respond differently from those growing in nature since the growth conditions such as light, air and soil temperature and temperature variations are different between the growth chamber and the field (Gusta & Wisniewski 2013).

**CBF cold-response pathway**

CBF gene discoveries are the most significant in the field of low-temperature adaptation and signal transduction. CBFs are discovered in all important field crops and some vegetable species (Sanghera et al. 2011). CBF genes have been identified in a wide range of herbaceous (Mizoi et al. 2012) and woody plants (Wisniewski et al. 2014). Intensive studies in the expression of cold-response gene expression in Arabidopsis resulted in the identification of the CBF/DREB1 transcription factors family which have an essential role in cold acclimation and freezing stress tolerance in plants (Gilmour et al. 1998). These transcription factors bind to specific regulatory sequences in the promoters of cold and dehydration-responsive genes. These sequences are C-repeat (CRT: TGGCCCG AC) and dehydration-responsive elements (DRE: TACCG ACAT). Both of these sequences contain the highly conserved core 5-bp sequence of CGGAC, which has the capacity to regulate transcription under drought, low temperature and salinity (Baker et al. 1994; Yamaguchi-Shinozaki & Shinozaki 1994; Gao et al. 2007). Therefore, CBF induces the expression of COR genes (the genes which contain the COR sequence) and these genes play an essential role in the improvement of plant cold tolerance (Mizoi et al. 2012). CBF/DREB1 is a family of transcription factors that belongs to the ERF/AP transcription family (Yamaguchi-Shinozaki & Shinozaki 2005). The finding of Yamasaki and Randall (2016) has confirmed the initial steps in the CBF/DREB1 pathway in soybean but the contributions to the lack of GmCBF/CBF1 transcripts and/or protein, which are not sufficient to up-regulate downstream genes, absence or co-activators or presence of strong negatively acting transcriptional regulators or lack of appropriate CBF/DREB1-responsive element in the promoters of critical cognate soybean genes. Thomashow (2001) has characterized three different cold-inducible CBF/DREB1 genes in Arabidopsis described as: CBF1/DREB1B, CBF2/DREB1C and CBF3/DREB1A. CBF3/DREB1A and CBF/DREB1B are induced at the same time and earlier than CBF2/DREB1C (Medina et al. 2011). More recently Monroe et al. (2016) screened 477 wild accessions from Arabidopsis for the variation in CBF genes; they found that CBF sequences variation is strongly associated with winter temperatures. The types that come from warmer winters showed significant access to the CBF polymorphisms. RNAi lines targeting all three CBF genes in Arabidopsis thaliana were obtained in eight different accessions in order to evaluate the quantitative influence of CBF expression on plant freezing tolerance. Outstanding differences were observed between different accessions in terms of the effects that reduced CBF expression had on freezing tolerance, though effects on growth were mostly very minor to draw steady conclusions (Gery et al. 2011). The authors reported that the analysis of the relationship between different types of CBFs revealed a tight co-regulation between CBF1 and CBF3, whereas the relationship between CBF2 and CBF1 or CBF3 expression levels was intensely related to the genetic background of the RNAi lines. However, while three closely related CBF genes have been reported in Arabidopsis, wheat and some other cereals have undertaken an extension in this family (Pearce et al. 2013). Badawi et al. (2007) reported that there are at least 15 CBF genes in each of the three hexaploid wheat genomes.

The CBF/DREB1 over-expression transcriptome analysis in transgenic plants demonstrated that about 12% of COR genes are controlled by CBF/DREB1 in Arabidopsis. However, among the three CBF factors no specific target was observed (Zeller et al. 2009; Matsui et al. 2010). The expression of these genes in transgenic plants activated downstream cold-responsive genes (COR genes) even at warm temperature improving the cold tolerance of these plants (Liu et al. 1998; Kasuga et al. 1999). The CBF/DREB1 pathway has been identified and described in several plant species, indicating that the CBF transcriptional cascade under the cold stress is highly conserved in the plant kingdom (Jaglo et al. 2001). A significant relation between the expression of CBFS/DREBS and changes in the temperature has been demonstrated, indicating that the lower the temperature the higher the expression of these genes. However, the expression can desensitize at a given low temperature (Zarka et al. 2003).

The increase in transcription levels of three CBFS (CBF 1, 2 and 3) has been observed within 15 min exposure to low temperatures. The rapid response to the cold effect strongly suggests that the ‘thermometer’ and signal ‘transducer’ of low temperature and which regulates the expressions of CBFS is present at warm temperatures. In this context, Gilmour et al. (1998) demonstrated that a transcription factor,
tentatively designed inducer of CBF expression (ICE), is present at warm temperature. They also reported that ICE recognizes DNA-regulatory elements in the CBF promoter and called these 'ICE boxes'. It was also reported that either 'ICE' or proteins with which it interacts are activated under the effect of low temperature and as a consequence the promoter of CBF genes was suggested to be activated and responsive to low temperature (Zarka et al. 2003). Chinnusamy et al. (2003) reported that ICE1, which is a nuclear gene encoding a MYC-like bHLH protein, has a crucial role in regulating some CBF genes. The role of ICE1-CBF/DREB1 singling pathway in increasing cold tolerance has been investigated in different plant species such as A. thaliana (Welling & Palva 2008; Chen et al. 2009; Fursova et al. 2009; Yang et al. 2011), Cucumis sativa (Liu et al. 2010), Triticum aestivum (Miller et al. 2006; Moran et al. 2011), Hordeum vulgare L. (Stockinger et al. 2007; Campoli et al. 2009), Solanum tuberosum (Behnam et al. 2007), eucalyptus (Navarro et al. 2009), Nicotiana plumbaginifolia (Gutha & Reddy 2008), apple (Ardump et al. 2014), etc. Badawi et al. (2008) reported that there are two ICE1 homologs, TaICE1A1 and TaICE1B7, in wheat and they up-regulate the expression of CBF group IV genes in wheat. Several studies have reported the existence of the CBF/DREB pathway and its role in increasing the cold tolerance of rice plants (Ito et al. 2006; Wang et al. 2008; Yang et al. 2009; Zhang et al. 2009; Cruz et al. 2013). Cold stress induces the expression of OsICE1 and OsICE2 in rice, leading to the up-regulation of OsDREB1B, OSHsfA3 and OsTPP1 (Nakamura et al. 2011). Zhao et al. (2013) indicated that the chilling tolerance of banana plant (Musa acuminate) was improved by jasmonate (IA) through the induction of MaMYC2. However, MaMYC2 interact with MalICE1, activating the cold signaling of the CBF-dependent pathway. Soltéz et al. (2013) reported that TaCBF14 and TaCBF15 play an essential role in cold tolerance of wheat. Moreover, they reported that these genes improved the cold tolerance of transgenic plants by several degrees lower than control plants. At least five CBF genes have been identified in both apple (Malus domestica) and peach (Prunus persica) that exhibit a variety of patterns of expression in response to low temperatures (Wisniewski et al. 2014, 2015).

Fursova et al. (2009) indicated that the over-expression of ICE2, which is a homolog to ICE1, improves the cold tolerance and the expression of CBF/DREB1B in Arabidopsis. However, it was demonstrated that the hyperacetylation of histones H3 and H4 and DNA demethylation take place in the ICE-binding region of the promoter of maize (ZmDREB1) after cold treatment, escorted by chromatin decondensation (Hu et al. 2011). This suggests that chromatin remodeling could be one of the essential requirements for the up-regulation of CBF/DREB1 by ICE1. However, it should be mentioned that apart from ICE proteins, there are several proteins such as LOS4, HOS1 and LOS1 proteins that have been reported to have positive effects of the induction of CBF expression (Matthew 2005).

CBF proteins, which are DNA-binding proteins, recognize the DNA-regulatory element CRT/DRE that is located in the promoter of COR genes. Therefore the induction of the COR gene expression is controlled by CBF proteins (Baker et al. 1994; Yamaguchi-Shinozaki & Shinozaki 1994). These proteins (CBF 1, 2 and 3) are encoded by three CBF genes located in tandem in chromosome 4 and they are considered as transcription factors having masses of about 24 kDa. These proteins are about 90% identical in amino acid sequence and they contain a conserved DNA-binding motif of about 60 amino acids designated the AP2/ERF domain (Riechmann & Meyerowitz 1998). However, 144 AP2/ERF genes were found and described in Arabidopsis (Riechmann et al. 2000) and based on the comparisons of their DNA-binding domain, Sakuma et al. (2002) has classified them into 5 subgroups: AP2 subfamily (14 genes), RAV subfamily (6 genes), DREB/CBF subfamily (55 genes), ERF subfamily (65 genes) and the others which were considered as a 5th group consisting of 4 genes.

The up-regulation of COR (cold-regulated) genes through the activation of CRT/DRE elements in their promoters by CBF proteins is considered to be a small part of the CBF regulon since intensive studies, which have been conducted to determine the expression profiling of CBF, reported that a total of 109 genes were being assigned to CBF regulon (Fowler & Thomashow 2002; Vogel et al. 2005). However, according to the nature of proteins that are encoded, Matthew (2005) classified CBF regulon–assigned genes into four categories: the first group is the largest containing more than 50% of the proteins and these proteins have unknown function, the second group is the cryoprotective proteins which involves the COR proteins, the third group is the regulatory proteins and the last group is the biosynthetic proteins.

COR (cold-regulated) genes encode extremely hydrophilic proteins that are either novel or members of the dehydrins or LEA (late embryogensis abundant) proteins (Close 2006). LEA proteins are suggested to be important for membrane stabilization and prevent protein aggregation (Hundertmark & Hincha 2008). However, most COR protein functional activities are still hypothetical. Artus et al. (1996) reported some evidence that the CORa protein, which is one of the most well-studied COR proteins, supports the stabilizing of cell membranes against freezing damage. COR15a that encodes a 15 kDa polypeptide is targeted to the chloroplast where it is processed to 9.4 kDa mature polypeptide (Lin & Thomashow 1992). Steponkus et al. (1998) reported that the COR15a polypeptide could act directly as a cryoprotective agent improving the cold tolerance of plants. Moreover, it has been demonstrated that COR proteins reduce the tendency of membranes forming hexagonal-II structures which is a harmful non-bilayer construction that takes place due to cellular dehydration associated with freezing (Thomashow 1999). Bravo et al. (2003) and Hara et al. (2003) have reported that COR proteins also have a positive role in protecting other proteins against freeze–thaw inactivation in vitro. Moreover, it has been suggested that CORs could play a role as a hydration buffer that isolate ions and help to re-nature unfolded proteins (Bray 1993). The essential role of CORs in the cold tolerance of plants was confirmed by Artus et al. (1996) who reported that over-expression of COR15a in transgenic Arabidopsis improved chloroplast freezing tolerance by about 2°C in non-acclimated plants.

In addition to COR genes, heat shock proteins’ expression is also induced under the effect of low temperature (Timperio et al. 2008). It was also confirmed that bacterial cold shock proteins (CSPs) enhanced stress adaptation in multiple plant species by demonstrating improved stress tolerance in maize and rice. The expression of CspA and CspB in transgenic rice improved the growth of plants under the effect of a number of abiotic factors like cold, heat and water deficit. The expression of bacterial CSPs increased cold tolerance in transgenic Arabidopsis (Karlson & Imai 2003; Nakaminami
et al. 2006; Castiglioni et al. 2008). Some PR (pathogen-related) proteins such as PR1, PR2, PR5, PR10, PR11 and PR14 are approved to be up-regulated by low-temperature treatment (Seo et al. 2008; Zhang et al. 2010).

It should be mentioned that there is a potential connection between the cold acclimation pathway and other physiological process pathways in plants. For example, Dhillon et al. (2010) suggested a prospective relation between cold acclimation and vernalization because both the processes require an exposure to low non-freezing temperature. Moreover, the initial increase in cold tolerance observed when winter genotypes of cereals were exposed to low non-freezing was decreased with the continuous cold exposure (Fowler et al. 1996). Fowler and Limin (2004) reported that this observed decline in cold tolerance inversely parallel the accomplishment of the vernalization requirement. However, it was suggested that a controlling factor of freezing tolerance is related to a developmental alteration between the vegetative and reproductive stages and that the main vernalization gene (VRN-1) has an essential role in the decrease of the capacity of cold acclimation with plants’ development (Limin & Fowler 2006). Stockinger et al. (2007) reported that when the winter genotype of barley carrying vrn-1 allele is vernalized, the transcription levels of CBF are relatively decreased compared to non-vernalized plants. Pidal et al. (2009) reported that main differences between winter and spring cereals genotypes in terms of vrn-1 characterization were insertions and deletions in the vrn-1 regions located in the promoter and first intron of this gene. However, it has been reported that the main two loci which control low-temperature tolerance in wheat are the frost resistance Fr-1 and Fr-2 (Vágújfalvi et al. 2000). The locus of Fr-1 was reported to be closely connected to the vernalization locus Vrn1, which is well known to be the main controller of the transition of the vegetative to the reproductive meristem in response to low temperature (Laudencia-Chingcuancio et al. 2011). Baga et al. (2007) reported that Vrn-1 and Fr-1 loci seem to be in the same locus and that they have not been genetically uncoupled. The pleiotropic properties of the Vrn-1 locus clarify most of the related cold tolerance and winter habit (Dhillon et al. 2010). Moreover, it was reported that the Fr-2 locus contains a cluster of CBFs which are well known to have an essential role in the up-regulation of cold-induced genes (CORs) in several plant species (Campoli et al. 2009; Knox et al. 2010; Laudencia-Chingcuancio et al. 2011). Manipulating the expression of genes as response to low-temperature stress is followed by increase in metabolites, some of which are proved to have a protective role against the damage of low-temperature stress. However, plants experience changes in their pattern of gene expression and protein products when they undergo low-temperature stress and manipulating and engineering of CBF genes have shown potential practical application for breeding cold tolerance in crops and horticultural plants suitable for temperate regions (Sanghera et al. 2011).

The effect of phytohormones on plant cold tolerance

Plant hormone levels are altered under abiotic stresses and they decrease plant growth. Cold response is one of the most important environmental stresses that induces growth repression and reduced yield. Plant hormones activities induce the cascades of cold-responsive genes.

The plant hormone ABA plays an essential role as a chemical signal in response to biotic and abiotic stresses such as salt, cold, drought and wounding. ABA stimulates several changes in plant physiological, molecular and developmental progressions resulting in plant adaptation to the stress environment (Tön et al. 2009) as well as it is characterized as an intracellular messenger which has been proven to perform the critical function of the heart of signaling operation (Xue-Xuan et al. 2010). Abiotic stresses induce the synthesis of ABA which in turn activates the expression of stress-related genes and stomatal closure (Lee & Luan 2012).

ABA has an essential role in the induction of various stress signals controlling downstream stress responses. Therefore, it is necessary that plants adjust their ABA content constantly in response to changes in physiological and environmental conditions. Plants respond to stresses by two main mechanisms described as ABA-dependent and ABA-independent pathways. The expression of ABA-responsive genes could be induced by several transcription factors such as DREB2A/2B, AREB1, RD22BP1 and MYC/MYB through interacting with their corresponding cis-acting elements such as DRE/CRT, ABRE and MYCRS/MYBRS, respectively (Tuteja 2007). Koornneef et al. (1998) have reported ABA-deficient mutants namely aba1, aba2 and aba3 in Arabidopsis. However, while no differences were observed between the growth of these mutants and wild-type plants under no stress conditions, ABA-deficient mutants readily wilt and die under drought stress and showed poor growth under salt stress. ABA can also be essential for freezing tolerance through the induction of dehydration tolerance genes (Xiong et al. 2001).

Several ABA-induced genes such as Arabidopsis cold-regulated (COR) genes, RAB18, LTI78 and KIN2, are up-regulated under the effect of various abiotic stresses such as cold, salt and drought (Knight et al. 2004). However, although Shinozaki and Yamaguchi-Shinozaki (2000) described several signaling pathways including both ABA-dependent and ABA-independent pathways resulting in COR gene (cold-responsive genes) expression up-regulation, Thomashow (1999) and Shinozaki and Yamaguchi-Shinozaki (2000) reported cold effects on the induction of gene expression was mainly through an ABA-independent pathway. The existence of an ABRE cis-acting element (ABA-responsive element) is an essential requirement for the up-regulation of ABA-induced gene expression (Shinozaki & Yamaguchi-Shinozaki 2000). It has been demonstrated using genetic analysis that there is no strong connection between ABA-dependent and ABA-independent pathways; however, the mechanism involved could usually crosstalk or sometimes converge in the signaling pathway (Uno et al. 2000). In view of this information, calcium was suggested to be a strong candidate that can mediate such crosstalks since calcium concentration was observed to rapidly increase in the plant cells under the effect of ABA and various abiotic stresses such as drought, cold and salinity (Mahajan & Tuteja 2005). Moreover, various changes in plant molecular and biochemical such as the transcript accumulation of RD29A gene and the accumulation of proline were observed and mediated by both ABA-dependent and ABA-independent pathways (Tuteja 2007) (Figure 3).

Finkelstein et al. (2002) reported an important role of ABA in the induction of LEA gene expression. Bies-Etheve et al. (2008) also reported that the application of exogenous ABA induced the expression of specific LEA genes in plant
vegetative tissues. The expression of LEA genes during the development of seed and under abiotic stress was up-regulated by ABA (Dalal et al. 2009). It has also been reported that ABA induces the expression of LEA genes resulting in an increase in the desiccation tolerance of cultured embryos (Leung & Giraudat 1998). The role of ABA in the up-regulation of LEA genes is considered to be one of the mechanisms that ABA has to increase plant drought and freezing tolerance.

Salicylic acid (SA) has been reported to be accumulated under the chilling effect in different plant species (Wan et al. 2009; Kosová et al. 2012). Moreover, the application of SA improved the cold tolerance of several plant species such as potato, rice, maize, etc. (Ahmad & Prasad 2012). Nevertheless, the over-accumulation of SA in several mutants of Arabidopsis caused a freezing sensitivity (Miura & Ohta 2010). It seems that treatment duration and the concentration of SA play essential roles in the response of plant species.

Gibberellin (GA) is the other plant hormone altered in plants under cold stress. It has been evident that GA plays a crucial role in the plant growth-regulatory mechanisms. It has been found that GA is involved in the expression of CRT/DRE-binding factor gene which in turn confers tolerance to drought, salt and cold stress (Niu et al. 2014). Moreover, GA is associated with SA/JA balance in the CBF-mediated stress response (Niu et al. 2014). The key components of GA is the nuclear localized proteins (DELLA) that repress plant growth; however recent advances indicated that DELLA has a role in many plant growth aspects especially those that are affected by environmental stresses (Achard et al. 2008). Achard et al. (2008) investigated the relationship between CBF1 and GA pathways and indicated that constitutive expression of CBF1 leads to less bioactivity of GA resulting in dwarfism in plants and delay in flowering. RGL genes, which code for DELLA proteins, act to restrain growth, whereas GA promotes growth by overcoming DELLA-mediated growth restraint (Claeyss et al. 2014).

Plant phytohormone JA also plays an essential role as an important regulatory signal in plant cold tolerance (Cao et al. 2009; Sayyari et al. 2011; Zhao et al. 2013). It has been proved that the external application of JA significantly enhanced cold tolerance in plants with or without acclimatization. Moreover, blocking of the endogenous JA increased the sensitivity to the cold stress. It has been proved that JA up-regulated the CBF/DREB1 signaling pathway (Hu et al. 2013).

Other hormones such as ethylene and cytokinin (CK) also have an important role in cold tolerance. Although it has been found that CK content was decreased in plants exposed to stress, the next application of CK improved cold tolerance in plant. However, stress-related genes at transcriptional level pattern have been identified by CK but the exact role of CK in cold stress is still unknown (Zwack & Rashotte 2015). Ethylene was proved to increase during low temperature in grapevine (Sun et al. 2016). Finally, phytohormones engineering is very promising in plant response to environmental stresses; however, more research is still needed in this field (Wani et al. 2016).

The role of molybdenum (Mo) in abiotic stress tolerance

Molybdenum (Mo) is an essential trace element present in the soil and is required by both plants and animals. Many oxidation statuses of Mo ranging from zero to VI have been reported in agricultural soils. However, VI is considered to be the most existed state (Sun et al. 2009). Mo belongs to a group of trace elements that are required, by organisms in a minute amount; in spite of this, unavailability of Mo is lethal for microorganisms. Mo has to be bounded to a pterin in order to form a Mo co-factor (Mo-co) (Mendel & Bittner 2006). Mo is not biologically active but it forms a part of an organic complex called the Mo-co. Mo-co is very important for the Mo-requiring enzymes (molybdoenzymes) that have been reported in most biological systems (Williams & Frausto da Silva 2002) and all Mo-dependent enzymes (molybdoenzymes) hold a mononucleic element of Mo-co, which consists of Mo coordinated to an organic tricyclic pyranopterin moiety, referred to as molybdopterin. Mo-co in different variants represents the active compound at the catalytic site of all Mo-containing enzymes in nature (except nitrogenase), that is several enzymes catalyzing divers keys oxotransfer reactions in the global carbon, sulfur and nitrogen metabolism (Mendel & Bittner 2006). Furthermore, even if Mo is available in the medium, it has to be complexed by a special co-factor to confer catalytic activity to several enzymes. Mo is an important element for more than 40 enzymes, 4 of which have been found in plants (Mendel & Hansch 2002) and include nitrate reductase (NR) which is involved in nitrogen fixation and assimilation, xanthine dehydrogenase/oxidase (XDH) which plays an important role in purine catabolism, aldehyde oxidase (AO) that plays an essential part in the synthesis of indole-3 acetic acid and ABA and sulfate oxidase (SO) which have a role in sulfur metabolism (Kaiser et al. 2005). XDH and AO contain a monoxo-Mo-co which needs Mo-co insertion and then consequent sulfuration of the Mo center to activate the Mo-co protein complex. SO and NR have a dioxo-Mo-co which motivates the protein when it is inserted into the protein complex (Mendel & Hansch 2002).

Li et al. (2001) proposed that Mo is involved in the amelioration of wheat frost tolerance. Several mechanisms, of which Mo can improve the frost tolerance, have been suggested. However, the mechanism for this enhancement has not been definitively determined. Mo increases the activity of the anti-oxidative enzymes SOD, CAT and POX resulting in an increase in plant anti-oxidative defenses under low-temperature stress (Yu et al. 1999) since frost stress leads to the production of ROS that has cellular damage effects on plant tissues (Sattler et al. 2000). Sun et al. (2006) demonstrated that Mo application increases the capacity of scavenging the ROS resulting in the reduction in membrane damage of winter wheat under low temperature.

Another hypothesis proposed for the role of Mo in plant cold tolerance was that Mo increases the activity of AO which has an important role in the production of ABA. ABA triggers bZIP and the up-regulation of the ABA-dependent COR gene expression pathways (Sun et al. 2009) and thereby protects against frost damage. However, the role of Mo in the biosynthesis of ABA suggests a significant role of this element in plant drought and salinity tolerance.

A third mechanism is suggested to be through NR and enhancing nitrate assimilation since Mo has significantly increased NR activity in wheat as it has been found that NR activity significantly increased in Mo-treated wheat and was associated with protein accumulation (Yu et al. 1999; Hale et al. 2001; Hamdia et al. 2005). Al-Issawi et al. (2013) recently reported a fourth mechanism of Mo effects on the
plant cold tolerance through the up-regulation of the CBF pathway which in turns lead to COR up-regulation and improvements in frost tolerance.

Utilizing the infrared camera in cold tolerance in planta

Many economic inputs are used to provide considerable protection against freezing events in plants or parts of plants, for example, rowcovers, windmills, smudge pots or spraying water on plants. All these methods seem to be ineffective in giving the required protection to the plants; however very little is known about the mechanisms associated with freezing of plants in the field conditions; therefore this gave a chance to use the most effective method of frost protection which is the use of high resolution of infrared camera directly in the field (Wisniewski et al. 2008). Thermal infrared imaging techniques are very valuable as an aid in understanding the physiological effects of frost on plants and can be used both in the field and under artificial conditions. This technique is based on measuring the heat released when different fractions of water freeze within tissue (Quamme et al. 1972; Ketchie & Kammerreck 1987; Fuller & Wisniewski 1998). The thermal infrared camera produces an image in a way exactly analogous to forming images from visible light with familiar electronic imaging devices like video or digital cameras. Thermal cameras contain an array of detectors that convert infrared radiation (thermal energy) to electrical output signals whose amplitude is proportional to the amount of the energy detected. These output signals can be processed as a normal video image in gray-scale image of the scene. The gray-scale value in the images represents the amount of thermal energy detected from a given point in view and is proportional to the radiant temperature of that point. Hotter radiant temperatures are normally displayed as whiter tones in an image, and colder temperature as blacker tones (Colwell 1983). More recently, gray-scale images can be converted to color-enhanced images using computing software generating a range of color palettes. Since freezing events are temporal it is necessary to record the images over time either as video images or digital video equivalents. The determination of the exact location of initial ice formation (point of ice nucleation) and how it progresses throughout the plant (ice spread) is very important information that needs to be known prior to devising methods to protect plants. Wisniewski et al. (1997) were among the first to use infrared thermal imaging technology to determine where ice first begins to form in a plant and at what temperature, based on the principle of release of heat when ice is formed (exotherm which is the process that describes the chemical reaction when energy is released usually in the form of heat). By using infrared video thermography, these researchers observed a plant freezing and followed the progress of ice formation throughout the plant. Subsequently, ice formation has been tracked by using an infrared camera system in many species including apple (Malus ssp), peach (P. persica) and pear (Pyrus ssp) trees and bean (Vicia faba L.), potato (S. tuberosum), strawberry (Fragaria ssp) and wheat plants (T. aestivum L.) (Wisniewski et al. 1997; Fuller & Wisniewski 1998). Computer software has also been used to analyze recordings of infrared video tape to study all stages of ice formation and to quantify the magnitude of the exotherms observed and their duration (Al-Swedi et al. 2013). Furthermore, the speed of ice front travel can also be determined by determining the start and the end times of the whole tissue being frozen and barriers to ice spread being detected (Wisniewski et al. 1997; Pearce & Fuller 2001) (Figure 4).

Of significance have been the observations that stem nodes hindered ice travel but ice forms quickly along the stem of the plant; but slows as it approaches the nodes; also, while shoots freeze quickly, ice formation progresses very slowly into the roots. In peach trees the stem was frozen before flowers and ice does not automatically form in the flowers, when shoots have frozen. Such differential freezing in various plant structures can begin to answer field observations of differential frost damage following natural freezing events.

Since the discovery that some bacteria (e.g. Pseudomonas syringae) can stimulate ice nucleation these have been used in freezing experiments to aid ice formation during infrared observation experiments (Wisniewski et al. 1997; Fuller &
Wisniewski 1998; Pearce & Fuller 2001). Typically, plants, which were treated with droplets of INA bacteria began to freeze first compared with samples or plants treated with water droplets only. It was observed that the drop of water on the surface of a leaf froze independently, at least 2.5 minutes after the part of leaf containing the bacteria (Wisniewski et al. 1997). Fuller et al. (2007) have reported that the ice nucleation temperature was uniform with *P. syringae* but was lower in the presence of distilled water. The warmest temperatures that INA bacteria are active at −2°C whereas distilled water droplets can supercool significantly below this. Thus it would seem that in nature, plants will always supercool to some extent and it follows that eliminating INA bacterial cells from the plant tissue surfaces allows plants to protect themselves from freezing by enabling supercooling. This approach is being used in California to protect blossom in almond and peach orchards from spring radiation frosts (Lindow et al. 1982) but has not been used systematically elsewhere. Fuller et al. (2007) have demonstrated in vitro that the use of compounds that help shed surface water decreases the

![Figure 4. The use of IR thermography technique showing the pattern of ice nucleation events in wheat spikes of non-sprayed and non-acclimated plants. The plants were in a frost chamber and an IR camera was fixed in the front window of it while plants were subjected to a freezing temperature down to −6°C at a freezing rate of 2°C down each 2 hours. (Circles refer to the start points of ice nucleation and the arrows refer to its progress pattern) (Al-Issawi 2013).](image)

![Figure 5. Avoiding ice nucleation by the supercooling mechanism in wheat (temperature dropped down to −11°C and no ice nucleation events were experienced) (Al-Issawi 2013).](image)
incidence of dew and thereby increases the likelihood of supercooling and frost escape in potatoes, tomatoes and grapevine but these compounds have not been tested extensively in the field.

Infrared thermal imaging has been used by many researchers who have observed that while supercooling is a mechanism for avoiding frost damage (Figure 5), it does carry with it an inherent risk if the temperature drops too low and is followed by freezing.

Ice crystals that develop after substantial supercooling have the potential to cause more injury than ice crystals that form at higher temperature (Choi et al. 1999). Slowly formed ice crystals are smaller than those formed more rapidly and in addition, when ice forms slowly, water has time to escape from plant cells so that ice grows in the intercellular spaces and intracellular ice formation, which is lethal, is less probable.

Conclusion
The physiological and molecular mechanisms of plant cold tolerance were reviewed in this study. The important role of cold acclimation and the biochemical and molecular changes that take place through this process were revised. The regulation of cold-regulated genes (CORs) and the pathways that control this regulation were discussed. Moreover, the essential part of CBF gens in the up-regulation and the production of COR proteins was reviewed. Recent research has demonstrated the positive role of Mo in plant cold tolerance. The role of this element in the up-regulation of COR genes through both ABA-dependent and -independent pathways was revised. Moreover, the effects of ABA on the abiotic stress tolerance generally and on the cold tolerance especially were discussed in this review. The use of infrared camera as a tool to improve the understanding level of ice formation in plant tissues was explained. This study provides a comprehensive overview of the recent achievement in the field of plant cold tolerance.

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No potential conflict of interest was reported by the authors.

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